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BRITISH PHARMACOLOGICAL SOCIETY

WINTER MEETING, 7th to 8th JANUARY, 1960,
AT THE SCHOOL OF PHARMACY, BRUNSWICK SQUARE, LONDON

COMMUNICATIONS

1. **G. A. Feigen** and **E. M. Vaughan Williams** (*Department of Pharmacology, Oxford*).
Electrophysiological evidence for the release of histamine during an antigen-antibody reaction in isolated guinea-pig auricles.

2. **K. R. Butterworth** and **Monica Mann** (*Departments of Pharmacology, St. Mary's Hospital Medical School, London, W.2, and School of Pharmacy, London, W.C.*1).
Variations in the catechol amine content of the adrenal glands of normal cats.

3. **J. R. Hodges** and **Joan Vernikos** (*Department of Pharmacology, Royal Free Hospital School of Medicine, London, W.C.*1).
The role of corticoids in regulating pituitary adrenocorticotrophic activity.

4. **J. M. Barnes** (*Toxicology Research Unit, Medical Research Council Laboratories, Carshalton, Surrey*).
The toxicity of acrylamide.

5. **W. H. H. Andrews** and **I. del Rio Lozano** (*Department of Pharmacology, St. Mary's Hospital Medical School, London, W.2*).
The effect of the route of administration of some drugs upon their rate of destruction by the liver.

6. **K. Ahmed** and **J. J. Lewis** (*Experimental Pharmacology, The University, Glasgow, W.2*).
Pharmacology of Petalin ; a convulsant alkaloid.

7. **D. R. Davies, P. Holland** and **M. J. Rumens** (*C.D.E.E., Porton Down, Salisbury, Wilts.*).
The relationship between the chemical structure and neurotoxicity of alkyl organophosphorus compounds. (*Precirculated.*)

8. **Z. M. Bacq** and **J. Renson** (*Laboratoire de Pathologie et Thérapeutique Générales, Liége*).
The inhibitors of adrenaline and noradrenaline inactivation. (*Precirculated.*)

9. **R. Schneider, Hilary Bishop, B. Shaw** and **A. C. Frazer** (*Department of Medical Biochemistry and Pharmacology, The Medical School, Birmingham*, 15).
Further observations on the mechanism of the inhibitory effect of gluten on isolated intestine.

10. **T. J. Sullivan** (*introduced by* **R. S. Stacey**) (*Department of Pharmacology and Therapeutics, St. Thomas's Hospital Medical School, London, S.E.*1).
The effect of some antibiotics on the amount of 5-hydroxytryptamine in the small intestine and spleen of mice and rats.

11. **S. E. Smith** (*introduced by* **R. S. Stacey**) (*Department of Pharmacology and Therapeutics, St. Thomas's Hospital Medical School, London, S.E.*1).
Some actions of α-methyldopa.

12. **I. Laszlo** (*introduced by* **R. S. Stacey**) (*Department of Pharmacology and Therapeutics, St. Thomas's Hospital Medical School, London, S.E.*1).
Observations on the estimation of substance P in mouse brain. (*Precirculated.*)

13. **J. H. Mackintosh** and **M. R. A. Chance** (*Department of Medical Biochemistry and Pharmacology, The Medical School, Birmingham*, 15).
Heredity and environment in the control of the variance of barbiturate anaesthesia.

14. **W. G. Smith** (*Department of Pharmacy, Sunderland Technical College*).
The anti-anaphylactic activity of ethanolamine and choline. (*Precirculated.*)

15. **J. J. Reuse** (*Laboratoire de Pharmacodynamie et de Thérapeutique, Université de Bruxelles*).
The effects of reserpine upon convulsions in rabbits.

16. **T. B. B. Crawford** and **D. F. Sharman** (*Department of Pharmacology, University of Edinburgh*).
Some observations on the methods of estimating 5-hydroxytryptamine in brain.

17. **E. H. Leach** and **P. A. Nasmyth** (*Department of Physiology, Oxford, and Department of Pharmacology, St. Mary's Hospital Medical School, London, W.*2).
Potentiation of the effects of tyramine by choline xylyl ether.

18. **R. W. Foster** (*introduced by* **G. Brownlee**) (*Department of Pharmacology, University of London King's College, London, W.C.*2).
The action of sympathomimetic drugs on the isolated guinea-pig tracheal muscle.

19. **A. B. Wilson** (*introduced by* **G. Brownlee**) (*Department of Pharmacology, University of London King's College, London, W.C.*2).
A method of analysing the inhibitory effects of sympathomimetic amines.

20. **G. D. H. Leach** (*introduced by* **G. Brownlee**) (*Department of Pharmacology, University of London King's College, London, W.C.*2).
The effect of central nervous stimulants and depressants on the movement of the transmurally stimulated guinea-pig ileum.

21. **L. C. Blaber** (*introduced by* **G. A. H. Buttle**) (*Department of Pharmacology, School of Pharmacy, London, W.C.*1).
The antagonistic action of ambenonium and its methoxy analogue to competitive and depolarizing neuromuscular blocking drugs. (*Precirculated.*)

22. **J. A. Holgate** and **B. T. Warner** (*Department of Pharmacological Research, Parke, Davis & Co., Hounslow, Middlesex*).
Potency of antihistamines *in vivo* as assessed by guinea-pig bronchiolar resistance. (*Precirculated.*)

23. **H. O. J. Collier** and **Patricia G. Shorley** (*Department of Pharmacological Research, Parke, Davis & Co., Hounslow, Middlesex*).
Effects of anti-rheumatic agents on some responses of guinea-pigs to bradykinin. (*Precirculated.*)

24. **G. V. R. Born** and **Janet Bricknell** (*Nuffield Institute for Medical Research, Oxford*).
5-Hydroxytryptamine in smooth muscle and connective tissue.

25. **B. Uvnäs** (*Farmakologiska Institutionen, Karolinska Institutet, Stockholm*, 60).
Histamine, slow reacting substance (SRS), and mast cell degranulation.

DEMONSTRATIONS

1. **Z. M. Bacq** and **J. Renson** (*Laboratoire de Pathologie et Thérapeutique Générales, Liége*).
Sensitization to adrenaline and adrenergic nerve stimulation by metanephrine. (*Precirculated.*)

2. **E. Letley** and **G. Batterson** (*introduced by* **G. Brownlee**) (*Department of Pharmacology, University of London King's College, London, W.C.*2).
The changes in the flow of blood in denervated rat hind-limb muscles in relation to the inhibition by adrenaline and other substances of acetylcholine-produced contractures.

3. **P. S. J. Spencer** (*introduced by* **G. B. West**) (*Department of Pharmacology, School of Pharmacy, London, W.C.*1).
The effect of the thyroid on the histamine and 5-hydroxytryptamine toxicities in the rat and mouse. (*Precirculated.*)

4. **A. M. Barrett** (*introduced by* **G. A. H. Buttle**) (*Department of Pharmacology, School of Pharmacy, London, W.C.*1).
Some factors affecting blood levels of corticotrophin.

5. **Shirley A. P. Price** (*introduced by* **G. B. West**) (*Department of Pharmacology, School of Pharmacy, London, W.C.*1).
The estimation of, and the effects of drugs on, the 5-hydroxytryptophan decarboxylase in rat brain.

6. **G. B. West** (*Department of Pharmacology, School of Pharmacy, London, W.C.*1).
Indole derivatives in tomatoes.

7. **J. M. Telford** (*introduced by* **G. B. West**) (*Department of Pharmacology, School of Pharmacy, London, W.C.*1).
The action of some adrenal cortical hormones on histamine and 5-hydroxytryptamine metabolism. (*Precirculated.*)

8. **Eleanor J. Zaimis** (*Pharmacology Department, Royal Free Hospital School of Medicine, London, W.C.*1).

A simple test for an amphetamine-like action.

9. **P. A. Nasmyth** (*Department of Pharmacology, St. Mary's Hospital Medical School, London, W.*2).

The effect of choline xylyl ether on the chromaffine cells.

10. **Third Year Students** (*introduced by* **G. A. H. Buttle**) (*Department of Pharmacology, School of Pharmacy, London, W.C.*1).

Some student exercises.

11. **Eva Kovacs** and **G. A. H. Buttle** (*Department of Pharmacology, School of Pharmacy, London, W.C.*1).

Effect of serum from tumour-bearing animals on the growth of a human tumour in rats.

12. **H. Ghaleb** (*introduced by* **G. A. H. Buttle**) (*Department of Pharmacology, School of Pharmacy, London, W.C.*1, *and Royal Marsden Hospital, London, S.W.*3).

Binding of stilboestrol on to plasma proteins.

13. **A. W. Cuthbert** (*introduced by* **G. A. H. Buttle**) (*Department of Pharmacology, School of Pharmacy, London, W.C.*1).

Action of drugs on muscle in tissue culture.

Brit. J. Pharmacol. (1960), **15**, 4.

THE ACCUMULATION OF ISONIAZID IN TISSUES, AND THE COURSE OF EXPERIMENTAL TUBERCULOSIS

BY

J. VENULET, KRYSTYNA JAKIMOWSKA, M. JANOWIEC, AND ALICJA URBAŃSKA

From the Pharmacological Department, Drug Research Institute and Institute of Tuberculosis, Warsaw, Poland

(RECEIVED MAY 12, 1958)

In an attempt to find agents enhancing the infiltration of isoniazid into tissues, the authors have investigated the effect of the drug on oedema due to injection of egg white into the rat paw, when it was given alone and in conjunction with histamine, antazoline, carbachol, chlorpromazine, and a pyrogen. Isoniazid increased the oedema. All the substances, except antazoline, increased the rate at which isoniazid disappeared from the blood, and they all favoured accumulation of the drug in tissues. Treatment of experimental tuberculosis with subthreshold doses of isoniazid, in conjunction with the above compounds, gave better results than when isoniazid was used alone.

Of the numerous factors affecting the final results of tuberculosis treatment, the accessibility of the drugs to the site of infection is of fundamental importance. The duration of the disease and the attendant protective and toxic reactions, as for example fibrosis and caseation, are serious impediments to the drug reaching the infected areas. The positive results obtained when the disease is treated with a potent antitubercular drug in conjunction with either corticotrophin or cortisone are explained by the paradoxically favourable effects of inhibition of some protective processes.

The experiments described in this paper were designed to determine the degree to which some antitubercular drugs and other substances might modify the inflammatory reaction and the permeability of tissues. Their value in treating experimental tuberculosis was also explored.

METHODS

The volume of extracellular fluid was measured in rabbits by the thiocyanate method (Odier, 1948), and the volume of circulating blood by the method of Griesbach (1921) and Heilmeyer (1929). Oedema of the rat paw was induced by injection into the paw of 0.05 ml. of egg white diluted 1 in 3 (Gross, 1950). Isoniazid concentrations were determined in rabbit blood and in rat lungs, liver, and kidney by the method of Deeb and Vitagliano (1955). In order to eliminate the opalescence in liver samples glycogen was hydrolysed with diastase.

Experimental tuberculosis was produced in albino mice weighing about 20 g. and in guinea-pigs weighing about 350 g. The mice were injected intravenously with 0.1 mg. of strain $H_{37}Rv$ and the guinea-pigs subcutaneously with 0.3 mg. of strain Sz. The drugs were administered subcutaneously daily from the day after inoculation until all the control animals had died. Then the surviving test animals were killed and the degree of the tubercular lesions was estimated microscopically. Three factors were determined with respect to the control group: (*a*) the death rate in each group, (*b*) the mean survival time, and (*c*) the index of tuberculosis.

The substances used in the experiments comprised: Isoniazid (isonicotinic acid hydrazide), histamine, antazoline (Antistine, Ciba), chlorpromazine (Largactil, Specia), carbachol (Doryl, Merck), and lipopolysaccharide from *Escherichia coli* (pyrogen).

RESULTS

Tissue reactions connected with alterations in permeability were studied in the rat paw, where oedema was produced by injection of egg white. Groups of ten rats were given one of the following preparations 30 min. before injection of the protein: isoniazid (0.05 g./kg., subcutaneously), chlorpromazine (1 mg./kg., subcutaneously) and bacterial lipopolysaccharide (0.1 or 1.0 μg./kg., intravenously). The results are shown in Figs. 1 and 2.

The oedema was increased and prolonged by isoniazid and decreased by chlorpromazine. Bacterial lipopolysaccharide in doses of 0.1 μg./kg. increased the oedema, but with larger doses the effect of the egg white was reduced. When given with isoniazid the effects of chlor-

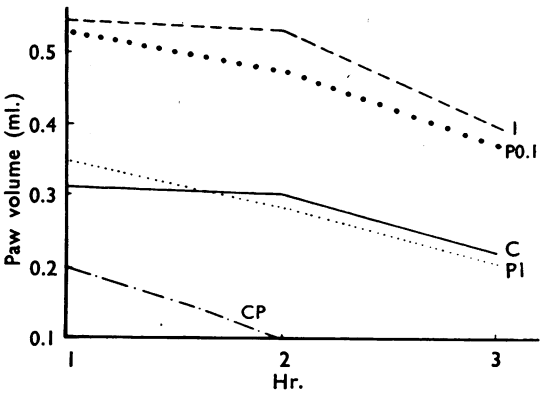

FIG. 1.—Effect of some agents on the volume of the rat paw. Each preparation was injected subcutaneously 30 min. before injection of egg white, which was used to produce oedema. C, control; I, 0.05 g./kg. isoniazid; P1 and P0.1, 1 μg./kg. and 0.1 μg./kg. respectively of bacterial lipopolysaccharide (pyrogen); CP, 1 mg./kg. chlorpromazine. For I, $P=0.005$; for P1, $P=0.8$; for P0.1, $P=0.04$; for CP, $P<0.001$.

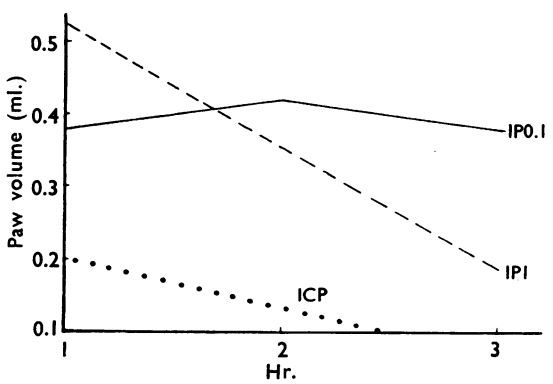

FIG. 2.—The effect of isoniazid combined with some agents on the volume of the rat paw. The combinations, which were 0.05 g./kg. isoniazid with 0.1 μg./kg. (IP0.1) and 1 μg./kg. (IP1) of bacterial lipopolysaccharide (pyrogen) and with 1 mg./kg. chlorpromazine (ICP), were injected subcutaneously 30 min. before injection of egg white, which was used to produce oedema. For IP0.1, $P=0.04$; for IP1, $P=0.8$; for ICP, $P>0.3$.

promazine and the pyrogen were similar to those obtained when they were given alone.

The effect of isoniazid on the distribution of extracellular fluid in rabbits was also studied. For this purpose, the volumes of total extracellular fluid and of plasma were measured in 10 animals before and after they had been injected with isoniazid (10 mg./kg.) twice daily for two days. The results are given in Table I, in which the coefficient I denotes the ratio of extracellular fluid to plasma.

TABLE I

DISTRIBUTION OF WATER IN CONTROL RABBITS AND IN RABBITS TREATED WITH ISONIAZID (10 mg./kg.)

I = extracellular fluid volume/plasma volume.

Control		Isoniazid	
Extracellular Volume (ml./kg.)	Plasma Volume (ml./kg.)	Extracellular Volume (ml./kg.)	Plasma Volume (ml./kg.)
252	66	307	78
I = 3·8		I = 3·9	

Isoniazid raised the overall retention of water in the animals without, however, modifying the rate of its distribution in tissues and blood.

Further experiments were aimed at determining the contents of isoniazid in tissues and in blood as modified by various agents. Groups of 10 rabbits were given histamine (0.5 mg./kg., subcutaneously), antazoline (5 mg./kg., subcutaneously), carbachol (15 μg./kg., subcutaneously), or chlorpromazine (1 mg./kg., subcutaneously), 60 min. before 20 mg./kg. of isoniazid, while in other groups bacterial lipopolysaccharide (0.01 μg./kg., intravenously) was given 30 min. before isoniazid and histamine was given simultaneously with isoniazid. The level of isoniazid in the blood 4, 30, 60, and 120 min. after injection is shown in Figs. 3 and 4.

All the preparations, except antazoline, significantly reduced the concentration of isoniazid in the blood. The effect of histamine was obviously dependent upon the timing of its administration. When it was given 1 hr. before isoniazid it increased the tissue permeability to such an extent that isoniazid immediately began to disappear from the blood. Even after 4 min. the concentration of isoniazid was much below that of the controls. When histamine and isoniazid were administered simultaneously the initial values were the same as for the controls. However, subsequently, the concentration of isoniazid fell rapidly and approached the values obtained in the experiments in which histamine was given before isoniazid.

For determining the isoniazid content in rat tissues, the animals were divided into groups of

ten. Group 1 served as a control. Group 2 received histamine, 0.5 mg./kg. subcutaneously 30 min. before, and 0.2 mg./kg. together with isoniazid. Group 3 received antazoline, 5 mg./kg. subcutaneously 30 min. before, and 0.5 mg./kg. intravenously together with isoniazid. Group 4 received carbachol, 15 μg./kg. subcutaneously 30 min. before, and 10 μg./kg. together with isoniazid. Group 5 was given chlorpromazine, 1 mg./kg. subcutaneously 30 min. before, and 0.25 mg./kg. intravenously at the same time as isoniazid. Group 6 was given the pyrogen, 0.1 μg./kg. intravenously 30 min. before isoniazid. The animals were sacrificed 30 min. after the injection of isoniazid. The circulatory system was rapidly perfused through the aorta with warm Ringer solution, and samples of the lung, liver and kidney were taken for isoniazid determinations. The results are shown in Figs. 3 and 4, together with curves illustrating the concentrations of isoniazid in blood.

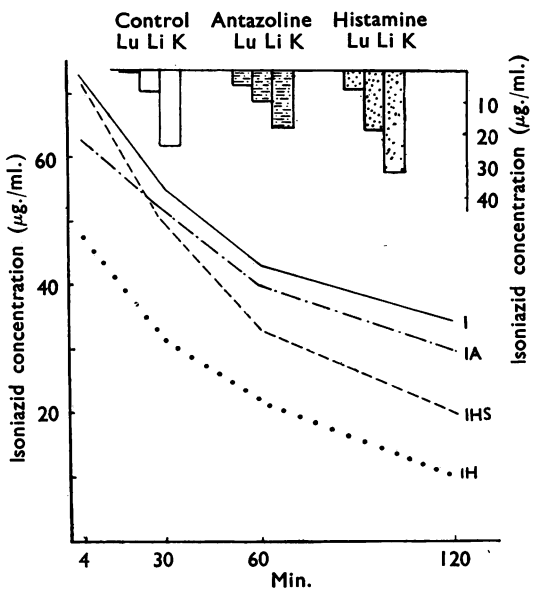

FIG. 3.—Effects of histamine and antazoline on the concentration of isoniazid in blood of rabbits and tissues of rats. The curves represent the concentration of isoniazid in the blood after injection of 20 mg./kg. isoniazid alone (I) and followed 60 min. later by 5 mg./kg. antazoline (IA), and by 0·5 mg./kg. histamine (IH). 20 mg./kg. isoniazid administered simultaneously with 0.5 mg./kg. histamine (IHS). For IA, $P=0.434$; for IHS, $P=0.015$; for IH, $P=0.001$. The columns represent the concentration of isoniazid in μg./g. in the lungs (Lu), liver (Li) and kidneys (K). For antazoline, $P=0.694$; for histamine, $P<0.001$.

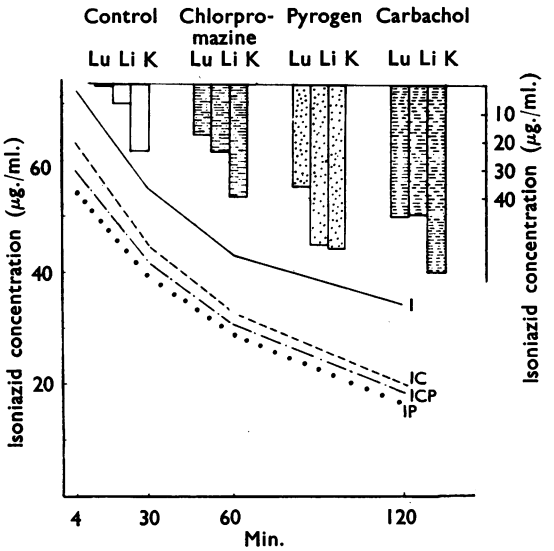

FIG. 4.—Effects of cabachol, chlorpromazine and bacterial lipopolysaccharide (pyrogen) on the concentration of isoniazid in blood of rabbits and tissues of rats. The curves represent the concentration of isoniazid in the blood after injection of 20 mg./kg. isoniazid alone (I) and followed 60 min. later by 15 μg./kg. carbachol (IC), 1 mg./kg. chlorpromazine (ICP) and by 0.01 μg./kg. bacterial lipopolysaccharide (pyrogen) (IP). For IC, $P=0.018$; for ICP, $P=0.018$; for IP, $P=0.010$. The columns represent the concentration of isoniazid in μg./g. in the lungs (Lu), liver (Li) and kidneys (K). For chlorpromazine, $P<0.001$; for bacterial lipopolysaccharide, $P<0.001$; for carbachol, $P<0.001$.

Since carbachol and the pyrogen proved very active in aiding infiltration of isoniazid into tissues, additional determinations with these two substances were carried out 1 hr. after the isoniazid injections. The results are shown in Figs. 5 and 6.

The isoniazid levels in various tissues differed according to the drugs used. Histamine, carbachol, bacterial lipopolysaccharide, and chlorpromazine significantly increased the content of isoniazid in all tissues. Consequently the reduction of isoniazid in the blood was attended by an increased infiltration into the tissues. This accounts, at least partly, for its disappearance from the blood. In spite of considerable dosage, only traces of isoniazid were found in the lungs after 30 min. when it was given alone. Even after 1 hr. the concentration was still lower than after the pyrogen. The highest concentration of isoniazid was found in the kidneys, and this is likely to be related to their excretory function.

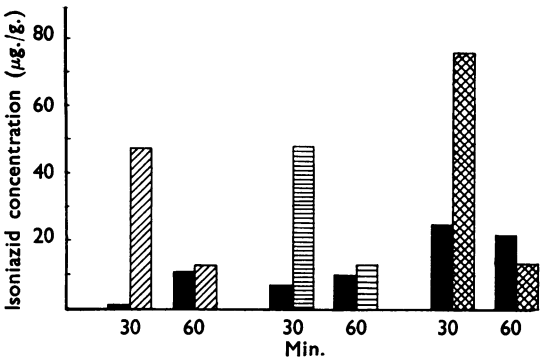

FIG. 5.—The effect of 15 μg./kg. carbachol on the concentration of isoniazid in lungs (cross-hatched), liver (horizontal hatched), and kidneys (double cross-hatched) of rats 30 and 60 min. after injection of 20 mg./kg. isoniazid. Black columns represent the concentrations after isoniazid treatment alone.

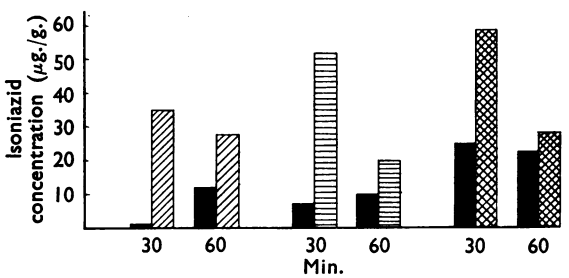

FIG. 6.—The effect of 0.01 μg./kg. bacterial lipopolysaccharide (pyrogen) on the concentration of isoniazid in lungs (cross-hatched), liver (horizontal hatched) and kidneys (double cross-hatched) of rats 30 and 60 min. after injection of 20 mg./kg. isoniazid. Black columns represent the concentrations after isoniazid treatment alone.

In the kidney the effects of the pyrogen and carbachol were most pronounced. In the control animals the concentration of isoniazid in the liver was only one-third of that in the kidneys.

When examined 1 hr. after the administration of isoniazid, the effects of carbachol and bacterial lipopolysaccharide were considerably smaller.

The overall pattern of these observations prompted us to test their possible usefulness in treating experimental tuberculosis in mice and guinea-pigs. In carrying out these experiments isoniazid was given in a dose below that currently used. It was considered that this dose was subthreshold, that is, inadequate for controlling the disease. The course of the experiments and the results obtained are shown in Tables II and III.

TABLE II

THE TREATMENT OF EXPERIMENTAL TUBERCULOSIS IN EIGHT GROUPS, EACH OF TEN MICE, FOR 35 DAYS, WITH ISONIAZID ALONE AND IN CONJUNCTION WITH BACTERIAL LIPOPOLYSACCHARIDE, HISTAMINE OR CARBACHOL

Injections were subcutaneous. INH=isoniazid.

Group of Animals	Preparation	Dose/kg.	Mortality in Group	Average Time of Survival (Days)	Index of Tubercular Changes
I	Control	—	10/10	28·1	118
II	INH	1·0 mg.	9/10	26·9	52
III	INH+ pyrogen	1·0 mg.+ 1·0 μg.	6/10	28·4	41
IV	INH+ histamine	1·0 mg.+ 0·5 μg.	2/10	35·0	43
V	INH+ carbachol	1·0 mg.+ 20·0 μg.	4/10	27·6	31
VI	Pyrogen	1·0 μg.	10/10	18·3	72
VII	Histamine	0·5 μg.	10/10	20·5	111
VIII	Carbachol	20·0 μg.	9/10	16·4	42

TABLE III

THE TREATMENT OF EXPERIMENTAL TUBERCULOSIS IN EIGHT GROUPS, EACH OF TEN GUINEA-PIGS, FOR 62 DAYS, WITH ISONIAZID ALONE AND IN CONJUNCTION WITH BACTERIAL LIPOPOLYSACCHARIDE, HISTAMINE OR CARBACHOL

Injections were subcutaneous. INH=isoniazid.

Group of Animals	Preparation	Dose per kg.	Mortality in Group	Average Time of Survival (Days)	Index of Tubercular Changes
I	Control	—	10/10	51·1	117
II	INH	1·0 mg.	6/10	52·9	61
III	INH+ pyrogen	1·0 mg.+ 0·5 μg.	1/10	56·9	44
IV	INH+ histamine	1·0 mg.+ 0·2 μg.	2/10	56·9	41
V	INH+ carbachol	1·0 mg.+ 10·0 μg.	1/10	59·8	41
VI	Pyrogen	0·5 μg.	10/10	46·4	89
VII	Histamine	0·2 μg.	10/10	40·2	91
VIII	Carbachol	10·0 μg.	9/10	45·2	96

In evaluating the results two criteria were used: the time of survival and the index of tubercular changes. The second criterion was used only in those instances where the survival time suggested that the results were not worse than those of controls. The first criterion has only a negative value in that it is helpful to show in which groups the results were worse than the control groups. It cannot be a definitive criterion since the proper estimation of the survival time is hampered by the killing of all the animals at the moment of death of the last animal in the control group. In the first experiment, results statistically superior to those of the controls were obtained from animals treated with isoniazid alone, and with isoniazid given in conjunction with bacterial lipopolysaccharide, histamine or carbachol. Isoniazid given with carbachol proved superior to isoniazid given alone; when given in conjunction with the pyrogen or histamine it was also slightly more effective. This is of interest because the pyrogen, histamine and carbachol significantly shortened survival time when used alone.

From Table III it can be seen that in guinea-pigs the treatment with isoniazid combined with the pyrogen or histamine or carbachol gave a reduced mortality rate and a lengthening of survival time. The Tubercular Index was significantly smaller than in the control group. Bacterial lipopolysaccharide and histamine given without isoniazid did not aggravate the course of the disease as they did in the experiment with mice recorded in Table II. All groups treated by these three combinations gave better results than the group treated with isoniazid alone, though the difference was not statistically significant at the 95% level.

Discussion

Oedema of the rat's paw due to injection of egg white provided a suitable criterion for assessing the alteration in tissue permeability due to antitubercular drugs. Isoniazid increased and prolonged the oedema and in other experiments streptomycin and *p*-aminosalicylic acid had a similar effect (Jakimowska and Venulet, 1957). To some extent the results reported here are inconsistent with those of Theobald (1954), who found isoniazid to inhibit oedema production in analogous experiments with dextran. This discrepancy may be explained by a difference in the mechanism of formation of the two kinds of oedema.

Histamine, carbachol, bacterial lipopolysaccharide and chlorpromazine considerably facilitated the accumulation of isoniazid in all the tissues examined, though the results with carbachol and pyrogen in which tissue concentrations were estimated after both 60 and 30 min. indicate that these effects are of short duration. Nevertheless the concentration of antitubercular drugs in the tissues might be of therapeutic value, and this was further examined by treating infected mice and guinea-pigs with isoniazid alone and in conjunction with those drugs which increased tissue permeability. From these experiments and from others (in which a total of 360 mice and 240 guinea-pigs were used), increasing tissue permeability appeared to be a valuable complement to isoniazid treatment. The relative effectiveness of the drugs used is not yet quite certain, but carbachol appeared to give somewhat superior results to the pyrogen and histamine. However, the side-effects of carbachol and histamine are sufficiently profound to limit their value while bacterial lipopolysaccharide with its milder effects is worthy of further investigation.

Previous work on the effect of carbachol, histamine, and bacterial lipopolysaccharide on the course of tuberculosis sheds little light on the problems raised by the present work because they have been used alone and not in conjunction with an antitubercular drug. The effects of histamine are usually believed to be unfavourable and those of antihistamine drugs favourable (Garbiński, 1953; Judd and Henderson, 1949; Duca and Scudi, 1949). Mitchell (1956) points out that there is some evidence that histamine stimulates the hypophyseal-adrenal system and this alone might lead to unfavourable results, but numerous authors have found combined treatment with isoniazid and corticotrophin or cortisone to be effective (Fortier and Favez, 1952; Quiring, Perlia, and Nicole, 1953; Favez and Fortier, 1953). Our results suggest that, in addition, histamine has an important direct action on tissues and vessels. In the light of our experiments antazoline is of no value and chlorpromazine has an intermediate activity, perhaps aiding permeability by its ganglionic blocking and sympatholytic actions. Kellentei and Föllders (1954) showed that acetylcholine enhances the permeability of the blood-brain barrier and carbachol favours the accumulation of phenytoin in the brain. This may well be due to a similar mechanism (Porszasz, Venulet, and Gibiszer-Porszasz, 1954).

The role of bacterial lipopolysaccharide is most difficult to evaluate, since little work has been performed on substances of this type. Recently, Dubos and Schaedler (1956) showed the favourable effects exerted by bacterial lipopolysaccharides on the course of tuberculosis in mice.

This preparation distinctly raises the level of plasma β- and γ-globulins (Piechocki and Venulet, 1957), but the effect of pyrogens on permeability was hitherto unknown. In this connexion, it is interesting that Rowley (1956) calls attention to the positive role of pyrogens in immunity.

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THE EFFECT OF SUBSTANCE P ON THE SUPERIOR CERVICAL GANGLION OF THE CAT

BY

D. BELESLIN, B. RADMANOVIĆ AND V. VARAGIĆ

From the Department of Pharmacology, Medical Faculty, University of Belgrade, Yugoslavia

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Intra-arterial injections of substance P in doses from 10 to 30 units potentiated the response of the nictitating membrane to submaximal stimulation of the preganglionic sympathetic nerve, while higher doses (30 to 100 units) usually depressed the response. The stimulating action of acetylcholine on the superior cervical ganglion (as judged by the response of the nictitating membrane) was also potentiated by substance P. The responses of the nictitating membrane to adrenaline, noradrenaline and tyramine were potentiated by substance P as well. It is concluded that the potentiating effect of substance P on the response of the nictitating membrane to submaximal stimulation of the preganglionic sympathetic nerve is probably due to sensitization of acetylcholine receptors in the postsynaptic neurone. The present experiments do not explain how substance P potentiated the response to sympathomimetic amines.

It has been shown that substance P may restore peristalsis when injected into the lumen of the isolated guinea-pig ileum in which the peristaltic reflex has been abolished by fatigue, by external or internal application of 5-hydroxytryptamine, or by lowering the temperature of the bath (Beleslin and Varagić, 1958). Substance P, when acting on the outside of the isolated guinea-pig ileum, blocks the peristaltic reflex (Beleslin and Varagić, 1959). In previous work some evidence was obtained that this block of peristalsis might be at least partly produced by the action of substance P on the intestinal ganglia. On the other hand, Lechner and Lembeck (1958) recorded the electroencephalogram of the cortex and of the hippocampus in rabbits after an injection of 30 to 100 units of substance P into the carotid artery and found that there was a decrease in amplitude and an increase in frequency which was most pronounced in 1 to 2 min. It was therefore of interest to investigate the effect of substance P on autonomic ganglia.

METHODS

Cats of both sexes, weighing 1.5 to 3 kg., were used. After inducing anaesthesia with ether, 80 mg./kg. of chloralose was injected intravenously. Intra-arterial injections were made into the central end of the lingual artery while occluding the external carotid artery. Thus the injected substance was diverted towards the superior cervical ganglion.

The contractions of the nictitating membrane were recorded with an isotonic lever fitted with a frontal writing point magnifying the movements of the membrane ten times. The cervical sympathetic chain was divided and, when stimulated electrically, its peripheral end was placed on shielded electrodes and covered with liquid paraffin. For stimulation an electronic stimulator delivering square wave pulses was used. The pulses had a duration of 0.8 msec. and a frequency between 4 and 15 per sec.

In some experiments the intra-arterial injections were made without occluding the external carotid artery, thus allowing the injected substance to act directly on the nictitating membrane.

Substance P was extracted and purified according to the method described by Zetler (1956).

The following substances were used: substance P, acetylcholine hydrochloride, adrenaline hydrochloride, noradrenaline bitartrate and tyramine hydrochloride. With the exception of substance P all doses are expressed in terms of the salts.

RESULTS

Substance P and Sympathetic Nervous Stimulation. — The intra-arterial injection of substance P in doses from 10 to 100 units changed the response of the nictitating membrane to submaximal stimulation of the preganglionic sympathetic nerve. With the stimulation in periods of 5 sec. every minute, a series of contractions of the nictitating membrane was recorded as shown in Fig. 1. At C the external carotid artery was occluded, and an intra-arterial injection of 18 units of substance P increased the response of the nictitating membrane to the preganglionic stimulation. The external carotid artery was

opened (at O) 10 min. after the injection of substance P, thus allowing the substance to reach the nictitating membrane in a high concentration. The response to preganglionic stimulation was now reduced and 8 min. later was completely abolished. The complete block lasted 15 min. after which the response gradually returned to normal. The amount of substance P which was injected in this experiment did not itself stimulate the ganglion and caused no contraction of the nictitating membrane in the absence of preganglionic stimulation. This type of response was obtained in five out of eleven experiments.

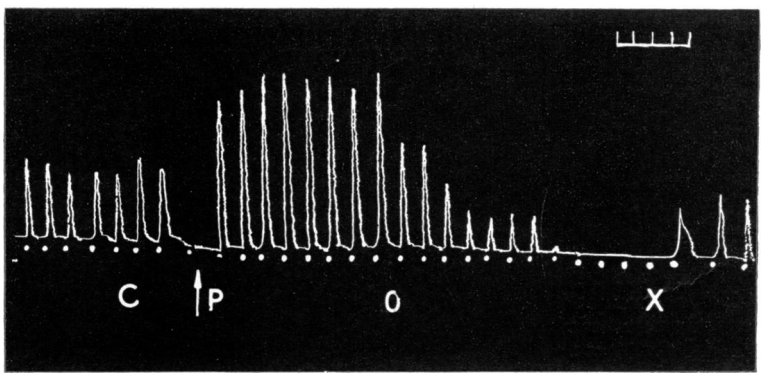

FIG. 1.—Cat, chloralose. Contractions of the nictitating membrane. At dots: submaximal preganglionic stimulation of the cervical sympathetic nerve (5 pulses/sec., 0·8 msec., 7 mA) for 5 sec. every min. At **C**, the external carotid artery was occluded. At **P**, 18 units of substance P was injected into the lingual artery. At **O**, the external carotid artery was opened. At **X**, the kymograph was stopped for 15 min. Time, 1 min.

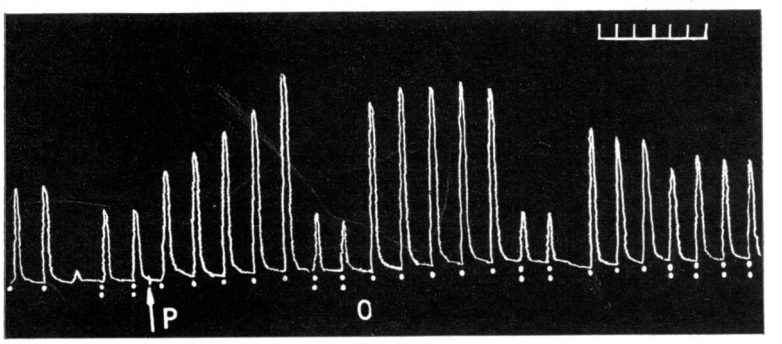

FIG. 2.—Cat, chloralose. Contractions of the nictitating membrane. The external carotid artery was occluded. At every single dot: submaximal preganglionic stimulation of the cervical sympathetic nerve (5 pulses/sec., 0·8 msec., 2 mA) for 10 sec. every 90 sec. At every two dots: submaximal postganglionic stimulation of the cervical sympathetic nerve. At **O**, the external carotid artery was opened. At **P**, 18 units of substance P was injected into the lingual artery. Time, 1 min.

The response of the nictitating membrane to stimulation of the postganglionic sympathetic nerve did not change significantly after intra-arterial injection of substance P. A typical result is shown in Fig. 2. The response of the nictitating membrane to preganglionic stimulation was potentiated immediately after injection of substance P. On the other hand, the response to postganglionic stimulation was unchanged. At O the external carotid artery was opened. After 10 min. the response to preganglionic stimulation was still potentiated but gradually declined, whereas the response to postganglionic stimulation was potentiated, as shown on the right-hand side of the figure.

In some experiments no initial increase in the response to preganglionic stimulation was observed. In these experiments the intra-arterial injection of substance P in doses from 30 to 100 units caused only a gradual decline in the response which sometimes was followed by a complete block. Such a result is shown in Fig. 3 (here the block of the response lasted for more than 45 min.). In four experiments a delayed potentiation of the effect of preganglionic sympathetic stimulation was observed. This effect was observed 30 to 120 min. after injection of substance P.

Substance P and Acetylcholine.—It has been shown previously that some substances may potentiate the response of the superior cervical ganglion to acetylcholine. Thus Konzett (1952; cited by Trendelenburg, 1956) found that histamine potentiated the response of the perfused superior cervical ganglion to acetylcholine. Trendelenburg (1956) confirmed this and found that both 5-hydroxytryptamine and pilocarpine shared this action. In the present experiments substance P also poten-

tiated the response of the nictitating membrane to acetylcholine. A typical result is shown in Fig. 4. The intra-arterial injection of 30 μg. of acetylcholine while the external carotid artery was occluded caused a contraction of the nictitating membrane due to a direct effect on the ganglion cells. These contractions of the nictitating membrane were increased after intra-arterial injection of 100 units of substance P. This effect was clear 12 min. after injection of substance P and it was regularly observed.

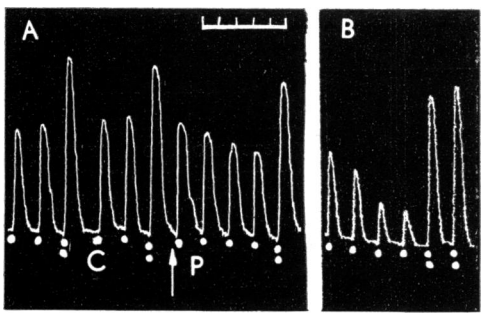

FIG. 3.—Cat, chloralose. Contractions of the nictitating membrane. At every single dot: submaximal preganglionic stimulation of the cervical sympathetic nerve (4 pulses/sec., 0·8 msec., 1·2 mA) for 10 sec. every 90 sec. At every two dots: submaximal postganglionic stimulation of the cervical sympathetic nerve (4 pulses/sec., 0·8 msec., 0·1 V.) for 10 sec. every 90 sec. At **C** the external carotid artery was occluded. At **P**, 100 units of substance P was injected into the lingual artery. **B** was taken 20 min. after the injection of substance P. Time, 1 min.

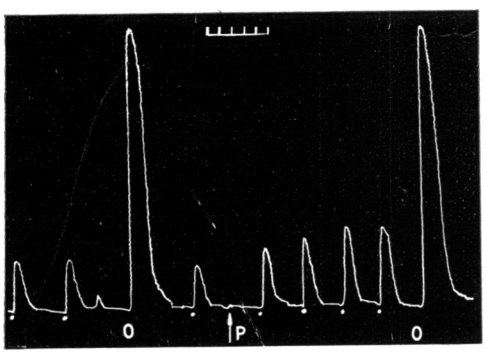

FIG. 4.—Cat, chloralose. Contractions of the nictitating membrane. At dots, 30 μg. of acetylcholine was injected into the lingual artery while the external carotid artery was occluded. At **O**, the same dose of acetylcholine was given while the external carotid artery was patent. At **P**, 100 units of substance P was injected into the lingual artery. Time, 1 min.

Substance P and Sympathomimetic Amines.—The effect of substance P on the response of the nictitating membrane to sympathomimetic amines was examined while the external carotid artery was patent, thus allowing the sympathomimetic drugs to act directly on the nictitating membrane. In the majority of the experiments it was found that the intra-arterial injection of substance P potentiated the response of the nictitating membrane to noradrenaline, adrenaline and tyramine. Fig. 5 shows such a result in which the response to noradrenaline was potentiated. This effect did not appear immediately after injection of substance P, but after an interval usually of 15 to 20 min.

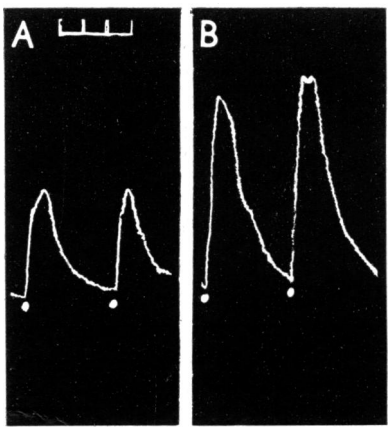

FIG. 5.—Cat, chloralose. Contractions of the nictitating membrane produced by intra-arterial injections of 20 μg. noradrenaline at the dots (the external carotid artery open). **A**, control. **B**, 20 min. after injection of 18 units of substance P into the lingual artery while the external carotid artery was open. Time, 1 min.

DISCUSSION

These experiments show that ganglionic transmission in the superior cervical ganglion of the cat may be potentiated or depressed by substance P. The potentiating effect was usually observed after small doses of substance P (10 to 30 units) and depression was usually produced by higher doses (100 units). It is known from the work of Trendelenburg (1956) that a variety of chemically unrelated substances may potentiate or depress ganglionic transmission. These substances can be divided into three groups : the nicotine-like substances (choline esters and nicotine), the sympathomimetic amines (adrenaline and noradrenaline) and a third group of drugs (including histamine, 5-hydroxytryptamine

and pilocarpine). Although all these substances potentiate sympathetic synaptic transmission, depression was obtained only with drugs of the first two groups. Substance P is known to be a polypeptide and hence chemically unrelated to any of these substances, but it can alter synaptic transmission in the sympathetic ganglion.

Substance P was also found to potentiate the response of the ganglion cells to intra-arterial injections of acetylcholine. This effect may be explained by the sensitization of acetylcholine receptors in the postsynaptic neurone. On the other hand, depression by substance P may be due to inhibition of the acetylcholine receptors or to non-specific actions of impurities in the substance P itself.

It is very difficult to explain the mechanism by which substance P potentiates the response of the nictitating membrane to the sympathomimetic amines which were used in the present experiments. The potentiating effect of substance P on the response of the nictitating membrane to postganglionic stimulation, which was occasionally seen, is in accordance with the finding that the response to noradrenaline was also potentiated.

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THE PHARMACOLOGICAL ACTIONS OF (*m*-HYDROXYPHENETHYL)TRIMETHYLAMMONIUM (LEPTODACTYLINE)

BY

V. ERSPAMER AND A. GLÄSSER

From the Institute of Pharmacology, University of Parma, and the Farmitalia S.p.A. Research Laboratories, Milan, Italy

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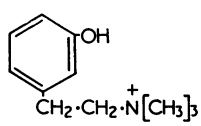
Leptodactyline is a naturally occurring quaternary ammonium base, which is found in large amounts in the skin of some South American amphibians of the genus *Leptodactylus*. It is the first example of a *m*-hydroxyphenylalkylamine in the living organism. In vertebrates it had powerful nicotinic actions and a marked neuromuscular blocking effect. This was considered, on the basis of experimental evidence, to be of the " depolarizing " type.

Leptodactyline [(*m*-hydroxyphenethyl)trimethylammonium] occurs naturally in the skin of some amphibian species of the genus *Leptodactylus*. It is the first *m*-hydroxyphenylalkylamine so far discovered in the living organism. It seems very probable that its remote precursor is *m*-tyrosine, an amino acid as yet unknown in nature.



Leptodactyline was first identified in extracts of *Leptodactylus* skin by Erspamer and Vialli (1952); its isolation and the determination of its chemical constitution were carried out by Erspamer (1959), and the proposed formula was confirmed by synthesis (Pasini, unpublished studies).

The present paper gives an account of the main pharmacological properties of leptodactyline.

METHODS

Leptodactyline Samples. — Both natural and synthetic leptodactyline picrate (molecular weight, 408.4; 1 mg. is equivalent to 0.44 mg. of the base) were used throughout the experiments. The doses given are of the picrate. The natural product was obtained as described by Erspamer (1959); the synthetic product was prepared by Dr. C. Pasini, of the Farmitalia Research Laboratories, Milan. The different samples of natural and synthetic leptodactyline showed the same chemical and paper-chromatographic characteristics and possessed exactly the same biological activity.

Pharmacological Methods.—The pharmacological methods used in the present investigation are in part identical to those described in our papers on murexine (Erspamer and Glässer, 1957, 1958).

The anaesthetics were chloralose (70 mg./kg.), pentobarbitone (30 mg./kg.), butallylonal [5-(2-bromoallyl)-5-s-butylbarbituric acid, Pernocton; 60 mg./kg.], and urethane (1 to 1.3 g./kg.), each given intravenously. Cats and dogs received intravenous injections into the femoral vein, rabbits into the marginal vein of the ear or the jugular vein, and rats and mice into the tail vein. Dogs were also given injections into the carotid artery through a plastic cannula in the thyroid artery. Injections were intravenous unless otherwise stated.

Blood pressure was measured from the femoral or carotid artery. Respiration was recorded by a tambour connected to a tracheal cannula. Adequate oxygenation of the blood during apnoea was maintained by a respiratory pump.

Neuromuscular blocking action was tested on the cat, dog, rabbit, and rat nerve-gastrocnemius preparations, prepared as described by Burn (1950). Maximal stimuli (rectangular current pulses of 7 msec. duration) were applied to the sciatic nerve through a unipolar electrode at a rate of 5 to 10 pulses/min., and the contractions were recorded semi-isometrically.

The rat phrenic nerve-diaphragm preparation, as described by Bülbring (1946), was used in a few experiments. The drug was left in contact with the tissue for 3 to 5 min.

The isolated frog rectus abdominis muscle, the leech dorsal muscle, the rabbit and guinea-pig atrium, and the guinea-pig, rabbit, rat, and cat intestine were prepared and employed in the usual manner.

Functional adrenalectomy was carried out as described by Holmstedt and Whittaker (1958), and

carotid sinus denervation by the procedure of Gollwitzer-Meier and Pinotti (1943).

RESULTS

Pharmacological Actions

General Effects

The basic action of leptodactyline in mammals is to paralyse skeletal muscle and to stimulate ganglia powerfully. Death seems to be caused mainly by anoxia due to paralysis of the respiratory muscles; it cannot, however, be excluded that the consequences of ganglionic stimulation may contribute to death.

Muscular paralysis and respiratory depression were generally preceded or accompanied by short-lived polypnoea and by muscular twitches and fasciculations all over the body, salivary hypersecretion, lachrymation, mydriasis alternated with myosis, intestinal borborygmi, defaecation and micturition. When death occurred muscular twitchings and fasciculations persisted for several minutes after death.

The LD50 of leptodactyline picrate in mice was 3.3 mg./kg. by intravenous route, and approximately 325 mg./kg. by mouth; the dose causing head drop in rabbits (ED50) was approximately 0.2 mg./kg., intravenously.

In birds leptodactyline, like all depolarizing muscle relaxants, provoked contracture (extension cramp of the legs and opisthotonos) instead of muscular paralysis. Myosis alternated with mydriasis and evacuation of the bowels was sometimes observed at the same time. In pigeons the minimum active intravenous dose was 20 to 25 μg./kg. and the LD50 120 to 180 μg./kg. Frogs and fishes were paralysed, like mammals. The paralysing dose (ED50) of leptodactyline in frogs, following injection into the dorsal lymphatic sac, was approximately 0.5 mg./kg. The righting reflex disappeared after 5 to 10 min., and returned to normal after 20 to 35 min.

Neuromuscular Block

Sciatic Nerve-Gastrocnemius Muscle Preparation.—A comparison of the paralysing action of different doses of leptodactyline on cat, dog, rabbit, and rat gastrocnemius is given in Table I. Neuromuscular transmission was less sensitive to leptodactyline in the dog than in the cat, while that in the rabbit and in the rat was still less sensitive. In this respect leptodactyline behaved like murexine.

As with all other blocking agents, the repeated administration of leptodactyline at brief intervals produced a cumulative effect. As with suxamethonium and murexine, the twitch reduction was sometimes preceded by a short-lived increase in the tension of the muscle, as shown by the potentiation of some twitches, and, between the contractions, fasciculations of the muscle could be seen. Tubocurarine could antagonize the muscle-relaxant action of leptodactyline. Intravenous injection of 100 μg./kg. of the drug, given during

TABLE I

THE PARALYSING ACTION OF LEPTODACTYLINE PICRATE ON THE SCIATIC NERVE-GASTROCNEMIUS MUSCLE PREPARATION OF DIFFERENT SPECIES

Twitch reductions are the maximum changes expressed as the % of control twitch. Recovery times refer to 50% recovery. — means no experiments. 0 means no depression of twitch.

Dose of Leptodactyline (μg./kg.)	Cat		Dog		Rabbit		Rat	
	Twitch Reduction (%)	Recovery Time (min.)	Twitch Reduction (%)	Recovery Time (min.)	Twitch Reduction (%)	Recovery Time (min.)	Twitch Reduction (%)	Recovery Time (min.)
20	2–15	—	—	—	—	—	—	—
40	10–15	½–2	—	—	—	—	—	—
50	35	2	20	2	0	—	—	—
80	60–90	2	—	—	0	—	0	—
100	85–90	3	70–80	2½	0	—	0–5	—
150	90–95	3	—	—	0	—	—	—
200	95–99	4–6	—	—	5–10	1	10	2–3
400	100	6	95	5	15–45	2	25	5
1,000	100	20	95–98	12	75–99	4–5	40	8–9
1,500	100	50	—	—	—	—	—	—
2,000	100	60	—	—	—	—	72	21

an intravenous infusion of 45 μg./kg./min. leptodactyline which depressed the twitch by 75%, promptly abolished muscular paralysis. The intravenous injection of 5 mg./kg. of choline chloride during the recovery from muscle relaxation produced by 300 to 500 μg./kg. of leptodactyline caused a transient increase of the depressed muscular twitch.

Rat Diaphragm.—Leptodactyline (7 μg./ml.) reduced by 15% the amplitude of muscular twitches. With 15 μg. and 30 μg./ml. the reduction was 30 and 80% respectively. It may

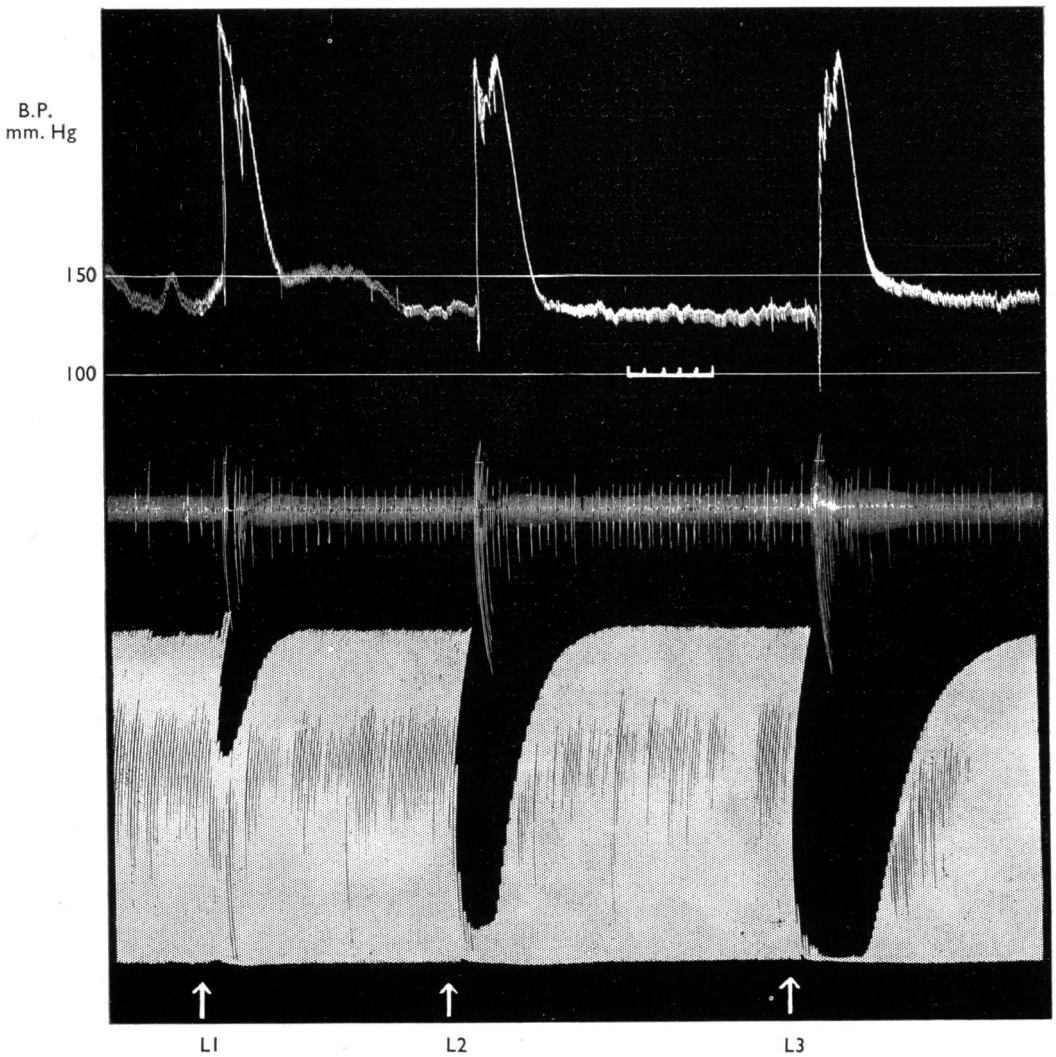


FIG. 1.—Cat anaesthetized with chloralose. Upper tracing, blood pressure. Middle tracing, respiration. Lower tracing, twitches of the gastrocnemius muscle in response to single maximal stimuli to the sciatic nerve. Time, 1 min. Leptodactyline picrate produced a sharp rise in blood pressure, a short-lived stimulation of respiration and a neuromuscular block. The pressure response was maximal with all the three doses of leptodactyline tested (50, 100 and 200 μg./kg., at **L1, L2** and **L3**), respiratory stimulation and neuromuscular block increased with the dose. With 200 μg./kg. there was no impairment of respiratory movements, in spite of a nearly complete neuromuscular block of the gastrocnemius.

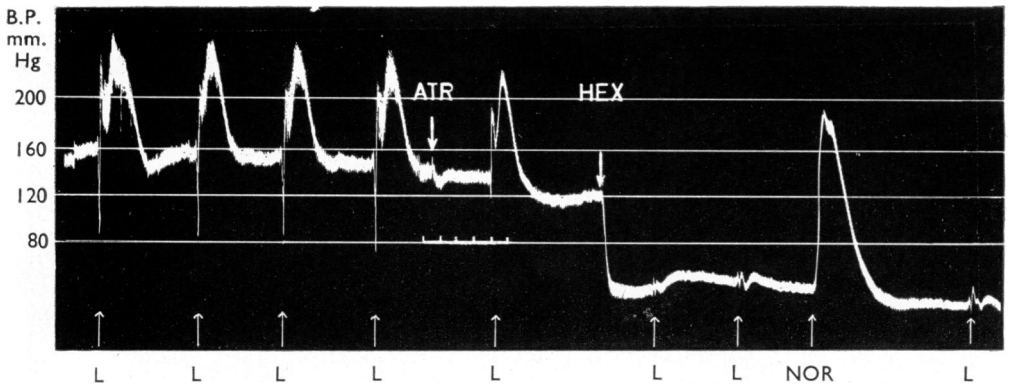


FIG. 2.—Blood pressure of a dog anaesthetized with pentobarbitone. At **L**, 30 μg./kg. leptodactyline picrate; at **ATR**, 0.5 mg./kg. atropine sulphate; at **HEX**, 4 mg./kg. hexamethonium bromide; at **NOR**, 1.5 μg./kg. noradrenaline. All the injections were given intravenously. Time, 1 min. The pressure response to leptodactyline showed no tachyphylaxis. The short-lived primary pressure fall was abolished by atropine, and the pressure rise by hexamethonium.

be seen that this preparation was not very sensitive to leptodactyline.

Blood Pressure

Cat.—In cats anaesthetized with chloralose, rapid intravenous injections of leptodactyline caused a very transient hypotension (10 to 70 mm. Hg, according to the dose), followed by a more sustained rise of blood pressure (Fig. 1). With high doses of leptodactyline (100 μg./kg. and more) the blood pressure rose by 80 to 130 mm. Hg, but the sharp pressure rise was generally followed by a secondary fall lasting 10 to 20 min. The pressure changes elicited by low doses of leptodactyline were not accompanied by changes in the heart rate; moderate or high doses, on the contrary, produced bradycardia, eventually followed by a period of tachycardia. The minimum dose of leptodactyline active on the blood pressure of the intact cat was 1 to 2 μg./kg.

In contrast to the intact animal, the spinal cat nearly always showed a simple rise of blood pressure after intravenous leptodactyline. Very often the rise was proportional to the dose administered. Only in some cats was hypertension preceded by a weak, fleeting fall of blood pressure.

Atropine sulphate (1 mg./kg.) abolished the initial fall of blood pressure; hexamethonium bromide (2 to 5 mg./kg.) and diethylaminomethyl-benzo-1,4-dioxan (Prosympal, 1 to 5 mg./kg.) reduced or abolished the leptodactyline hypertension. In the spinal cat, the rise of blood pressure produced by 25 to 50 μg./kg. of leptodactyline was reduced by about 25% following functional adrenalectomy. This may signify that only 25% of leptodactyline hypertension is due to release of medullary catecholamines.

Dog.—In dogs anaesthetized with chloralose, pentobarbitone, or butallylonal the usual response to intravenous leptodactyline was an abrupt fall of blood pressure lasting a few seconds, followed by a sharp, more sustained rise (Fig. 2). However, with very low doses of leptodactyline, hypotension, with no change in the heart rate, was sometimes the only response. With moderate doses of leptodactyline (20 to 50 μg./kg.) hypotension of 40 to 60 mm. Hg was followed by hypertension of 40 to 100 mm. Hg. The heart rate was at first reduced, then increased.

Often hypertension was roughly proportional in intensity and duration to the dose of leptodactyline, and repeated doses of the same magnitude elicited similar responses.

As in the cat, the short-lived primary hypotension was antagonized by atropine (0.5 mg./kg.) and the rise of blood pressure by hexamethonium (3 mg./kg.) (Fig. 2). Prosympal reduced the hypertension, leaving the primary hypotension completely unaffected. Bilateral vagotomy and denervation of the carotid sinuses caused very little, if any, effect either on the initial fall or the subsequent rise of blood pressure due to leptodactyline. Intracarotid injections of leptodactyline produced approximately the same initial hypotension as intravenous injections, but were several times less effective in causing the rise of blood pressure, which was also somewhat delayed (Fig. 3).

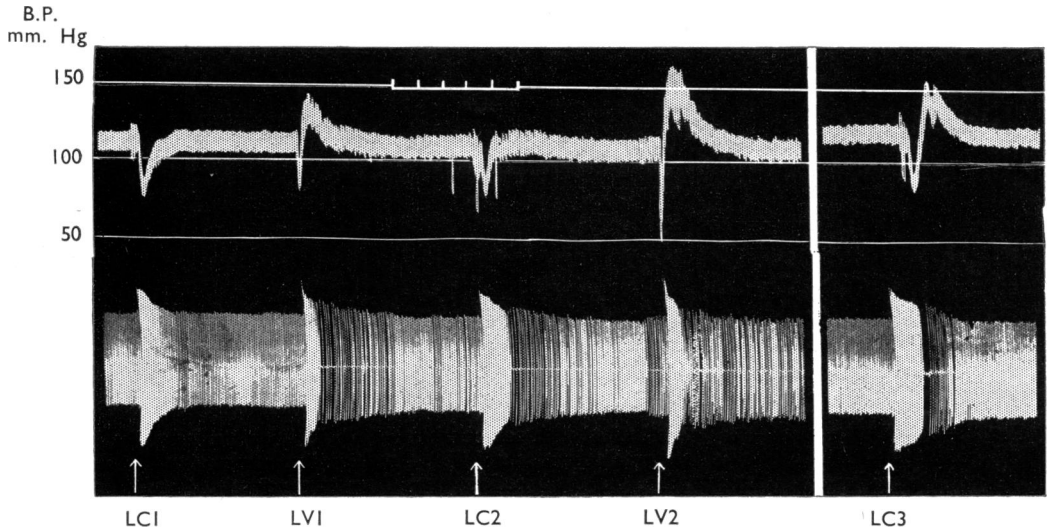


FIG. 3.—Dog anaesthetized with butallylonal. Upper tracing, blood pressure. Lower tracing, respiration. At **LC1, LC2** and **LC3**, 30, 60 and 150 μg./kg. leptodactyline picrate into the carotid artery; at **LV1** and **LV2**, 30 and 60 μg./kg. leptodactyline intravenously. Time, 1 min. The primary hypotensive phase produced by leptodactyline remained apparently unchanged following injection into the carotid artery; the hypertensive phase, on the contrary, was lacking or reduced in intensity and delayed in time. Respiratory stimulation was more intense and prolonged following injection of leptodactyline into the carotid artery.

It appears probable that the initial hypotension is essentially due to a cardiac action of leptodactyline and that the hypertension is due both to ganglionic stimulation and to release of pressor amines from the body stores. Reflex or direct stimulation of the vasomotor centres in the medulla does not play any important role.

Rabbit.—In rabbits anaesthetized with urethane leptodactyline nearly always provoked a more or less pronounced hypotension (20 to 70 mm. Hg). This was transient with small doses (2 to 20 μg./kg.), and more persistent with high doses (50 to 500 μg./kg.). The first fall of blood pressure was often followed, after return to the starting level, by a secondary, long-lasting fall. Rarely a slight, short-lived rise of blood pressure was interposed between the two hypotensive phases. Hexamethonium (5 mg./kg.) had no effect on the hypotension after leptodactyline.

Heart

Guinea-pig Atrium. — This preparation was considerably more sensitive to leptodactyline than the rabbit atrium. The predominant effect was a reduction in the amplitude of beat: 4 to 10 min. following 0.5 μg./ml. the reduction was 25 to 60%, and after 2 μg./ml. it was 55 to 95%. The frequency was similarly reduced, by 9 and 50%, respectively, at the above concentrations. A concentration of 1 μg./ml. of leptodactyline was equipotent to 0.1 μg./ml. acetylcholine bromide.

Following 0.1 μg./ml. atropine sulphate weak concentrations of leptodactyline were ineffective; high concentrations (10 μg./ml.) caused a moderate increase in the frequency (15 to 30%) and a more conspicuous increase in the amplitude (40 to 100%) of beat. This effect was similar to that produced by 1 to 3 μg./ml. nicotine tartrate. Very high concentrations of hexamethonium (50 to 300 μg./ml.) were required to abolish the depressive effects of leptodactyline on the normal atrium: much smaller concentrations (0.1–0.2 μg./ml.), on the contrary, could reduce or abolish the stimulant effects of leptodactyline on the atropinized atrium (Fig. 4).

Rabbit Atrium.—The isolated rabbit atrium responded to leptodactyline (1 to 50 μg./ml.) with a prolonged reduction in the frequency of contractions, which often persisted for some time after washing with fresh nutrient solution. The amplitude of beat was moderately reduced with weak concentrations (1 to 5 μg./ml.) of

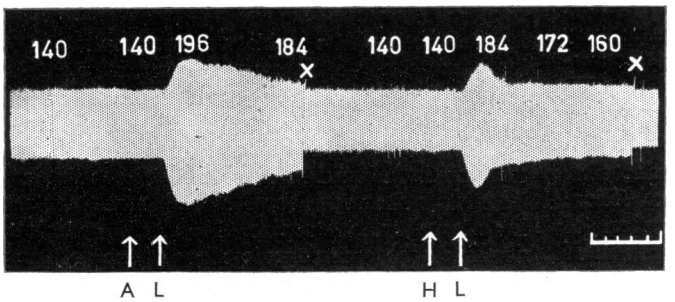


FIG. 4.—Guinea-pig atrium, suspended in oxygenated Ringer-Locke solution at 30°. At **L**, 10 μg./ml. leptodactyline picrate; at **A**, 0.1 μg./ml. atropine sulphate; at **H**, 0.01 μg./ml. hexamethonium bromide + 0.1 μg./ml. atropine sulphate; at **X**, washing. Time, 1 min. After atropine, leptodactyline produced an increase in amplitude and frequency of beats. This effect was considerably reduced by low doses of hexamethonium.

leptodactyline. With higher concentrations the initial negative inotropic effect turned into a positive effect within a few minutes. After 5 to 15 min. the amplitude of beat was sometimes increased, sometimes decreased.

Following atropine sulphate (1 μg./ml.), leptodactyline was completely inactive in concentrations of 1 to 3 μg./ml. In concentrations of 5 μg./ml., on the contrary, it regularly caused a marked increase both in the frequency and the amplitude of beat. The first effect was persistent, the second often turned into a decrease in the amplitude of beat.

High concentrations of hexamethonium tartrate (100 to 200 μg./ml.) produced a complete block of the leptodactyline effects, with the exception of a slight, transient increase in the frequency of contractions. Similarly, all the effects of leptodactyline were opposed by the simultaneous administration of atropine and Prosympal (20 μg./ml.). The stimulant effect of 15 to 20 μg./ml. leptodactyline on the atropinized rabbit atrium was quantitatively similar to that after 10 μg./ml. nicotine tartrate.

It seems highly probable that the negative effects of leptodactyline on the isolated atria are due to a stimulation of parasympathetic ganglia, and the positive effects to stimulation of sympathetic ganglia, or, more likely, to a release of catecholamines from stores within the heart. In fact, Glässer (unpublished observations) has found that stimulation by leptodactyline is reduced or abolished in atria taken from animals treated with reserpine, exactly as it is for stimulation by nicotine (Burn and Rand, 1958).

Respiration

Cat.—In cats anaesthetized with chloralose the minimum intravenous dose of leptodactyline

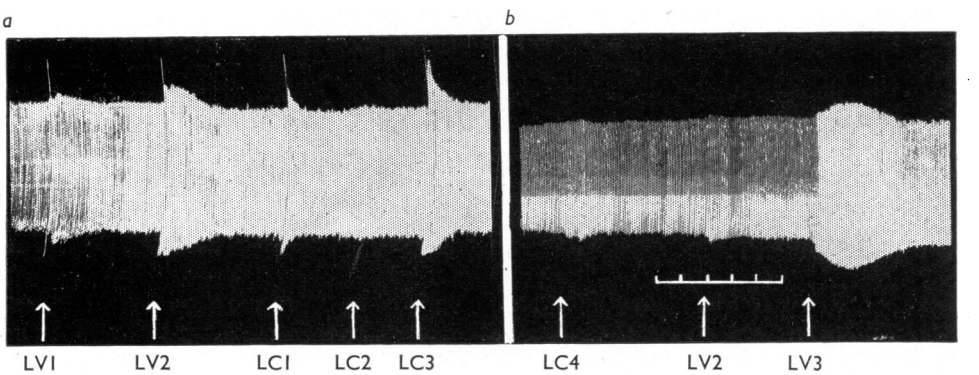


FIG. 5.—Respiration of a dog anaesthetized with butallylonal. At **LV1**, **LV2** and **LV3**, 7, 15 and 50 μg./kg. leptodactyline picrate intravenously; at **LC1**, **LC2**, **LC3** and **LC4**, 0.05, 0.5, 1.5 and 2.5 μg./kg. leptodactyline into the carotid artery. Between (*a*) and (*b*) section of both vagi and denervation of the carotid sinus areas. Time, 1 min. Leptodactyline was ten to fifteen times more active on respiration when given into the carotid artery than when given intravenously; denervation of the carotid sinus areas abolished the prompt respiratory stimulation produced by leptodactyline. With high doses of the substance a more sustained respiratory stimulation could be observed.

which affected respiration was 10 to 20 μg./kg. The usual response was a short-lived respiratory stimulation, followed by a period of slight depression (Fig. 1). High doses of leptodactyline (500 μg./kg. and more) regularly provoked respiratory arrest due to paralysis of the skeletal musculature.

Dog.—The minimum dose of leptodactyline active on respiration was 1 to 5 μg./kg. There was a very short-lived intense stimulation of respiratory movements, followed by depression or short-lasting apnoea.

Injections of leptodactyline into the carotid artery were ten to fifteen times more active than intravenous injections (Fig. 5); on the other hand, denervation of the carotid sinus areas completely abolished the respiratory response. It is therefore concluded that the above effect of leptodactyline on respiration is brought about by stimulation of the chemoreceptors of the carotid bodies. The secondary respiratory depression was probably a consequence of hyperventilation.

Following denervation of the carotid sinus areas, leptodactyline sometimes produced a different respiratory response; a stimulation which was slower in onset and more prolonged than that previously described.

Rabbit.—The usual response to leptodactyline (10 μg./kg. and more) in this species was a short-lasting apnoea followed by a brief period of respiratory stimulation.

Smooth Muscle

Intestine. — The minimum concentration of leptodactyline which caused contraction of the guinea-pig small and large intestine and the frog stomach was approximately 0.5 to 1 μg./ml. There was no good dose/response relationship and often tachyphylaxis could be observed. The rabbit large intestine showed the same sensitivity to leptodactyline. Atropine (1 μg./ml.) completely abolished the spasmogenic effect of the substance, which then produced a transient decrease in tone and reduction or suppression of spontaneous movements (Fig. 6).

The cat colon responded to leptodactyline with a contraction which was often satisfactorily proportional to the dose. Atropine (1 μg./ml.) abolished this effect; hexamethonium tartrate (0.2 to 1 mg./ml.) and mecamylamine hydrochloride (5 to 25 μg./ml.) consistently reduced it.

The results obtained on the intestinal smooth muscle, which are very similar to those produced by nicotine, are to be interpreted as the expression of a stimulation of intramural ganglia.

Other Tissues

Frog Rectus Abdominis. — Leptodactyline induced, in very low concentrations (minimum active concentration 0.01 to 0.03 μg./ml.), a contracture in the frog rectus. There was a very satisfactory dose/response relationship. Therefore, the frog rectus may be considered a very convenient and suitable preparation for the quantitative estimation of leptodactyline. The leptodactyline contracture was very similar to that produced by the methonium compounds and murexine, but differed from that provoked by acetylcholine, which had a more rapid onset and reached a plateau much more readily (Fig. 7). The relative potencies in stimulating the frog rectus following a 3 min. contact were: leptodactyline picrate, 100; murexine dipicrate, 6 to 8; acetylcholine bromide, 20 to 25; nicotine tartrate, 1 to 2.

Eserine salicylate (0.01 mg./ml.) did not potentiate the response to leptodactyline. Tubocurarine showed

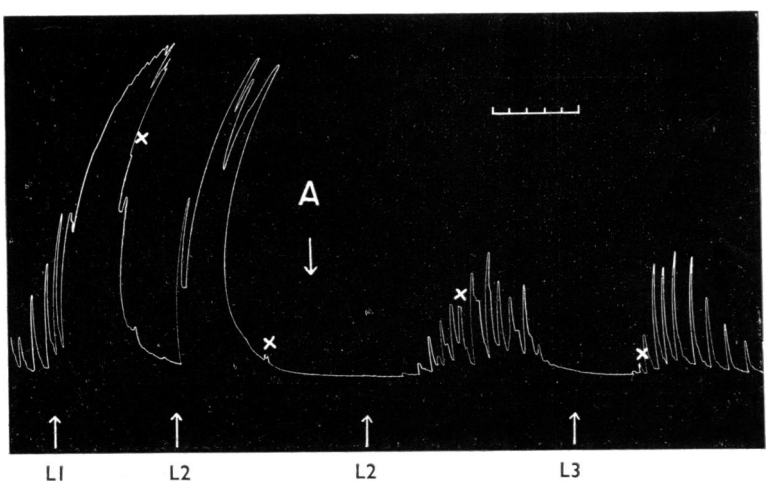


FIG. 6.—Isolated rabbit colon suspended in Tyrode solution at 37°. At **L1, L2,** and **L3,** 3, 10 and 30 μg./ml. leptodactyline picrate; at **A,** atropine sulphate, 1 μg./ml.; at **X,** washing. Time, 1 min. Atropine abolished the stimulant effect of leptodactyline on the rabbit colon. Tachyphylaxis was evident before atropinization.

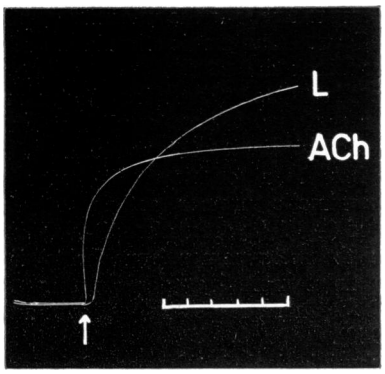


FIG. 7.—Frog rectus abdominis preparation. At arrow 0.2 μg./ml. leptodactyline picrate (**L**) and then, after washing and relaxation of the muscle, 1 μg. ml. acetylcholine bromide (**ACh**). Time, 1 min. The response caused by leptodactyline had a slower onset compared with that caused by acetylcholine and did not reach a plateau.

a strong antagonistic action: after 0.125 and 1.25 μg./ml. the contracture caused by 0.05 μg./ml. leptodactyline was reduced by 50 and 85% of the control, respectively.

Leech Muscle. — Leptodactyline caused a contracture of this preparation which closely resembled that produced by murexine (Erspamer and Glässer, 1957). The minimum active concentration was 0.5 to 1 μg./ml. There was, in several instances, an excellent dose/response relationship (Fig. 8).

Fate of Leptodactyline in the Organism

Three groups of four rats, each weighing 800 to 950 g., were given 10 mg. leptodactyline per rat, one group orally and two groups subcutaneously. Urine was collected first for a 6 hr. period, and then for a second period of 18 hr. The activity of the urine on the frog rectus abdominis was determined, and expressed in terms of leptodactyline; moreover, concentrated urine was submitted to paper chromatography.

The urinary activities, expressed in absolute values of leptodactyline and in percentages of the drug administered, were as follows:

Oral	0– 6 hr. period	42 μg.	(0·42%)
	7–24 ,, ,,	96 ,,	(0·96%)
	Total	138 ,,	(1·38%)
Subcutaneous	0– 6 ,, ,,	2,900 ,,	(29·0%)
	7–24 ,, ,,	400 ,,	(4·0%)
	Total	3,300 ,,	(33·0%)
Subcutaneous	0– 6 ,, ,,	1,300 ,,	(13·0%)
	7–24 ,, ,,	180 ,,	(1·8%)
	Total	1,480 ,,	(14·8%)

It may be seen that leptodactyline was largely destroyed in the organism, even when administered by parenteral route. Since monoamine oxidase does not seem able to attack quaternary ammonium bases (Blaschko, 1952) it is probable that other enzyme systems are involved in the inactivation of leptodactyline.

Paper chromatograms of concentrated urine demonstrated the presence of unaltered leptodactyline and of two unidentified metabolites of the substance, still bearing a free hydroxyl group in the *meta*-position. The R_F values on Whatman No. 1 paper, using the ascending technique and diazotized sulphanilic acid as a spraying reagent, were: (*a*) for the butanol + acetic acid + water (4:1:5) solvent system, leptodactyline 0.58, metabolite I 0.68, metabolite II 0.90; and (*b*) for the butanol + 25% aqueous methylamine (8:3) solvent system, leptodactyline 0.15, metabolite II 0.28.

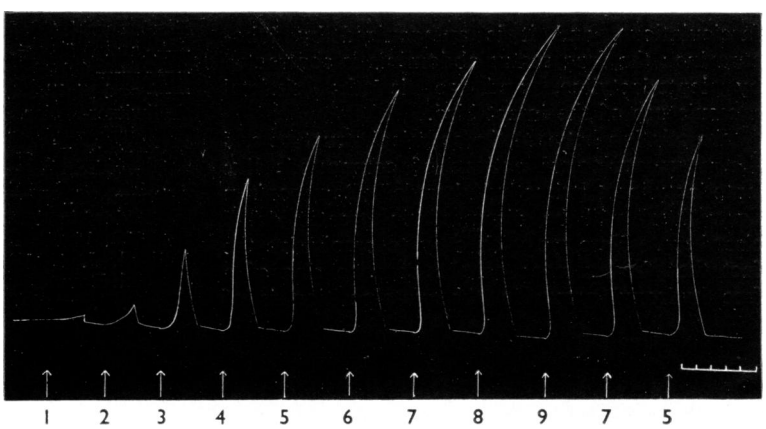


FIG. 8.—Leech muscle preparation. Contractions produced by increasing concentrations of leptodactyline picrate (in μg./ml.). Time, 1 min. There was a good dose response relationship.

Discussion

Leptodactyline is the first *m*-hydroxyphenylalkylamine to be found in the living organism, specifically in the skin of a vertebrate. It is highly probable that *m*-tyrosine represents the parent amino acid of leptodactyline. Blaschko (1950, 1958) and Mitoma, Posner, Bogdanski, and Udenfriend (1957) have shown that *m*-tyrosine, in sharp contrast to the widely distributed *p*-tyrosine, is a substrate, both *in vitro* and *in vivo*, for mammalian decarboxylase. The resultant *m*-tyramine might easily be transformed into its *N*-methylated derivatives by transmethylating enzymes which seem to be particularly active and abundant in the skin of amphibians, as shown by the occurrence in this skin of a whole series of *N*-methylated derivatives of 5-hydroxytryptamine. Bufotenidine is the trimethylammonium derivative of 5-hydroxytryptamine, as leptodactyline is the trimethylammonium derivative of the hitherto unknown *m*-tyramine.

With the description of leptodactyline and of *o*-tyrosine (Dennell, 1956), all the three isomers of hydroxyphenylalanine (that is, the three tyrosines) may be considered naturally occurring amino acids, capable of giving origin to highly active derivatives. As one of them, leptodactyline possesses the pharmacological properties shown by other natural or synthetic *N*-methylated hydroxyphenylalkylamines (such as hordenine, candicine, and coryneine). In fact, leptodactyline causes both a powerful nicotinic stimulation at autonomic ganglia and the neuromuscular junction, and a considerable neuromuscular block. Muscarinic effects seem to be lacking.

All the available experimental evidence suggests that leptodactyline should be listed among the blocking agents acting by depolarization: the neuromuscular block produced by the substance is preceded and followed in mammals by muscular fasciculations, and it is antagonized by tubocurarine; the frog rectus abdominis is contracted by leptodactyline, and so is the skeletal musculature of the intact bird.

The relation between the chemical structure and the pharmacological actions of compounds like leptodactyline [trimethyl(phenylalkyl)ammonium derivatives] will be discussed in greater detail elsewhere. Here we wish only to emphasize the fact that the presence on the benzene ring of a free hydroxyl group in the *meta*-position is of decisive importance for the intensity of pharmacological effects. Ganglionic stimulation and neuromuscular block are ten to twenty times greater for leptodactyline than for (*p*-hydroxyphenethyl)trimethylammonium (candicine) and (*o*-hydroxyphenethyl)trimethylammonium. Something similar has been observed in regard to the sympathomimetic pressor activity of the three isomers of (±)-1-(hydroxyphenyl)-2-methylaminoethanol: the *meta*-isomer (synephrine) is six and eighteen times more hypertensive than the *para*- and *ortho*-isomers, respectively (Bovet and Bovet-Nitti, 1948).

The physiological significance of the occurrence of leptodactyline in *Leptodactylus* skin is obscure, as is that of the hydroxyindolyl alkylamines and catecholamines in the skin of other amphibian species. It is rather doubtful whether it can serve for defence, since leptodactyline, at least pure leptodactyline, is almost completely inactivated, and in consequence largely ineffective when administered by oral route.

It seems possible that, in common with other amphibian extracts (Vellard, 1959), extracts of *Leptodactylus* skin have also been used by the natives of South America in the preparation of some "curares."

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RECIPROCAL POTENTIATING ACTION OF DEPOLARIZING DRUGS ON THE ISOLATED FROG RECTUS ABDOMINIS MUSCLE

BY

J. H. FLEISHER, J. P. CORRIGAN, AND J. W. HOWARD

From the Biochemical Research Division, U.S. Army Chemical Warfare Laboratories, Army Chemical Center, Maryland, U.S.A.

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Subthreshold concentrations of the depolarizing agents, acetylcholine and decamethonium, potentiated isotonic contractures of the isolated frog rectus abdominis muscle elicited by effective doses of either of these drugs or by choline in the absence of the anticholinesterases, sarin, or dyflos. Comparable potentiation of the contracture produced by decamethonium or choline could be obtained by alternate stimulation with acetylcholine in the absence of dyflos or sarin. Furthermore, dyflos alone did not effect potentiation in the absence of alternate stimulation with acetylcholine. Finally, the inhibitory action of tubocurarine, as measured by depression of the contracture elicited by decamethonium and acetylcholine, was not antagonized by sarin. It was concluded that the enhancement of the response to acetylcholine produced by dyflos and by sarin was brought about exclusively by their action as inhibitors of cholinesterase. The ability of dyflos and of sarin to potentiate contractures elicited by decamethonium or by choline may be attributed to a facilitation of the action of the depolarizing compounds by subthreshold concentrations of acetylcholine available in the presence of these anticholinesterases.

Cohen and Posthumus (1955) observed that dyflos (di-isopropyl phosphorofluoridate) and sarin (isopropyl methylphosphonofluoridate) potentiate the effects of decamethonium and choline on the isolated frog rectus abdominis muscle. Because decamethonium and choline are not esters, and are therefore not hydrolysed by cholinesterase, they concluded that dyflos, and other anticholinesterases, promote a "sensitization" of acetylcholine receptors independent of any inhibition of cholinesterase activity. They further inferred that direct, non-enzymatic sensitization "is at least partly responsible for the enhanced response of the muscle toward acetylcholine after the administration of anticholinesterases." In view of the fundamental nature of their conclusions concerning the mechanism of action of anticholinesterases, a similar study on the frog rectus has been conducted in our laboratory.

METHODS

Frog Ringer Solution. — Composition: NaCl, 0.56%; KCl, 0.0075%; $CaCl_2$, 0.01%; dextrose 0.2%. The solution was buffered with 0.02 M-tris-(hydroxymethyl-amino)methane (Tris buffer) at *p*H 7.2 and was bubbled with oxygen.

Depolarizing Drugs. — Acetylcholine chloride, choline chloride, and decamethonium bromide were made up in frog Ringer solution. The test solutions of acetylcholine were freshly prepared before each experiment.

Anticholinesterase Drugs. — Dyflos (di-isopropyl phosphorofluoridate, DFP) [Merck Ampoules]. Sarin (isopropyl methylphosphonofluoridate), obtained from Chemical Research Division of Chemical Warfare Laboratories; purity was 95% or better according to an analysis of acidity. All solutions of anticholinesterases were made up freshly before use in frog Ringer solution.

Measurement of Isotonic Contracture. — The method of preparation of the isolated muscle, addition of reagents and measurement of muscle contracture have been previously described (Fleisher, Howard, and Corrigan, 1958). Responses of the muscle to any dose of a stimulating drug were repeated until successive measurements yielded an identical rate of contracture (± 1 mm./min.) before the addition of a second depolarizing substance (for example, see Table I).

Results

Effect of Subthreshold Concentrations of Depolarizing Agents upon the Contracture Produced by Addition of a Second Depolarizing Agent

A reproducible rate of contracture of the muscle was obtained to a particular dose of decamethonium to serve as a control for the response elicited by the same dose of decamethonium in the presence of a subthreshold concentration of acetylcholine. Control measurements showed no contraction following addition of subthreshold concentrations of acetylcholine, decamethonium and choline which were approximately half of the minimal effective dose.

TABLE I

POTENTIATING EFFECT OF SUBTHRESHOLD CONCENTRATIONS OF DEPOLARIZING SUBSTANCES ON THE CONTRACTURE PRODUCED BY THE ADDITION OF A SECOND DEPOLARIZING AGENT ON ISOLATED FROG RECTUS ABDOMINIS MUSCLE

Expt. No.	Dose (μg./ml.)	Contracture (mm./min.)
1	50 μg. decamethonium	20
	50 ,, ,, ...	20
	0·05 μg. acetylcholine for 1·5 min.	No contracture
	Followed by 50 μg. decamethonium	27
	0·05 μg. acetylcholine for 1.5 min.	No. contracture
	Followed by 20 μg. decamethonium	20
2	1 μg. acetylcholine	18·0
	1 ,, ,, ...	18·5
	6 μg. decamethonium for 1·5 min.	No contracture
	Followed by 1 μg. acetylcholine ..	28·5
3	1,000 μg. choline	21
	1,000 ,, ,, ...	21
	0·05 μg. acetylcholine for 1·5 min.	No contracture
	Followed by 1,000 μg. of choline	26·5
	0·05 μg. acetylcholine for 1·5 min.	No contracture
	Followed by 800 μg. of choline ..	21
4	1 μg. acetylcholine	20·5
	1 ,, ,, ...	20
	70 μg. choline for 1·5 min. ..	No contracture
	Followed by 1 μg. acetylcholine ..	23

Results shown in Table I indicate that an increased response to decamethonium may be produced by as little as 0.05 μg./ml. of acetylcholine (Expt. No. 1). Similarly the responses to acetylcholine and to choline were potentiated by the presence of subthreshold concentrations of decamethonium and of acetylcholine (Expt. Nos. 2 and 3).

There was little or no potentiation to acetylcholine in the presence of a subthreshold concentration of choline (Expt. No. 4).

Effect of Sarin on the Potentiation of Acetylcholine Produced by Decamethonium.—A constant initial response was obtained to acetylcholine and then in the presence of a subthreshold amount of decamethonium. After washing, the response to further addition of acetylcholine was elicited twice in the absence of decamethonium at the usual interval of 1 min. Table II shows that the enhancement of response disappears rapidly after the decamethonium is washed out.

Incubation with sarin 5×10^{-7} M for 1.5 hr., and testing in the presence of 10^{-7} M sarin, resulted in a ten-fold sensitization of the muscle to acetylcholine, without its response to decamethonium

TABLE II

EFFECT OF SARIN ON THE RESPONSE PRODUCED BY THE ADDITION OF ACETYLCHOLINE TO ISOLATED FROG RECTUS ABDOMINIS MUSCLE IN THE PRESENCE OF A SUBTHRESHOLD CONCENTRATION OF DECAMETHONIUM

In B the muscle was incubated with sarin, 5×10^{-7}M for 1.5 hr. followed by measurement of responses in 10^{-7}M-sarin.

	Dose (μg./ml.)	Contracture (mm./min.)
A.	Before sarin	
	1 μg. acetylcholine	22
	1 ,, ,, ...	23
	2 μg. of decamethonium for 1·5 min.	No contracture
	Followed by 1 μg. acetylcholine	32
	1 μg. acetylcholine	26
	1 ,, ,, ...	24
B.	After sarin	
	0·1 μg. acetylcholine	24
	0·1 ,, ,, ...	25
	2 μg. of decamethonium for 1·5 min.	No contracture
	Followed by 0·1 μg. acetylcholine	36
	0·1 μg. acetylcholine	20

being affected (Table II). Addition of acetylcholine to the sarin-treated muscle in the presence of previously added decamethonium showed further potentiation over and above that produced by sarin. After washing, the response elicited by acetylcholine, in the continued presence of 10^{-7} M sarin but in the absence of decamethonium, was less than the initial response after sarin.

Effect of Sarin on the Potentiation Produced by Acetylcholine Toward Choline.—The responses of the muscle to choline were observed before and after incubation of the muscle with sarin 10^{-5} M. The response was increased after incubation with sarin, but returned to the control value during the next two additions of choline to the muscle despite the continued presence of the anticholinesterase. When choline was added in the presence of a subthreshold concentration of acetylcholine, the response was comparable to that obtained immediately after incubation with sarin (Table III).

Effect of a Previous Response to Acetylcholine on the Response to Decamethonium or Choline in the Absence of and After Incubation with Anticholinesterase

In the experiments of Cohen and Posthumus (1955) the frog rectus muscle was made to contract to acetylcholine, decamethonium, and choline in that order, using frog Ringer solution to wash the tissue between contractions. After incubation with dyflos or sarin, the same procedure was repeated. By this method they observed a 1.5 to 2-fold enhancement of response towards decamethonium and a 4-fold enhancement towards choline after incubation with the anticholinesterases mentioned.

These results have been confirmed in the present experiments. However, additional experiments were performed in which contractures were also obtained by decamethonium and choline before measuring those elicited by acetylcholine. The results of representative experiments are shown in Tables IV and V. Expt. No. 1 in each table shows that the control responses obtained to decamethonium and to choline are potentiated after a contracture elicited with acetylcholine in the absence of dyflos. This potentiation was temporary, since further responses, obtained by either decamethonium or choline not preceded

TABLE III

EFFECT OF SARIN ON THE RESPONSE PRODUCED BY THE ADDITION OF CHOLINE TO ISOLATED FROG RECTUS ABDOMINIS MUSCLE. EFFECT OF A SUBTHRESHOLD CONCENTRATION OF ACETYLCHOLINE

In B the muscle was incubated with 10^{-5}M-sarin for 30 min. followed by measurement of responses in 10^{-7}M-sarin.

Dose (μg./ml.)	Contracture (mm./min.)
A. Before sarin	
1,000 μg. choline	30·0
1,000 ,, ,, ...	29·5
B. After sarin	
1,000 μg. choline	34·5
1,000 ,, ,, ...	32·5
1,000 ,, ,, ...	29·5
0·005 μg. acetylcholine for 1·5 min.	No contracture
Followed by 1,000 μg. choline	34·0
0·005 μg. acetylcholine for 1·5 min.	No contracture
Followed by 800 μg. choline ..	29·5

TABLE IV

EFFECT OF ACETYLCHOLINE ON THE CONTRACTURE PRODUCED BY DECAMETHONIUM IN THE ABSENCE OF AND AFTER INCUBATION WITH DYFLOS

In B the muscle was incubated in 10^{-5}M dyflos for one hour followed by measurement of responses in 10^{-6}M dyflos.

Expt. No.	Dose (μg./ml.)	Contracture (mm./min.)
1	Not treated with dyflos	
	21 μg. decamethonium ..	24
	21 ,, ,, ..	24
	1·25 μg. acetylcholine ..	44
	21 μg. decamethonium ..	36
	1·25 μg. acetylcholine ..	50
	10·5 μg. decamethonium ..	25
	21 ,, ,, ..	33·5
	21 ,, ,, ..	26
2	A. Before dyflos	
	21 μg. decamethonium ..	19
	21 ,, ,, ..	17
	B. After dyflos	
	21 μg. decamethonium ..	19
	21 ,, ,, ..	18
	0·15 μg. acetylcholine ..	37
	21 μg. decamethonium ..	24
	0·15 μg. acetylcholine ..	34
	14 μg. decamethonium ..	16
	21 ,, ,, ..	20·5
	21 ,, ,, ..	19

TABLE V

EFFECT OF A PREVIOUS RESPONSE TO ACETYLCHOLINE ON THE CONTRACTURE PRODUCED BY CHOLINE IN THE ABSENCE OF AND AFTER INCUBATION WITH DYFLOS

In 2B the muscle was incubated with 10^{-5}M dyflos for one hour followed by measurement of responses in 10^{-6}M dyflos.

Expt. No.	Dose (μg./ml.)	Contracture (mm./min.)
1	Not treated with dyflos	
	800 μg. choline	23
	1,000 ,, ,, ...	29
	2 μg. acetylcholine	21·5
	1,000 μg. choline	36·5
	2 μg. acetylcholine	22·5
	800 μg. choline	30
	1,000 ,, ,, ...	31·5
	1,000 ,, ,, ...	29
2	A. Before dyflos	
	1,000 μg. choline	22
	1,000 ,, ,, ...	22·5
	B. After dyflos	
	1,000 μg. choline	22·5
	0·4 μg. acetylcholine	33·5
	1,000 μg. choline	27·5
	0·4 μg. acetylcholine	32
	800 μg. choline	23
	1,000 ,, ,, ...	22

TABLE VI

EFFECT OF TUBOCURARINE UPON THE RESPONSE OF THE ISOLATED FROG RECTUS MUSCLE TO DECAMETHONIUM

In the experiments with sarin the rectus muscle was incubated for 30 min. with 10^{-5}M sarin followed by measurement of responses in 5×10^{-7}M sarin.

Expt. No.	Contracture Produced by 25 μg./ml. of Decamethonium			
	Before Sarin		With Sarin	
	Without Tubocurarine	With 2×10^{-7}M Tubocurarine	Without Tubocurarine	With 2×10^{-7}M Tubocurarine
	(mm./min.)	(mm./min.)	(mm./min.)	(mm./min).
1	18	11	18	11
2	23	14	21	13
3	19	11	20	11

TABLE VII

EFFECT OF TUBOCURARINE UPON THE RESPONSE OF THE ISOLATED FROG RECTUS MUSCLE TO ACETYLCHOLINE

In the experiments with sarin the rectus muscle was incubated for 30 min. with 10^{-5}M-sarin followed by measurement of responses in 10^{-6}M-sarin.

Expt. No.	Before Sarin			With Sarin		
	Acetylcholine Concentration (μg./ml.)		Desensitization Ratio	Acetylcholine Concentration (μg./ml.)		Desensitization Ratio
	No Tubocurarine	With 5×10^{-7}M Tubocurarine		No Tubocurarine	With 5×10^{-7}M Tubocurarine	
1	1·5	3·5	2·3	0·08	0·2	2·5
2	1·5	5·0	3·3	0·075	0·25	3·3
3	1·0	3·0	3·0	0·07	0·20	2·9
4	2·0	5·5	2·8	0·20	0·60	3·0

by a response to acetylcholine, decreased to the control value.

Expt. No. 2 in Tables IV and V shows that no potentiation over the control responses upon the addition of decamethonium and choline respectively after incubation with dyflos occurred. Subsequent measurement of a response to decamethonium or choline following a contracture elicited by acetylcholine yielded a degree of potentiation no greater than that obtained in the absence of anticholinesterase. This potentiation, like that obtained following an acetylcholine response in the absence of dyflos, faded rapidly if alternate stimulation with acetylcholine is not maintained. Similar results were obtained with other rectus muscles undergoing incubation with sarin instead of dyflos.

Effect of Sarin upon the Response to Decamethonium and Acetylcholine of the Rectus Muscle Treated with Tubocurarine, Decamethonium and Acetylcholine

A reproducible rate of contracture in response to the addition of 25 μg./ml. of decamethonium was first obtained. Then the response of the rectus muscle to the same concentration of decamethonium in the presence of 2×10^{-7} M tubocurarine was measured, followed by washing with frog Ringer solution until the initial response was again obtained. The muscle was then

incubated with 10^{-5} M sarin for 30 min. and the response to 25 μg./ml. of decamethonium again elicited in the presence of 5×10^{-7} M sarin. As in earlier experiments, the rectus muscle displayed no enhanced response after incubation with sarin. The rate of contracture to 25 μg./ml. of decamethonium was then measured in the presence of 5×10^{-7} M sarin and 2×10^{-7} M tubocurarine. If the anticholinesterase had a direct potentiating action upon receptor sites in the muscle some antagonism of the previous depression of the decamethonium response by tubocurarine, in the presence of an excess of cholinesterase inhibitor, could be expected. Table VI shows that no such antagonism was demonstrable for the rectus muscles of three frogs treated in the manner described.

In a similar experiment, the effect of sarin upon the response of curarized rectus muscle to acetylcholine was determined. The concentration of acetylcholine needed to yield a constant response was determined for each of the conditions noted in Table VII. For example, in Expt. No. 1 the presence of 5×10^{-7} M tubocurarine necessitated 2.3 times the initial concentration of 1.5 μg./ml. of acetylcholine to maintain the constant response (36 ± 1 mm./min.) obtained before treatment with sarin. The ratio of the doses required to give equal responses before and after tubocurarine is called the "desensitization ratio." After incubating with sarin, only 1/19 of the original concentration of acetylcholine (0.08 μg./ml.) was required to give the initial contracture. But after the addition of tubocurarine (5×10^{-7} M) to the medium containing the sarin, the concentration of acetylcholine required to maintain the initial contracture increased 2.5-fold over that needed in sarin alone. The other experiments shown in Table VII, carried out in the same way, agreed with Expt. No. 1—that is, they show no direct antagonism of sarin to tubocurarine over and above that due to its property of inactivating cholinesterase.

Discussion

The frog rectus abdominis muscle is believed to contain slow muscle fibres which respond to acetylcholine (and other depolarizing substances) with slow contractile changes (Brecht and Epple, 1952; Kuffler and Vaughan Williams, 1953). Such slow muscle fibre systems in frog ilio-fibularis muscle show true mechanical facilitation—that is, during a series of stimuli successive nerve volleys add increasing amounts of tension (Kuffler and Vaughan Williams, 1953).

The application of an appropriate concentration of acetylcholine (or other suitable depolarizing agent) mimics the effect of stimulating the nerve supply of such muscles (Burgen and MacIntosh, 1955). Consequently it may be postulated that the potentiation between subthreshold concentrations of one depolarizing agent and an effective concentration of another develops by a facilitatory mechanism comparable to that demonstrated for the frog ilio-fibularis muscles. This explanation would include the potentiation of the muscle response to acetylcholine by decamethonium before treatment with dyflos. It serves also to explain the other examples of potentiation developed in the presence of subthreshold concentrations of the depolarizing agents studied. In addition, the presence of anticholinesterases was found to be unnecessary for the potentiation produced by the presence of subthreshold concentrations of acetylcholine and decamethonium, as well as butyryl and succinyl choline (Fleisher, Corrigan and Howard—unpublished observations), on the response elicited by addition of effective doses of the same drugs and of choline to frog rectus muscle.

Cohen and Posthumus (1955) observed no further sensitization by decamethonium toward acetylcholine after treatment with anticholinesterases. We found a significant potentiation in the response of sarin-treated muscle to acetylcholine in the presence of a subthreshold concentration of decamethonium (Table II) over and above the increased sensitization to acetylcholine induced indirectly by inactivation of cholinesterase (Miquel, 1946). Table II also shows that the relative increase in response to acetylcholine developed in the presence of identical subthreshold concentrations of decamethonium is the same before and after sarin. If the anticholinesterase possessed the property of sensitizing the receptor sites so as to yield an enhanced response to decamethonium, then a significantly greater effect of subthreshold doses of decamethonium could have been expected after incubation with sarin. The small and temporary potentiation obtained to choline following sarin incubation (Table III) may be attributed to accumulation of endogenous acetylcholine in the presence of an anticholinesterase (Fatt and Katz, 1952).

In addition sarin showed no antagonism to tubocurarine over and above the anticurare activity derived from its anticholinesterase action. That is, the inhibitory action of tubocurarine on receptor sites as measured by its depression of the response of the rectus muscle to decamethonium

or acetylcholine was not antagonized by the cholinesterase inhibitor (Tables VI and VII). It would be expected that if sarin acted on the rectus muscle exclusively as an anticholinesterase, and tubocurarine specifically reduced the depolarizing action of applied acetylcholine (del Castillo and Katz, 1956), then the degree of desensitization to acetylcholine produced in the rectus muscle by identical partially curarizing concentrations of tubocurarine would be approximately the same with and without sarin. The results in Table VII strongly support this conclusion for the rectus muscle under the conditions tested. The direct depressant action of some anticholinesterases on receptor sites observed by other investigators has usually been found with concentrations considerably greater than those required for complete inhibition of cholinesterase activity (Eccles and MacFarlane, 1949; Kirschner and Stone, 1951; and Axelsson, Gjone, and Naess, 1957).

The consistent potentiation of the contracture of the muscle elicited by decamethonium and choline following a previous response to acetylcholine and washing, before incubation with an anticholinesterase, may be explained on the basis of a residual sensitization of receptors, or subthreshold depolarization following the response to stimulation with acetylcholine. This hypersensitivity was transient, and usually disappeared after 20 to 30 min. soaking in frog Ringer. This explanation is supported by the observation of a chemical component in slow muscle-fibre activity which can restore tension or shortening after temporary collapse (Kuffler and Vaughan Williams, 1953).

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ASSAY OF LYSERGIC ACID DIETHYLAMIDE AND ITS PASSAGE FROM BLOOD INTO THE PERFUSED CEREBRAL VENTRICLES

BY

M. DRAŠKOCI

From the National Institute for Medical Research, Mill Hill, London

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On the isolated rat uterus, lysergic acid diethylamide had an oxytocic action in a concentration of 2×10^{-8}; in smaller concentrations (10^{-9} to 10^{-10}), which had no stimulating effect of their own, it potentiated acetylcholine-induced contractions. This potentiating effect was made the basis for assaying minute amounts of lysergic acid diethylamide. The method was used to assay this substance in plasma of cats during its intravenous infusion at a rate of 10 μg./min./kg. During these infusions 0.4 to 2 ng./min. of lysergic acid diethylamide passed into the cerebral ventricles perfused with a salt solution of a composition resembling that of cerebrospinal fluid.

The present experiments show that minute doses of lysergic acid diethylamide augment acetylcholine-induced contractions of the isolated rat uterus, and this effect can provide the basis for a simple, sensitive method of assay of lysergic acid diethylamide. This method was used in experiments on cats during which lysergic acid diethylamide was infused intravenously, to measure the plasma concentration of the drug and to detect its appearance in the fluid with which the cerebral ventricles and the subarachnoidal space of the brain were perfused.

Apart from determinations of labelled lysergic acid diethylamide, only two methods have been used previously for the assay of this drug. Lanz, Cerletti and Rothlin (1955) used the inhibition which lysergic acid diethylamide exerts on the contractions of the rat uterus induced by 5-hydroxytryptamine, and they were able to assay amounts of 1 to 2 ng./ml. The disadvantage of this method derives from the persistence of the effect of the lysergic acid diethylamide so that 1.5 hr. have to elapse between two tests. Axelrod, Brady, Witkop and Evarts (1957) isolated the lysergic acid diethylamide from sodium chloride-saturated biological material by extraction into heptane. The lysergic acid diethylamide in the heptane extract was then returned to diluted HCl and estimated spectrofluorometrically. In this way, as little as 1 ng./ml. could be detected.

There are two reports on the plasma concentration of lysergic acid diethylamide, and one on its passage into the cerebrospinal fluid after single intravenous injections of this substance. Lanz *et al.* (1955) determined the plasma concentration in mice after injection of 35 mg./kg. Axelrod *et al.* (1957) injected smaller amounts in cats and monkeys. After the injection the concentration in the blood fell quickly, but, even 90 min. after an intravenous injection of 1 mg./kg. into a cat, the plasma concentration was 1.75 μg./g. At this time the cerebrospinal fluid contained 0.36 μg./g. which was only a little lower than the concentration in the brain, namely 0.52 μg./g. In a monkey a maximal concentration of nearly 50 ng./ml. was reached in the lumbar cerebrospinal fluid 10 min. after an intravenous injection of 0.2 mg./kg. lysergic acid diethylamide. The concentration in the cerebrospinal fluid then decreased slowly but remained about the same as that found for the unbound form of this substance in plasma. Axelrod *et al.* (1957) concluded that there was little hindrance to the passage of lysergic acid diethylamide across the blood brain barrier.

METHODS

Isolated Rat Uterus.—Albino rats weighing about 200 g. were injected intramuscularly with 1 mg./kg. stilboestrol 24 hr. before they were killed, and one uterine horn was suspended in a bath containing 5 ml. of the solution which was used by Gaddum, Peart and Vogt (1949) for the assay of adrenaline on the rat uterus. This solution contained NaCl 9, KCl 0.42, $CaCl_2$ 0.06, $NaHCO_3$ 0.50 and dextrose 0.5 g./l. of distilled water. The bath fluid was bubbled with oxygen and was maintained at 30°. The quantities of acetylcholine refer to the chloride.

Cat Experiments.—These experiments were carried out on cats which weighed 2.3 to 2.5 kg. and which were anaesthetized by the intraperitoneal injection of 35 mg./kg. of pentobarbitone sodium. The trachea was cannulated and artificial ventilation was usually administered during the experiment. The arterial blood pressure was recorded from the right femoral artery. The cerebral ventricles were perfused from a cannula implanted into the left lateral ventricle to either the cannulated cisterna or the cannulated aqueduct as described by Bhattacharya and Feldberg (1958). The solution used for perfusion was that introduced by Merlis (1940); it resembles normal cerebrospinal fluid and contains NaCl 8.1, KCl 0.25, $CaCl_2$ 0.14, $MgCl_2$ 0.11, $NaHCO_3$ 1.76, Na_2HPO_4 0.07, urea 0.13 and glucose 0.61 g./l. The rate of infusion was 0.1 ml./min. and the effluent was collected for 30 min. or 1 hr. These samples were kept at 4° until tested on the rat uterus, usually on the same day. Lysergic acid diethylamide was infused at a rate of 0.2 ml./min. into the left femoral vein using a continuous slow injector (C. F. Palmer, Ltd.). For each experiment a fresh solution of lysergic acid diethylamide in saline was prepared containing in 0.2 ml. 10 μg./kg. To estimate the lysergic acid diethylamide content of blood, 2 ml. samples of arterial blood were removed from the left femoral artery with a siliconed glass syringe, brought into a siliconed test-tube containing a small amount of heparin, and centrifuged. The plasma was removed and kept in siliconed glass vessels until diluted and tested on the rat uterus.

Results

Lysergic Acid Diethylamide on Rat Uterus

Two effects of lysergic acid diethylamide were obtained on the rat uterus. The drug sensitized the preparation to the action of acetylcholine and, in larger doses, it caused rhythmic contractions. The sensitizing effect on the acetylcholine contractions was usually obtained with 0.5 ng. to 1 ng./ml. but sometimes with as little as 0.1 ng./ml. In these experiments, acetylcholine was given every 2 min. and kept in the bath for 30 sec. The doses used varied between 0.03 and 0.05 μg./ml. and gave threshold responses. Two procedures were used for adding lysergic acid diethylamide. In the first, the same amount of lysergic acid diethylamide was given twice after washing out

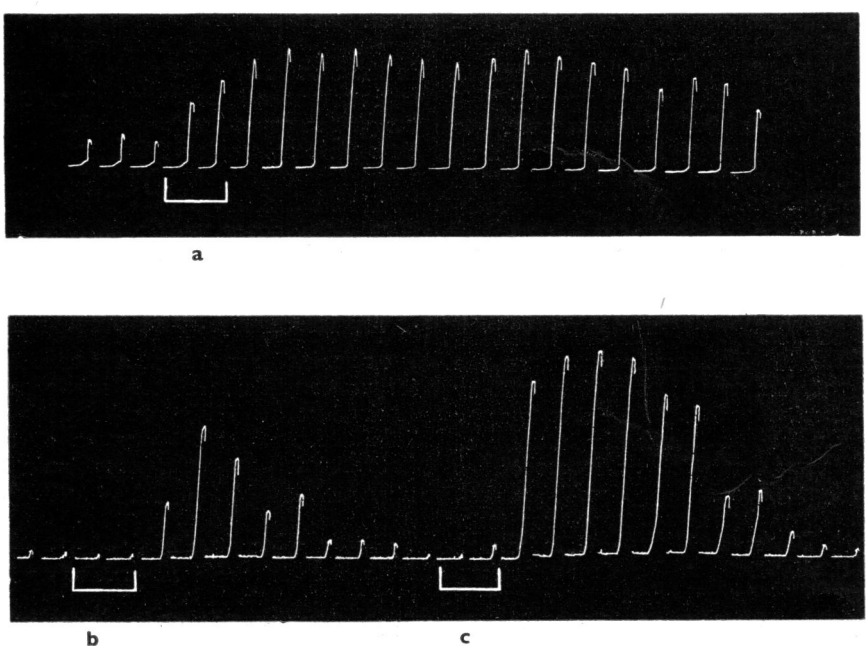

FIG. 1.—Records of contractions of the rat uterus suspended in 5 ml. bath in response to acetylcholine. Upper tracing : the effect of 0.4 ng./ml. of lysergic acid diethylamide (at **a**) on contractions produced by 0.05 μg./ml. of acetylcholine. Lower tracing: a record made with another uterus showing effect of 0.5 ng./ml. (at **b**) and of 1 ng./ml. (at **c**) of lysergic acid diethylamide on contractions produced by 0.036 μg./ml. of acetylcholine. For details about timing, see text.

two successive doses of acetylcholine, so that the time of contact of the lysergic acid diethylamide with the preparation was about 4 min. In the second, lysergic acid diethylamide was kept in the bath for 5 min. during which time no acetylcholine was given. Then after 5 min. acetylcholine was added to the bath without washing. Both drugs were washed out 30 sec. later, and the testing for acetylcholine was continued.

Fig. 1 (upper tracing) shows the long-lasting sensitizing effect of 0.4 ng./ml. of lysergic acid diethylamide (at **a**) on small contractions produced by 0.05 μg./ml. of acetylcholine using the first procedure. The lower record was obtained from another preparation and illustrates the sensitizing effect of 0.5 and 1 ng./ml. of lysergic acid diethylamide (at **b** and **c** respectively) on threshold contractions produced by 0.036 μg./ml. of acetylcholine. The effect of 1 ng./ml. was more pronounced and longer lasting than that of 0.5 ng./ml. of lysergic acid diethylamide.

The second procedure was used in the experiment shown in Fig. 2. The sensitization of the response to 0.04 μg./ml. of acetylcholine increased with increasing doses of lysergic acid diethylamide ; at **a**, 1, and at **b**, 2 ng./ml. respectively were added to the bath for 5.5 min. When the dose of lysergic acid diethylamide was increased to 20 ng./ml. (at **c**), there was not only a further augmentation of the acetylcholine responses but also rhythmic contractions appeared. This latter response is shown in the lower record. The rhythmic contractions started in the 5-min. period at **c** during which 20 ng./ml. of lysergic acid diethylamide was kept in the bath and increased during the subsequent 15 min., then gradually decreased but were still present at the end of the record about 1 hr. after washing out the lysergic acid diethyl-

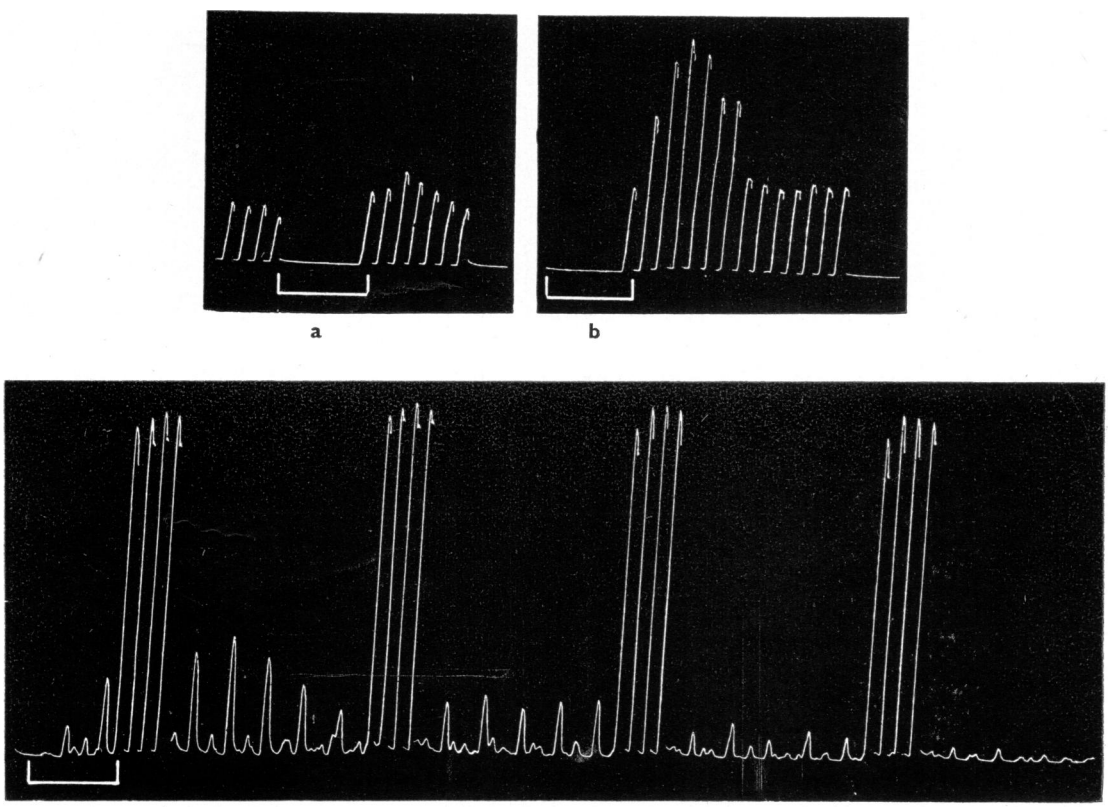

FIG. 2.—Records of the contractions of rat uterus suspended in 5 ml. bath in response to 0.04 μg./ml. of acetylcholine. At **a**, 1 ng./ml. ; at **b**, 2 ng./ml. ; and at **c**, 20 ng./ml. of lysergic acid diethylamide added to the bath for 5.5 min. In the lower tracing, the testing with acetylcholine was discontinued after each group of four acetylcholine contractions for periods of about 15 min. during which the rhythmic activity is seen.

amide. The augmentation of the responses to 0.04 μg./ml. of acetylcholine by this concentration of lysergic acid diethylamide is shown by four groups of four acetylcholine contractions. Between each group the testing of acetylcholine was discontinued for about 15 min. in order to show the rhythmic contractions. It will be seen that the augmentation of the acetylcholine responses persisted for at least 1 hr.

The potentiating effect of minute doses of lysergic acid diethylamide on the acetylcholine responses can be used as a method for the assay of lysergic acid diethylamide. Two saline solutions, which contained 260 and 600 ng./ml. lysergic acid diethylamide, when assayed without knowing their lysergic acid diethylamide contents gave estimated values of 230 and 670 ng./ml. respectively.

Since plasma influenced the acetylcholine responses of the rat uterus this fact had to be taken into account when assaying lysergic acid diethylamide in plasma. When cat plasma, which had been collected in siliconed glass tubes containing a small amount of heparin, was added in volumes of 0.1 ml. to the 5 ml. bath, the acetylcholine responses increased. Sometimes the augmentation was slight, sometimes it was pronounced, but it always differed from the effect of lysergic acid diethylamide in that the augmentation persisted only for as long as the plasma remained in the bath. When the preparation had been repeatedly treated with small doses of lysergic acid diethylamide, plasma sometimes produced its effect in volumes smaller than 0.1 ml. In one experiment, a strong effect was obtained with as little as 0.005 ml. (Fig. 3 at **b**). This effect of

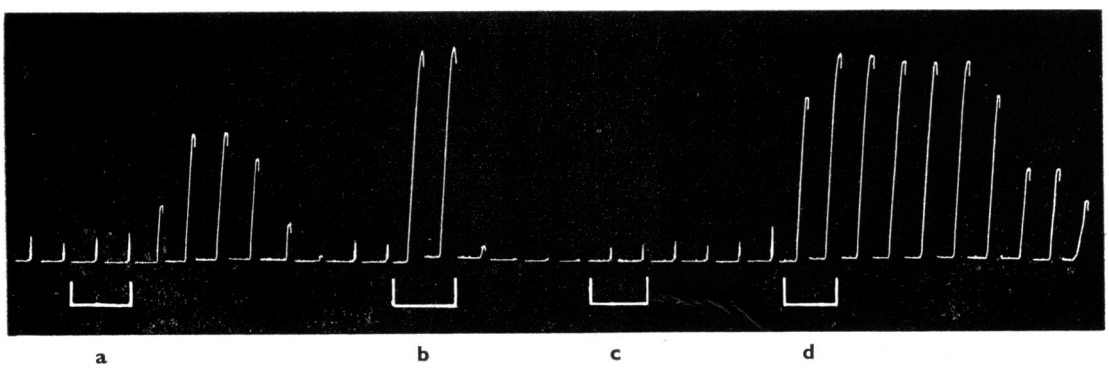

FIG. 3.—Record of the contractions of rat uterus suspended in 5 ml. bath in response to 0.036 μg./ml. of acetylcholine. At **a**, the effect of 0.5 ng./ml. of lysergic acid diethylamide. At **b**, 0.005 ml. of normal cat plasma was added to the 5 ml. bath. At **c** and **d**, 0.00125 ml. and 0.0025 ml. respectively of plasma obtained after an intravenous infusion into the cat of 10 μg./min./kg. of lysergic acid diethylamide. For details of timing, see text.

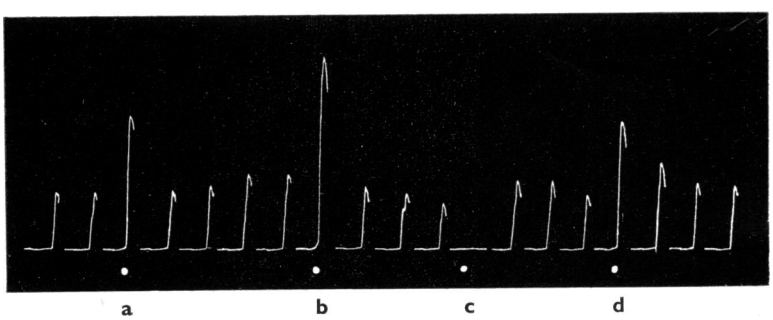

FIG. 4.—Records of the contractions of rat uterus suspended in 5 ml. bath in response to 0.025 μg./ml. of acetylcholine. At the white dots 0.1 ng./ml. (at **a** and **d**) and 0.15 ng./ml. (at **b**) of 5-hydroxytryptamine were added to the bath 30 sec. before the acetylcholine. At the white dot at **c**, 0.2 ng./ml. of 5-hydroxytryptamine given alone. For details of timing, see text.

plasma never interfered with the assay of plasma samples obtained after intravenous infusion of lysergic acid diethylamide even if the amounts of plasma that had to be added to the bath were sufficiently large to produce the short lasting augmentation. In the assay shown in Fig. 3 the volumes of plasma required were, however, so small that they did not sensitize the preparation. The effect of 0.00125 ml. of this plasma was less and that of 0.0025 ml. was more than that of 2.5 ng. of lysergic acid diethylamide which was tested before and after (not shown in Fig. 3) the plasma samples. Thus 1 ml. of this plasma contained more than 1 and less than 2 ng. of lysergic acid diethylamide.

5-Hydroxytryptamine caused augmentation of the response of the rat uterus to acetylcholine in doses too small to contract the uterus, namely 0.1 to 0.2 ng./ml. As shown in Fig. 4, the augmentation, like that produced by plasma occurred only whilst the 5-hydroxytryptamine was present in the bath. The synergism between 5-hydroxytryptamine and acetylcholine on the rat uterus has recently been observed by Barlow and Khan (1959). The augmentation produced by plasma cannot be due to 5-hydroxytryptamine since the effect of 5-hydroxytryptamine was abolished by lysergic acid diethylamide whereas that of plasma was often more pronounced after treatment of the uterus with small doses of lysergic acid diethylamide.

Intravenous Infusion of Lysergic Acid Diethylamide into Cats

An intravenous infusion of 10 μg./min./kg. of lysergic acid diethylamide into an anaesthetized and artificially-ventilated cat caused, within 10 to 20 min., a gradually increasing dilatation of the pupils; within 1 to 2 hr. the dilatation became maximal. In one cat in which the nictitating membranes were fully relaxed before the lysergic acid diethylamide infusion, they were gradually retracted during the first 10 min. of the infusion. The infusion of lysergic acid diethylamide caused a gradually-developing fall in arterial blood pressure of 20 to 60 mm. during 1 hr. and some slowing of the heart rate. These are well known effects of lysergic acid diethylamide observed on intravenous injection of single doses (Rothlin, 1957).

Plasma Concentration of Lysergic Acid Diethylamide.—In five cats the plasma of samples of blood removed after 1 or 2 hr. intravenous infusion of 10 μg./min./kg. of lysergic acid diethylamide contained between 1 and 2 μg./ml.

TABLE 1

CONCENTRATION OF LYSERGIC ACID DIETHYLAMIDE IN PLASMA AND ITS OUTPUT FROM THE PERFUSED CEREBRAL VENTRICLES DURING ITS INTRAVENOUS INFUSION INTO A CAT

The bold numerals refer to the output into aqueductal, the other numerals to the output into cisternal effluent. Lysergic acid diethylamide was infused intravenously at 10 μg./min./kg.

Expt. No.	Plasma Concentration (μg./ml.) After				Output (ng./min.) During			
	1st hr.	2nd hr.	3rd hr.	4th hr.	1st hr.	2nd hr.	3rd hr.	4th hr.
1	2	2			0·6	1·2		
2	1·5	1·3			>0·9	1·5		
3	1·5	1·8			**>0·5**	**0·4**		
4	1·5	2			**1·9**	**1·4**		
5	1				**0·2**	**0·4**		
6	—	4·5	5	5	0·5	0·9	**0·8**	**<1·0**

of lysergic acid diethylamide. In one cat in which blood samples were removed 2, 3 and 4 hr. after the infusion of lysergic acid diethylamide, the plasma concentration varied between 4.5 and 5 μg./ml. These results are shown in Table I.

Appearance of Lysergic Acid Diethylamide in Cisternal and Aqueductal Effluent.—During the intravenous infusion of 10 μg./min./kg. of lysergic acid diethylamide, the substance appeared in the fluid with which the cerebral ventricles and the subarachnoidal space of the brain were perfused. There was no obvious difference in the output of lysergic acid diethylamide whether perfusion was from the lateral ventricle to the cisterna or from the lateral ventricle to the aqueduct. The output varied from 0.4 to 1.9 ng./min. This is shown in Table I. In Expt. 6 in Table I, perfusion was for the first 2 hr. from the lateral ventricle to the cisterna. The lysergic acid diethylamide output was 0.5 ng./min. during the first and 0.9 ng./min. during the second hour. The subarachnoidal space was then excluded from the perfusion by collecting the outflow from the cannulated aqueduct. During the first hour the output of lysergic acid diethylamide in the aqueductal effluent fell slightly to 0.8 ng./min. but rose in the second hour to over 1 ng./min.

These results suggest that all or nearly all the lysergic acid diethylamide in the cisternal effluent had entered the perfusion fluid on its passage through the lateral and third ventricles. It is

doubtful whether there is also a direct passage of lysergic acid diethylamide from the blood into the subarachnoidal space.

Discussion

An oxytocic effect of lysergic acid diethylamide has so far apparently been observed only on the rabbit uterus, on which the effect is slightly weaker than that of ergometrine (Rothlin and Cerletti, 1956; Rothlin, 1957). The present experiments show that lysergic acid diethylamide, in a concentration of 2×10^{-8}, had an oxytocic action also on the isolated rat uterus. In even smaller concentrations (10^{-9} to 10^{-10}), in which it had no direct stimulating effect, it potentiated the contractions produced by acetylcholine. Although lysergic acid diethylamide inhibits pseudocholinesterase (Thompson, Tickner, and Webster, 1955), it is unlikely that the potentiation can be explained by this property since the rat pseudocholinesterase is relatively insensitive to lysergic acid diethylamide and the pseudocholinesterase plays little or no part in the destruction of acetylcholine by living tissue (Shelley, 1955; Admiraal, Myers, and Houten, 1955).

The potentiating effect on the acetylcholine contractions provides a simple and sensitive method for the assay of minute amounts of lysergic acid diethylamide. Using this method of assay it could be established that lysergic acid diethylamide can pass from the blood into the fluid perfusing the cerebral ventricles during an intravenous infusion of this substance.

In previous experiments (Draškoci, Feldberg, and Haranath, 1959) the passage of adrenaline into the perfused cerebral ventricles has been examined under similar experimental conditions but with the intravenous infusion of larger amounts of adrenaline (40 μg./min./kg.). As far as it is possible to compare the results obtained with these two substances infused in different concentrations, there seems to be no great quantitative difference in the passage from the blood into the perfused cerebral ventricles. Adrenaline passed also into the perfused subarachnoidal space, and in fact in greater amounts than into the perfused cerebral ventricles. This was particularly evident from the experiments in which various parts of the subarachnoidal space were perfused without the cerebral ventricles. For lysergic acid diethylamide, no definite evidence of its direct passage from the blood into the subarachnoidal space was found, but no experiments were carried out with parts of the subarachnoidal space separately perfused during its intravenous infusion. It is therefore not possible to say whether the lysergic acid diethylamide, which Axelrod *et al.* (1957) detected in the lumbar cerebrospinal fluid of monkeys after an intravenous injection of this substance, had entered these spaces directly from the blood or by way of the cerebral ventricles.

I am grateful to Sir Charles Harington for granting me the opportunity of working in the National Institute for Medical Research, and to Dr. W. Feldberg for his advice and encouragement. The lysergic acid diethylamide used in these experiments was a gift from Messrs. Sandoz.

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Brit. J. Pharmacol. (1960), **15**, 35.

THE EFFECT OF IPRONIAZID AND IMIPRAMINE ON THE BLOOD PLATELET 5-HYDROXYTRYPTAMINE LEVEL IN MAN

BY

ELIZABETH F. MARSHALL, G. S. STIRLING, A. C. TAIT AND A. TODRICK

From the Department of Clinical Research, Crichton Royal, Dumfries

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Observations are reported on the blood platelet 5-hydroxytryptamine content of six patients receiving imipramine, *N*-(γ-dimethylaminopropyl)-iminodibenzyl hydrochloride. The response was a fall to a level of one-sixth of the original in three weeks, with little change thereafter. This is in sharp contrast to the action of iproniazid which caused a rise of some 200% in the blood platelet 5-hydroxytryptamine level over the same period. Imipramine in a concentration of 1 mg./ml. had no inhibitory action on 5-hydroxytryptophan decarboxylase; 8·0 μg./ml. of imipramine suppressed two-thirds of the *in vitro* uptake of 5-hydroxytryptamine (2·5 μg./ml.) by normal human platelets.

The recently developed pharmacological approach to the treatment of disordered mental states, particularly schizophrenia, with the so-called tranquillizing or ataractic drugs has undoubtedly achieved results (Brill and Patton, 1957), although the individual response is unpredictable and the mode of action is still not clear. Nevertheless, the degree of success has been sufficient to cause the adoption of a similar approach to the treatment of the other major functional psychosis, the manic-depressive syndrome. Of the two aspects of this illness, the depressive is the more frequent in the ratio of 10 to 1 and therefore calls for more urgent attack.

Iproniazid, developed for the treatment of tuberculosis, was among the first drugs to achieve improvement of mood in psychotic depression; it has been known for some time as a powerful inhibitor of amine oxidase (Zeller, Barsky, Fouts, Kircheimer and van Orden, 1952). Imipramine, *N*-(γ-dimethylaminopropyl)-iminodibenzyl hydrochloride (G.22355, Tofranil, Geigy), has more recently been claimed to be of value in the treatment of typical endogenous depressions (Kuhn, 1957, 1958; Kielholz and Battegay, 1958).

The knowledge that iproniazid was an inhibitor of amine oxidase suggested that it might cause an increase in 5-hydroxytryptamine *in vivo*, and when trials of this drug were instituted it was decided to follow the blood platelet 5-hydroxytryptamine levels. Pletscher and Bernstein (1958) have since shown that the expected increase does in fact occur. Imipramine was similarly studied to determine whether it also caused a rise of blood platelet 5-hydroxytryptamine.

METHODS

Blood was withdrawn from an arm vein into a siliconed 10 ml. syringe through a No. 20G needle. The needle was removed and 9 ml. of the blood transferred to a siliconed centrifuge tube containing 1.0 ml. of a 1.0% solution of disodium diaminoethanetetraacetate and 0.7% NaCl and mixed by slow inversion. After removal of blood for a platelet count, the tube was transferred to a chilled 15 ml. centrifuge bucket. This was prepared by placing the 15 ml. bucket without its rubber cushion inside a 50 ml. bucket containing its rubber cushion, filling the intermediate space with water, and storing the combination in a deep freeze cabinet (−15°) until required; the rubber cushion of the 15 ml. bucket was replaced immediately before use. The blood was centrifuged for 20 min. at 150 *g*. The platelet-rich plasma was withdrawn into a siliconed pointed centrifuge tube and centrifuged in a chilled bucket for 15 min. at 2,000 *g*. The button of platelets was freed from supernatant plasma and suspended in 7 ml. of physiological saline, care being taken to obtain a uniform suspension. A sample was taken for a platelet count from this suspension, which was then frozen to −15°.

Platelet counts were carried out by the method of Baar (1948). The recovery of platelets averaged 72%.

Estimation of 5-Hydroxytryptamine.—Estimations were carried out in duplicate on 3 ml. portions of the saline suspension by a fluorimetric method based on that of Udenfriend, Weissbach and Clark (1955), as modified by Brodie, Tomich, Kuntzman and Shore

(1957). In this method the suspension is brought to *p*H 10 with the addition of borate buffer, and shaken with *n*-butanol; heptane is then added to the butanol solution and the 5-hydroxytryptamine extracted from the solvent mixture into 0.1 N HCl. It is estimated in the Aminco-Bowman Spectrophotofluorimeter using exciting light of wavelength 295 mμ and measuring the fluorescence in the range 330 to 340 mμ.

The Locarte fluorimeter, Pattern LMF/2 (Laurence, 1957), does not incorporate monochromators, and filters had to be employed. Since it seemed likely to be difficult to obtain filters giving good transmission of the fluorescence with complete cut-off of the exciting light, use was made of the fact that the fluorescence shifts into the visible with a peak at 550 mμ if the 5-hydroxytryptamine solution is made strongly acid (Udenfriend, Bogdanski and Weissbach, 1955). The filters used have been, on the primary side, Chance OX7+2 cm. thickness of $NiSO_4$ solution (437.5 g./l.), and, on the secondary side, Chance OY3.

Other modifications have involved the retention of the buffer and solvent volumes given in the original paper of Udenfriend *et al.* (1955b) and the use of 0.5 M formate buffer *p*H 4 for the final extraction. There was a considerable reagent blank, equivalent to five to ten times the fluorescence from the original formate buffer; this appeared to be due to butanol. The procedure adopted to reduce this interference to a minimum was to measure the fluorescence of the formate buffer extracts against a reagent blank prepared by passing water through the extraction procedures. To the solutions, volume about 1.5 ml., 0.1 ml. of concentrated HCl was then added. This addition caused some reduction of the reagent blank fluorescence, which was brought back to the previous scale reading by increasing the sensitivity of the instrument. The standard and unknown 5-hydroxytryptamine fluorescences were then measured, the net values being obtained by deducting the readings obtained before adding HCl. A linear relationship between scale reading and 5-hydroxytryptamine concentration was thus obtained. Each group of estimations carried out included pairs of reagent blanks and 5-hydroxytryptamine standards. In order to reduce systematic errors, the duplicates from one platelet suspension were always estimated in separate groups.

Fluorimetric methods of estimation of 5-hydroxytryptamine have come in for criticism, particularly in relation to brain tissue. A comparison of the results obtained by the technique described above with those of other workers is therefore given in Table I.

The mean value for normal subjects obtained with the simple fluorimetric method is practically identical with the mean value obtained with the spectrophotofluorimeter. They are 25% higher than the mean value obtained by the bioassay technique; the difference, though probably significant, is not large and should not lead to grave errors in serial studies.

The Effect of Imipramine on the Estimation of 5-Hydroxytryptamine.—Another source of error may be the presence of imipramine in the blood. It was early noted that, in mixed aqueous solutions, 3 μg./ml. of imipramine could make the fluorimetric estimation of 5-hydroxytryptamine impossible, since imipramine itself fluoresced at *p*H 4 and the fluorescence disappeared on adding HCl. Herrmann and Pulver (personal communication) found that the blood imipramine concentration in animals receiving higher than therapeutic doses was not measurable by the only method available, that is, it was less than 1 μg./ml.

However, even the simplified procedure involves two steps, (1) the separation of the platelets from the blood and (2) the butanol extraction. In Table II, figures are given for the effect of varying concentrations of imipramine on the estimation of 5-hydroxytryptamine in an aqueous mixture of the two compounds subjected to butanol extraction. The

TABLE I

NORMAL BLOOD PLATELET 5-HYDROXYTRYPTAMINE LEVELS: COMPARISON OF RESULTS OF DIFFERENT WORKERS AND METHODS

Figures in brackets are standard deviations, except those marked with an asterisk, which are limits including 95% of observations.

Authors	Method	Description of Subjects	Number	Mean Platelet 5-Hydroxytryptamine of Blood (μg./ml.)
Feldstein, Hoagland, and Freeman (1959)	Spectrophoto-fluorimeter	Normal, male	16	0·19 (±0·08)
Hardisty and Stacey (1955)	Bioassay: rat uterus	Normal, male and female	35	0·16 (±0·06)
This paper	Filter fluorimeter	Normal, male	20	0·20 (±0·05)
Weiner and Udenfriend (1957)	Spectrophoto-fluorimeter	General hospital patients, representative selection, sex unspecified	94	0·23 (0·05–0·49)*
This paper	Filter fluorimeter	Mental hospital patients, male	86	0·24 (0·07–0·50)*

TABLE II

THE EFFECT OF IMIPRAMINE ON THE ESTIMATION OF 5-HYDROXYTRYPTAMINE EXTRACTED FROM AQUEOUS MIXTURES

Aqueous 5-hydroxytryptamine solutions (3·0 ml. samples) were extracted by standard procedure. Percentage recovery was based on parallel extracts of solutions in the absence of imipramine. Figures are means of four estimates, and refer to free base.

5-Hydroxytryptamine Concentration (μg./ml.)	Imipramine Concentration (μg./ml.)	5-Hydroxytryptamine as Percentage of that Extracted from a Solution Containing no Imipramine
0·144	7·5	101
0·144	3·8	100
0·144	1·9	95
0·036	30·0	17
0·036	7·5	85
0·036	1·9	89

higher of the two concentrations of 5-hydroxytryptamine employed is 75% of the normal mean level for platelet suspensions, the lower about 20%. Somewhat surprisingly, since imipramine is a base, the procedure appears to remove it, at any rate up to a concentration eight times the suggested upper limit in blood.

If the imipramine in the blood were equally distributed between the various components, the separation of the platelets, first from the erythrocytes and subsequently from the plasma, would reduce the concentration of imipramine in the saline suspension to one-four-hundredth of that in the original blood. However, since platelets are known to absorb amines, it did not appear wise to make this assumption. Therefore, in experiments on the effect of imipramine on the uptake of 5-hydroxytryptamine by normal platelets, a control was added to give a figure for the effect of imipramine on the estimation of endogenous 5-hydroxytryptamine from platelets. The results are summarized in Table III; they show that imipramine did not interfere with the estimation of 5-hydroxytryptamine in platelets.

Uptake of Exogenous 5-Hydroxytryptamine by Platelets.—The experimental technique was based on that of Born, Ingram, and Stacey (1958). To reduce natural variation, blood for this series of experiments was drawn only from four normal volunteers and no individual's platelets were subjected to the same set of experimental conditions twice. A volume of 22 ml. of blood was taken as previously described and mixed with 2 ml. of anticoagulant; of the platelet-rich plasma, isolated in the standard manner, 1.5 ml. was added to each of four siliconed pointed centrifuge tubes containing 0.5 ml. saline, and either imipramine,

TABLE III

THE EFFECT OF IMIPRAMINE ON THE ESTIMATION OF 5-HYDROXYTRYPTAMINE IN PLATELETS

Three parts of platelet-rich plasma were mixed with one part of saline suspension or drug in saline suspension; mixtures were incubated for 60 min. at 37°. Estimations were in duplicate on each mixture. Imipramine concentrations are as free base; 5-hydroxytryptamine percentages are means.

Imipramine Concentration (μg./ml.)	Number of Experiments	5-Hydroxytryptamine in Presence of Imipramine (Expressed as % 5-Hydroxytryptamine Estimated in Absence of Imipramine)
0·44	3	93
1·77	6	115
7·10	2	105

5-hydroxytryptamine or 5-hydroxytryptamine+imipramine. The final concentrations of imipramine used were 0.5, 2.0, and 8.0 μg./ml.; the 5-hydroxytryptamine concentration was 2.5 μg./ml. except in one set of experiments, where it was 10.0 μg./ml. The final dilutions of the compounds were in saline. After mixing carefully but thoroughly a sample was withdrawn for a platelet count. The tubes were loosely covered and placed vertically in a thermostat at 37° for 60 min., being shaken at sixty double oscillations (6 cm. amplitude) per min. They were then centrifuged under the standard conditions. Since 5-hydroxytryptamine was present in two of the tubes the methods described by Born *et al.* (1958) for the removal of as much plasma as possible were used; the platelets were then resuspended in 5 ml. of saline of which 2 ml. portions were taken for duplicate estimations of 5-hydroxytryptamine. A sample for a platelet count was taken immediately after resuspension.

Effect of Imipramine on 5-Hydroxytryptophan Decarboxylase. — The manometric technique of Davison and Sandler (1958) was employed.

Liver Function Tests.—Determinations of serum bilirubin, thymol turbidity, and alkaline phosphatase activity were carried out by standard methods (King and Wootton, 1956).

RESULTS

Except where otherwise stated, this study refers to male patients who were receiving either imipramine or iproniazid for therapeutic purposes.

Effect of Iproniazid on Platelet 5-Hydroxytryptamine.—Four patients received this drug in tablet form; the dose was raised over the course of 4 days to 225 mg./day. Two patients were taken off the drug after 11 and 13 days; the other two continued to receive it for 33 and 38 days.

The response of the blood platelet 5-hydroxytryptamine level to the drug, calculated as the percentage change from the mean for the pre-treatment values, is given in Fig. 1. Where drug treatment continued for an adequate length of time increases of 200% and upwards were observed. On stopping the drug a return to normal levels occurred.

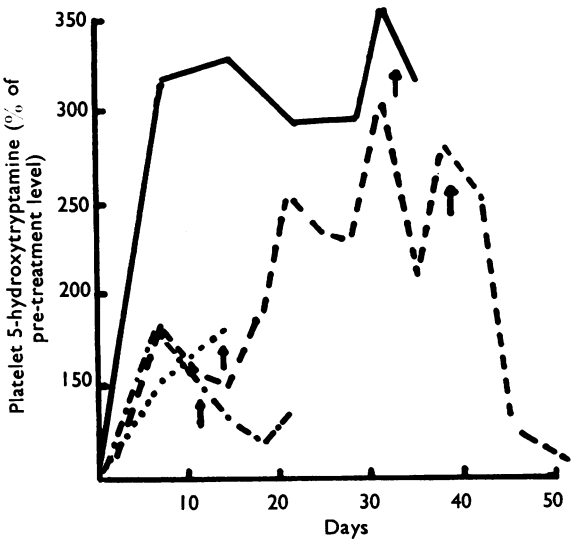

FIG. 1.—The effect of iproniazid on platelet 5-hydroxytryptamine in man. Each line represents one patient. Arrows indicate termination of treatment.

Effect of Imipramine on Platelet 5-Hydroxytryptamine.—Six patients received a standard course of imipramine hydrochloride, commencing with the parenteral therapy recommended for severe cases of depression but passing over to oral therapy at the maximum level of 300 mg./day by the 9th day. The detailed dosage schedule is given in Table IV. Subsequently the dose was adjusted to meet the patients' requirements and ranged from 150 to 300 mg./day.

There was no great variation in response between individuals when this was calculated in terms of the percentage change from the pre-treatment level. The mean values have therefore been given (Fig. 2). Imipramine caused a fall in platelet 5-hydroxytryptamine to 17% of the pre-treatment level after 3 weeks. There was no further significant change up to 6 weeks. A sharp rise in one patient to 50% of the pre-treatment level after 6 weeks was subsequently found to correlate with the patient's refusal to take the drug over a period of 10 days.

TABLE IV

DAILY DOSAGE SCHEDULE FOR IMIPRAMINE

Figures refer to mg. of imipramine hydrochloride. The total daily doses were distributed between three administrations.

Day	Injection mg.	Oral mg.	Total mg.
1	75	0	75
2	100	0	100
3	125	0	125
4	100	50	150
5	75	100	175
6	50	150	200
7	25	200	225
8	0	250	250
9	0	300	300

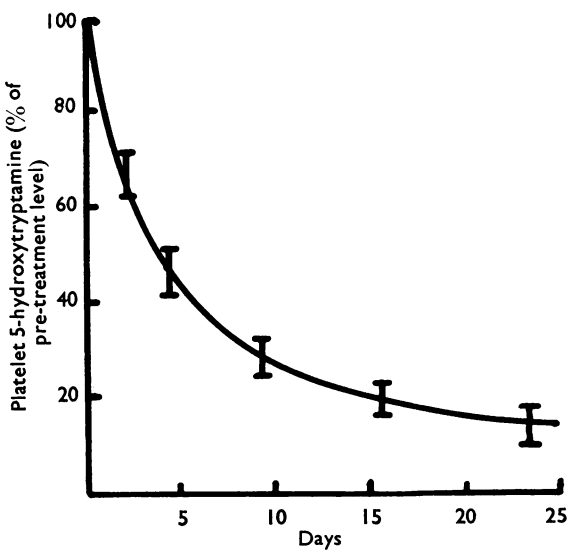

FIG. 2.—The effect of imipramine on platelet 5-hydroxytryptamine in man. Points represent mean percentage of pre-treatment level for six patients, with standard deviations of means.

Observations on the Mode of Action of Imipramine.—Investigation of the action of imipramine on 5-hydroxytryptophan decarboxylase gave negative results: there was no measurable inhibition of enzyme activity at an imipramine concentration of 3×10^{-3} M (1 mg./ml.).

In one patient, the level of excreted 5-hydroxyindoleacetic acid was investigated during a course of therapy. There was no fall in 5-hydroxyindoleacetic acid excretion over a

period of 3 weeks. It has recently been pointed out, however (Milne, 1959), that a fall in the level of excretion is not conclusive proof of decreased synthesis but may also be due to reduced urinary clearance.

In preliminary observations, comparison of the uptake of 5-hydroxytryptamine by normal platelets and by those from patients who had been on continuous imipramine therapy gave equivocal results. It was therefore decided to study the effect of imipramine on normal platelets *in vitro* in the first instance, in order to permit adequate controls with known concentrations of the compounds to be made.

The results of this series of experiments are given in Table V. The uptake of 5-hydroxytryptamine from a diluted plasma containing 2.5 μg./ml. averaged about 300% of the endogenous platelet level. An imipramine concentration of 8.0 μg./ml. reduced the uptake to one-third, and even 0.5 μg./ml. appeared to cause some inhibition. A four-fold increase in 5-hydroxytryptamine concentration markedly reduced the extent of the inhibition, though it raised the uninhibited uptake of 5-hydroxytryptamine by only 40%.

Liver Function Tests on Patients Receiving Iproniazid and Imipramine.—A frequent check is kept on the possibility of liver damage in patients receiving iproniazid (Pare and Sandler, 1959). Liver function tests were done not on the six male patients receiving imipramine but on nine female patients receiving much the same dosage. The results are summarized in Table VI. The percentage of results of these tests above the recognized normal upper limits (Varley, 1954) averaged 22% for iproniazid and 4% for

TABLE V

THE UPTAKE OF 5-HYDROXYTRYPTAMINE BY HUMAN PLATELETS IN THE PRESENCE OF IMIPRAMINE

Platelet-rich plasma (1·5 ml.) was added to 5-hydroxytryptamine and imipramine in saline (0·5 ml.) and incubated for 60 min. at 37° with shaking (60 × 6 cm. double oscillations per min.). Initial 5-hydroxytryptamine contents are the means of values for saline controls and imipramine controls, as in Table III. Drug concentrations are as free base.

5-Hydroxytryptamine Concentration (μg./ml.)	Imipramine Concentration (μg./ml.)	5-Hydroxytryptamine Content (ng./10^8 Platelets)			Percentage Inhibition of Uptake Due to Imipramine
		Initial	After Incubation without Imipramine	After Incubation with Imipramine	
1·1	7·1	82	307	169	61
		40	160	76	70
		90	314	172	63
					Mean 65
1.1	1·77	79	283	168	56
		74	266	177	46
		123	291	200	54
					Mean 52
1·1	0·44	62	229	222	4
		48	361	173	60
		72	271	224	24
					Mean 29
4·3	1·77	56	305	273	13
		69	385	308	24
		115	399	428	−11
					Mean 9

TABLE VI

RESULTS OF LIVER FUNCTION TESTS ON PATIENTS RECEIVING IPRONIAZID AND IMIPRAMINE

Test	Percentage of Tests Giving Figures above Recognized Upper Limit of Normal for Bed Patients		Percentage of Tests Giving Figures Higher than: Bilirubin, 0·5; Thymol Turbidity, 2·0; Alkaline Phosphatase, 8·0	
	Iproniazid	Imipramine	Iproniazid	Imipramine
Bilirubin	17	0	35	15
Thymol turbidity	17	0	92	0
Alkaline phosphatase	33	12	54	19
All tests	22	4	60	11

imipramine. These limits refer to bed patients; the upper limits for normal individuals (and possibly for ambulant mental patients) might be somewhat lower. Varley (1954) gives an upper limit of 2.0 units for the thymol turbidity test in normal subjects; King and Wootton (1956) state that the great majority of normal bilirubin values are below 0.5 units. The percentage above these arbitrarily fixed and more stringent upper limits was 60% for iproniazid and 11% for imipramine.

DISCUSSION

The findings on iproniazid confirm those of Pletscher and Bernstein (1958). The observed fall in platelet 5-hydroxytryptamine in patients receiving imipramine is of interest. Aspects of the pharmacology of this compound have been studied (Bradley and Key, 1959; Mörsdorf and Bode, 1959; Domenjoz and Theobald, 1959), but the findings do not appear to relate directly to the present observation. Himwich (1959) quotes a personal communication by Costa that imipramine increases brain 5-hydroxytryptamine in the rabbit.

The results of the experiments *in vitro* suggest a possible mode of action of imipramine *in vivo*; they do not provide independent confirmation of the validity of the fluorimetric technique nor, assuming this to be valid, do they necessarily account for the quantitative aspects of the findings *in vivo*. An attempt to confirm the present findings using a bioassay technique is obviously desirable, but cannot be undertaken in this laboratory. The exact concentrations of neither imipramine nor 5-hydroxytryptamine in human plasma are known; a value of less than 1.0 μg./ml. has been quoted for imipramine (Herrmann and Pulver, personal communication) and figures of 0.002 and of less than 0.0001 μg./ml. have been given for 5-hydroxytryptamine (Humphrey and Jaques, 1954; Armin and Grant, 1957). The concentrations chosen for the experiments *in vitro* were considerably higher than the concentrations occurring *in vivo*. It appears to be the ratio of the concentrations which is important; however, no extrapolation can be attempted with so many factors unknown. Using lower concentrations of both compounds *in vitro* is not necessarily going to provide the answer, since *in vitro* uptake appears to stop within 75 to 90 min. (Born *et al.*, 1958), whereas *in vivo* the changes would hardly be significant in the first 24 to 48 hr.

Our results suggest that imipramine resembles reserpine in inhibiting the uptake of 5-hydroxytryptamine by platelets (Brodie *et al.*, 1957), but beneath this similarity there lie a number of differences in detail. Reserpine in therapeutic dosage reduces the platelet 5-hydroxytryptamine level to zero in 24 hr., whereas imipramine requires the same number of days to achieve its maximum effect; an equilibrium state appears then to be attained, rather than a complete inhibition. The experiments *in vitro* suggest that, unlike reserpine (Shore, Carlsson and Brodie, 1957), imipramine does not set free the endogenous 5-hydroxytryptamine. With imipramine, depletion may involve competition rather than destruction.

We are indebted to the Secretary of State for Scotland and his Advisory Committee on Medical Research for a grant for one of us (E. F. M.) and for the provision of a fluorimeter and technical assistance; also to the Crichton Royal for a grant from the Hospital Endowment Fund. We have to thank Mr. J. Girling for carrying out liver function tests, Geigy Pharmaceuticals Ltd. for the gift of pure imipramine hydrochloride, and our colleagues for regularly giving blood. The 5-hydroxytryptophan was obtained from the California Foundation for Biochemical Research.

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OXIDATION OF 5-HYDROXYTRYPTAMINE AND RELATED COMPOUNDS BY *MYTILUS* GILL PLATES

BY

H. BLASCHKO AND A. S. MILTON

From the Department of Pharmacology, University of Oxford

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Homogenates of gill plates of *Mytilus edulis* L. used oxygen when 5-hydroxytryptamine was added. The oxidation of 5-hydroxytryptamine was not due to the presence of an amine oxidase, but to that of an enzyme that catalysed the oxidation of other 5-hydroxyindoles (5-hydroxytryptophan, bufotenine). The oxidation was cyanide-sensitive, but was not inhibited by iproniazid. In the reaction a yellowish-brown substance was formed. The occurrence of an amine oxidase in the anterior retractor muscle of the byssus and in the digestive gland was confirmed.

A number of observations suggest that in molluscs 5-hydroxytryptamine acts as a hormone or mediator; these have been reviewed by Welsh (1955). In *Mytilus edulis*, the anterior retractor muscle of the byssus is inhibited by 5-hydroxytryptamine in very low concentrations (Twarog, 1954). Aiello (1957) reported that 5-hydroxytryptamine had a stimulating action on the ciliary movements in the gill epithelium of *Mytilus* and that extracts of gill plates had a similar effect.

Amine oxidase, a catalyst of the biological inactivation of 5-hydroxytryptamine, occurs in many molluscs. In *Mytilus*, it was not demonstrated in earlier studies (Blaschko and Hawkins, 1952), but more recently Blaschko and Hope (1957) found amine oxidase in homogenates of both the digestive gland and the anterior retractor muscle of the byssus, but no amine oxidase activity was found in the gill plates. Since in the work on *Mytilus* gill plates 5-hydroxytryptamine had not been used as substrate, it was decided to find out if gill plate extracts oxidized 5-hydroxytryptamine.

METHOD

Fresh specimens of *Mytilus edulis* L. were usually dissected immediately after arrival at our laboratory at Oxford. When necessary animals were kept aerated in sea water at 4°. Homogenates were usually prepared without adding fluid; they were dialysed, with frequent changes, against 0.067 M-sodium phosphate buffer of *p*H 7.4 for 2 to 4 hr.; dialysing against the phosphate buffer was then continued without changes overnight.

For the manometric experiments, two different types of conical manometer vessels were used. One had a capacity of 16 to 18 ml., the other 7.5 to 8.5 ml. In the larger vessels the volume of the reaction mixture was 2.0 or 3.0 ml., in the smaller vessels it was 0.7 ml. The substrates were tipped in from the side bulb to give an initial concentration of 0.5×10^{-2} M. The gas phase was either oxygen or, in all later experiments, air. The temperature of the manometer bath was 25°.

5-Hydroxytryptamine creatinine sulphate, 5-hydroxy-DL-tryptophan and iproniazid were obtained from Roche Products. We are grateful to Dr. R. B. Barlow and Dr. I. Khan for the sample of bufotenine hydrochloride.

RESULTS

When a dialysed homogenate of *Mytilus* gill plates was incubated with 0.5×10^{-2} M-5-hydroxytryptamine, a slow but steady uptake of oxygen occurred. No similar uptake of oxygen was found when the homogenate was incubated with tryptamine. In the presence of 5-hydroxytryptamine, a yellowish-brown colour soon developed and increased in intensity as the reaction proceeded.

To establish whether the oxidation of 5-hydroxytryptamine was due to the presence of an amine oxidase in the gill plate homogenates, a second experiment was carried out in which the homogenate was incubated with 5-hydroxytryptamine in the presence of either 10^{-3} M-iproniazid or 10^{-2} M-potassium cyanide, and also without any other substance. To ensure that the iproniazid was effective in inhibiting any amine oxidase present, all flasks were pre-incubated at 25° for 30 min. before tipping. In this experiment, the flask containing iproniazid showed the same

oxygen uptake, and the same development of brown colour, as that to which no inhibitor had been added. However, both the oxygen consumption and the development of colour were entirely suppressed in the presence of cyanide.

That the oxygen consumption in the presence of 5-hydroxytryptamine was due to an enzyme was supported by the observation that no uptake of oxygen occurred when 5-hydroxytryptamine was added to a gill plate homogenate after this had been heated in a boiling water bath for 30 min.

The observations reported made it unlikely that the catalyst involved in the oxidation of 5-hydroxytryptamine was amine oxidase. First, there was an absence of oxidation of tryptamine, usually a good substrate for the study of amine oxidase, and second, there was the lack of inhibition by iproniazid and also the inhibition by cyanide.

In another experiment, both phenethylamine and tyramine were incubated with the gill plate homogenate. Neither amine was oxidized. With tyramine, observations were continued for 180 min. to establish whether there was a phenolase present which required a prolonged incubation of a monophenolic amine; no oxygen uptake occurred within the period of observation.

The findings suggested that the oxidation of 5-hydroxytryptamine by the gill plate homogenates depended upon the presence of the phenolic hydroxyl group in position 5 on the indole nucleus. An experiment was therefore carried out in which both 5-hydroxytryptamine and 5-hydroxytryptophan were incubated. Oxygen uptake occurred not only in the flask incubated with 5-hydroxytryptamine, but also in the flask which contained 5-hydroxytryptophan; the rate of oxidation with the latter was a little slower than with 5-hydroxytryptamine. As with 5-hydroxytryptamine, a brown colour developed when 5-hydroxytryptophan was oxidized, but the rate of development of colour was also a little slower than with 5-hydroxytryptamine.

That the enzymic reaction was, in fact, characteristic of 5-hydroxyindoles was supported by the observation that bufotenine was also oxidized. In Fig. 1 an experiment is shown in which the rates of oxidation with 5-hydroxytryptamine were compared with 5-hydroxytryptophan and with bufotenine. Development of colour was seen with each of these substances.

Although there had been no oxygen uptake with tyramine, dopamine (3,4-dihydroxyphenethylamine) was oxidized; its rate of oxidation in two experiments was slightly less than that of 5-hydroxytryptamine. The oxidation of dopamine was also accompanied by a development of colour, but the greyish-black colour was in marked contrast with the yellowish-brown one formed when the 5-hydroxyindoles were oxidized.

In the earlier experiments, the gas phase during incubation was 100% oxygen, but it was found that there was no significant difference in the rate of the enzymic reaction when the manometers contained air. For this reason, in all later experiments, the gas phase was air. The independence of the rate of the enzymic reaction from the partial pressure of oxygen is also in contrast to what is known of the properties of amine oxidase: the latter is less active in air than in 100% oxygen (Kohn, 1937).

On high-speed centrifugation the activity was found to be retained in the supernatant fluid. In a preliminary experiment, a homogenate was centrifuged at 18,000 *g*; the enzymic activity of the supernatant fluid did not significantly differ from that of the original homogenate.

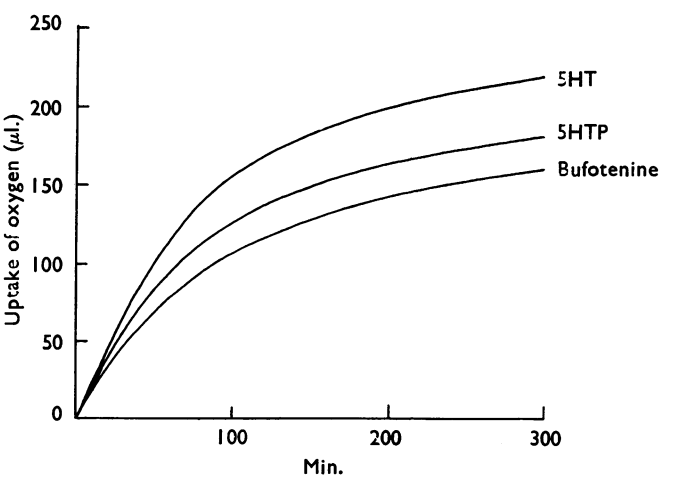


FIG. 1.—Oxidation of 5-hydroxytryptamine, 5-hydroxytryptophan and bufotenine by a preparation from gill plates of *Mytilus edulis* at 25° in air. Each flask contained 1.8 ml. of a resuspended sediment from a 25–55% ammonium sulphate precipitation. Substrate concentrations: 0.5×10^{-3} M. Abscissa: time in min. Ordinate: µl. oxygen used.

A further purification of the oxidase responsible for the enzymic reaction was carried out on a larger batch of homogenate as follows:

Ten dozen specimens of *Mytilus* were dissected to give 129 g. of gill plates. This material was stored at −12° overnight. After homogenizing, it was dialysed for 2 hr. against distilled water and for a further 2 hr. against the phosphate buffer. The homogenate was then centrifuged for 1 hr. at 40,000 r.p.m. in the angle head No. 40 of the Spinco ultracentrifuge. The supernatant fluid from this centrifugation—100 ml.—was used as starting material for two ammonium sulphate precipitations. In the first experiment, 50 ml. was used and three ammonium sulphate precipitates and a final supernatant fluid were obtained. The first sediment was the precipitate from adding ammonium sulphate to bring saturation to 25%; the second from 25 to 55%, and the third from 55 to 100%. Each of these sediments was suspended in 5 ml. of phosphate buffer and they, as well as the final supernatant fluid, were dialysed against the buffer for two hours.

No activity was present in the final supernatant fluid, but all three sediments contained enzymic activity. Most of the activity was recovered in the precipitate from the 25 to 55% saturation. The recoveries, in terms of the supernatant fluid obtained by high-speed centrifugation which had been the starting material for the ammonium sulphate precipitation, were as follows:

High-speed supernatant (starting material)	800	μl. O_2/hr.
Sediment from 0 to 25% saturation	80	„ „
„ „ 25 to 55% „	600	„ „
„ „ 55 to 100% „	437	„ „

The second batch of the same high-speed supernatant fluid was used for another ammonium sulphate precipitation at 30, 60, and 90% saturation; in this experiment the protein content of the various fractions was also determined.

The results summarized in Table I show that the greater part of the activity was recovered in the precipitate from the 30 to 60% saturation. The specific activity of this fraction was 2.09, as compared with 0.64 for the high-speed supernatant fluid, the starting material for the ammonium sulphate precipitation. In other words, the sediment from the 30 to 60% saturation contained 77% of the enzymic activity of the starting material, and at the same time a 3.3-fold purification had been achieved. The overall purification from the original homogenate must have been considerably greater, but this fraction still contained too much fibrous material to make the determination of protein useful.

The more highly purified enzyme preparations were used to find out how much oxygen was consumed in the oxidation reaction. For this experiment, 1.35 ml. of the resuspended sediment from the 30 to 60% ammonium sulphate precipitation, plus 0.45 ml. phosphate buffer, was incubated with 5.0 μmoles of 5-hydroxytryptamine (total reaction volume 2.0 ml.). The oxidation reaction, which proceeded rapidly at first but with a slowly declining rate later, was followed for 5 hr. It came to a standstill after 4 hr., when 100.5 μl. oxygen corresponding to 4.5 μmoles of oxygen had been taken up. In other words, 0.9 moles of oxygen had been consumed per mole of substrate added.

TABLE I

ENZYMIC ACTIVITY AGAINST 5-HYDROXYTRYPTAMINE, AND PROTEIN CONTENT OF THE HIGH-SPEED SUPERNATANT FLUID OBTAINED FROM HOMOGENATES OF THE GILL PLATES OF *MYTILUS EDULIS* AND OF FRACTIONS OBTAINED BY AMMONIUM SULPHATE PRECIPITATION

Protein content determined according to Layne (1957). One enzyme unit (E.U.) is defined as the amount of enzyme that causes uptake of 1 μl. oxygen in 30 min. The readings for the final supernatant were too small to allow an accurate measurement of enzymic activity and protein content.

Fraction	Ammonium Sulphate Percentage Saturation	Volume of Fraction ml.	E.U./ml.	Protein mg./ml.	Specific Activity (E.U./mg. Protein)	Total Enzyme Units
High-speed supernatant ..	—	37·5	8·9	13·8	0·64	334
Sediment I	0–30	6·7	4·4	30·0	0·15	29
„ II	30–60	7·5	34·3	16·4	2·1	257
„ III	60–90	12·7	6·4	3·4	1·9	81
Final supernatant	—	36	1·4 (?)	0·14 (?)	—	—

The most active sediment from the first ammonium sulphate precipitation, that from the 25–55% saturation, was used for a comparison of the rate of oxidation of 5-hydroxytryptamine and L-dopa (β-3,4-dihydroxyphenyl-L-alanine). With 0.63 ml. of the fraction in each flask and a total reaction volume of 0.7 ml., the rates of oxidation were:

	After 15 min.	After 30 min.
With 5×10^{-3} M-5-hydroxytryptamine	8.5 μl. O_2	19 μl. O_2
With 5×10^{-3} M-L-dopa ...	12 μl. O_2	26 μl. O_2

The experiment of Fig. 1, in which the rate of oxidation of different 5-hydroxyindoles is shown, was also carried out using the sediment from the 25–55% saturation with ammonium sulphate.

A few experiments were carried out on other *Mytilus* tissues. In one experiment with 37 specimens, the anterior retractor muscles of the byssus were dissected (weight of muscles 4.0 g.) and a dialysed homogenate in phosphate buffer was prepared (total volume of homogenate 10 ml.). Of this 1.6 ml. was used in each manometer flask. Upon incubation for 1 hr. with 10^{-2} M-tryptamine and 0.5×10^{-3} M-5-hydroxytryptamine, 12.5 μl. oxygen was consumed with tryptamine and 11.5 μl. oxygen with 5-hydroxytryptamine. The corresponding figures after 2 hr. incubation were 23.5 μl. and 21 μl. respectively. Here also a brown colour appeared, but, in contrast to the experiments with the gill plate homogenates, a strong bluish-white fluorescence was seen, particularly in the flask incubated with tryptamine. This experiment confirmed the occurrence of amine oxidase in the anterior retractor muscle, as already reported by Blaschko and Hope (1957). Both amines were also oxidized by a homogenate of the posterior retractor muscle of the byssus.

A homogenate was also prepared of the digestive glands of *Mytilus* (1 part of fresh tissue plus 2 parts of phosphate buffer); this homogenate also contained parts of the intestine and other adjoining tissues. Each manometer flask contained 1.6 ml. of the dialysed homogenate. Both tryptamine and 5-hydroxytryptamine were oxidized. In the same experiment, 10^{-3} M-iproniazid was also tested. The amounts of oxygen used in the first hour of incubation were:

With tryptamine	36 μl. oxygen
„ 5-hydroxytryptamine	19 μl. „
„ tryptamine plus iproniazid ...	1 μl. „
„ 5-hydroxytryptamine plus iproniazid	9.5 μl. „

In the experiment, the oxidation of tryptamine was more strongly inhibited by iproniazid than that of 5-hydroxytryptamine, suggesting that the oxidation of tryptamine was only due to amine oxidase but that the oxidation of 5-hydroxytryptamine was due in part to an iproniazid-resistant enzyme. Thus, it is possible that the digestive gland contains not only amine oxidase, but also the catalyst of the oxidation of 5-hydroxytryptamine found in the gill plates.

Homogenates of the gill plates, the gonads and the digestive glands of *Chlamys* (*Pecten*) *opercularis* were also prepared. Tryptamine was readily oxidized by all three homogenates, but an oxidation of 5-hydroxytryptamine occurred only with the gill plate homogenate.

Discussion

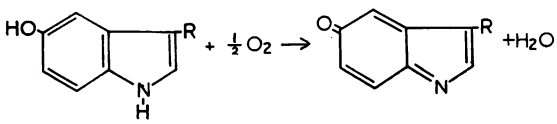
These experiments show that gill plates of *Mytilus edulis* contain an enzyme that oxidizes 5-hydroxytryptamine.

The absence of any oxygen uptake with tryptamine, phenethylamine and tyramine, the lack of inhibition by iproniazid, and the sensitivity to cyanide are all properties which show that the enzyme responsible for the oxidation is not amine oxidase. This view is supported by the observation that other 5-hydroxyindoles, 5-hydroxytryptophan and bufotenine, are similarly oxidized. All these compounds are characterized by the presence of the phenolic hydroxyl group in position 5 on the indole nucleus.

There is good reason to believe that the enzyme is of the phenolase type. First, there is the inhibition of the enzymic reaction by cyanide. Second, there is the fact that both dopamine and dopa are oxidized, the latter by a partly purified preparation of the enzyme. The lack of any uptake of oxygen with tyramine suggests that the enzyme is not a monophenoloxidase. It must be borne in mind that 5-hydroxytryptamine and other 5-hydroxyindoles are not strictly comparable to the monophenols; they are substituted derivatives of *p*-aminophenol, and should therefore be more similar to the *p*-diphenols.

It has recently been shown by Mansour (1958) that 5-hydroxytryptamine has an affinity for the phenol oxidase of the liver fluke, *Fasciola hepatica*, and that of the Harding-Passey mouse melanoma. Both enzymes are inhibited by 5-hydroxytryptamine. There was no oxidation of 5-hydroxytryptamine by the *Fasciola* enzyme, but with the melanoma Mansour's data show a very slight oxidation of 5-hydroxytryptamine. However, the chief difference between these preparations and that containing the *Mytilus* gill plate enzyme is that the latter rapidly oxidizes the 5-hydroxyindoles.

The nature of the enzymic reaction catalysed has not been studied. The most likely reaction is the formation of a quinone-imine, as already suggested by Blaschko and Philpot (1953), thus:



This reaction would require 0.5 moles of oxygen per mole of substrate. In our experiments, the total oxygen uptake was nearer one mole of oxygen per molecule of substrate; this could either be interpreted as due to a further oxidation of the product of the enzymic reaction, or to the accumulation of hydrogen peroxide, or to peroxidatic reactions in which the latter was used up.

An alternative possibility should not be forgotten: the introduction of a second phenolic hydroxyl group in the indole nucleus. There is no evidence to suggest any particular position for such a group; it may be mentioned that Carlisle (1956) has discussed the occurrence of an *ortho*-dihydroxytryptamine in the pericardial organ of Crustacea.

The relatively rapid action of a phenolase type of enzyme of molluscan origin on 5-hydroxytryptamine and related substances is of interest. It has often been discussed whether melanin-like pigments can arise from tryptophan, and it seems that the reaction here described makes it possible that pigments may arise by the oxidation of a 5-hydroxyindole. The colour of the incubation mixture was different from that produced by dopa or dopamine; it was yellowish-brown, and it may be mentioned here that we have seen a similar colour when 5-hydroxytryptamine was oxidized by silver oxide.

Whether 5-hydroxytryptamine has a regulatory function in the ciliary epithelium of the gill plates is unknown. The work of Aiello (1957), who observed an effect of 5-hydroxytryptamine on the ciliary rhythm, has already been mentioned. There is evidence of an antagonism of 5-hydroxytryptamine and acetylcholine in the molluscan heart and in the anterior retractor muscle of the byssus. It is known that in the gill plates acetylcholine has an effect on the ciliary rhythm and that the gill plates contain all the catalysts for the synthesis as well as the inactivation of acetylcholine (Bülbring, Burn and Shelley, 1953; Milton, 1959). In the light of these findings, the presence in the gill plates of a catalyst for the inactivation of 5-hydroxytryptamine is of particular interest.

We are grateful to the director and staff of the Marine Biological Laboratory at Plymouth who sent us a regular supply of fresh *Mytilus edulis* L. We are grateful for the support of the U.S. Air Force Office of Scientific Research in the later stages of this work.

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THE EFFECT OF PRECURSORS OF NORADRENALINE ON THE RESPONSE TO TYRAMINE AND SYMPATHETIC STIMULATION

BY

J. H. BURN AND M. J. RAND

From the Department of Pharmacology, University of Oxford

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Previous observations have shown that the effects of sympathetic stimulation and of tyramine were absent in the organs of animals treated with reserpine, but that they were restored by an infusion of noradrenaline. Observations are described showing that an infusion of adrenaline did not restore the pressor action of tyramine in the cat or in the rat, but that in the rat the pressor action was restored by an infusion of dopamine, or of (−)-dopa, or of *m*-tyrosine, or of phenylalanine. Observations are also described showing that the effect of postganglionic stimulation of the fibres to the nictitating membrane and to the iris was restored by an infusion of dopamine or of (−)-dopa; it was restored less well by an infusion of noradrenaline. An infusion of noradrenaline did not restore the action of tyramine on the denervated iris or on the denervated vessels of the cat's foreleg. An infusion of noradrenaline appeared to increase the effect of sympathetic stimulation of the hypogastric nerves to the uterus of the virgin cat about as much as an infusion of adrenaline. An infusion of noradrenaline restored the constrictor action of nicotine on the perfused vessels of the rabbit ear.

The pressor action of tyramine was shown by Carlsson, Rosengren, Bertler, and Nilsson (1957) to be absent in animals treated with reserpine, and we observed (Burn and Rand, 1958b) that it was restored by an intravenous infusion of noradrenaline. We also found that in normal dogs (not treated with reserpine), or in the perfused dog hindleg, the vasoconstrictor action of stimulation of the lumbar sympathetic chain was increased by an intravenous infusion of noradrenaline (Burn and Rand, 1960).

We have now carried out experiments to see if similar effects followed the infusion of adrenaline and of the precursors of noradrenaline, and have tested their effect on stimulation of sympathetic fibres to organs other than the blood vessels.

METHODS

In treating animals with reserpine a solution of reserpine 5 mg./ml. was prepared in a solution of ascorbic acid 20%. Cats received 0.6 ml. on two successive days; male rats of weight 200 g. received 0.3 ml. on two successive days. Observations were made on the blood pressure in the spinal preparation of cats and in the rats prepared as described by Muscholl and Vogt (1957), in which the brain and spinal cord of the rat are destroyed by pithing (Shipley and Tilden, 1947), and the rat receives 1 to 2 mg. atropine. In making intravenous infusions a motor-driven syringe (C. F. Palmer London, Ltd.) was used and a polythene tube was inserted into a vein other than the vein which was cannulated for injections. In the rat all infusions were made for 25 min. In the cat the duration varied from 18 to 30 min.

In experiments on the virgin cat uterus, the cat was anaesthetized with chloralose, after which the abdomen was opened and the cat was eviscerated. The ligament between the right ovary and the kidney was divided and a thread was tied to the ovary and connected to a lever writing on the drum. Sometimes stimulation was applied to both hypogastric nerves and sometimes to the right nerve only, using electrodes of a pattern already described (Burn and Rand, 1960). The nerves were dissected below the inferior mesenteric ganglion and were irrigated through the electrode-holder by a slow stream of oxygenated Krebs solution. The abdominal cavity was filled with Locke solution at 37° and maintained from a slow drip. Rabbit ears were perfused by the method described by Burn (1952) using the outflow recorder of Stephenson (1948).

RESULTS

Restoration of the Action of Tyramine.—Observations on the restoration of the pressor

TABLE I

INCREASE OF PRESSOR ACTION OF TYRAMINE IN SPINAL PREPARATIONS OF CATS TREATED WITH RESERPINE

Dose of tyramine=2 mg.

Substance Infused	Amount Infused	Pressor Response (mm. Hg)		Increase	Mean
		Initial	After Infusion		
Noradrenaline	0·1 mg.	20	48	28	19
		10	30	20	
		18	42	24	
		18	22	4	
	0·5 ,,	12	70	58	48
		10	52	42	
		8	70	62	
		20	60	40	
		2	46	44	
		18	64	46	
		19	65	46	
		22	72	50	
Adrenaline..	0·5 ,,	38	20	18	7
		12	20	8	
		32	32	0	
		6	22	16	
		12	32	18	
		16	32	16	
Dopamine ..	5 ,,	10	34	24	13
		20	26	6	
		16	26	10	
	10 ,,	26	72	44	34
		24	48	24	

action of tyramine were made in the cat and in the rat. The results for a series of experiments in the cat are given in Table I, in which the effect of infusing noradrenaline, adrenaline, and dopamine is seen. Adrenaline had little or no effect. This is illustrated in Fig. 1 in which a series of injections of 2 mg. tyramine was given before and after the intravenous infusion of 0.5 mg. adrenaline, and of 0.5 mg. noradrenaline, repeated twice. The pressor action of tyramine was increased only after the infusion of noradrenaline, and not after the infusion of adrenaline.

The increase in the pressor action after the infusion of noradrenaline was greatest for the first injection after the infusion, and then rapidly disappeared. This is shown in Fig. 2 in which the mean results of eight experiments are shown; from these it appears that even the second injection had little more effect than the injection before the infusion.

The effect of dopamine was more lasting, as is shown in Fig. 2, although the amount of dopamine infused was 10 mg. as compared with 0.5 mg. noradrenaline, that is to say was less than an equipressor amount.

Observations in rats have been summarized in part in Table II, where the increase in the pressor action of tyramine has been expressed, not in terms of the height of the rise, but in terms of the area of the pressor response recorded on the drum. Thus the first line of Table II gives the results of three experiments in which 5 μg. noradrenaline was infused. In the first of these the area of the pressor response to the first injection of 0.5 mg. tyramine after the infusion was 9.5 times as great as it was before the infusion. Table II shows that dopamine when given in only 10 times the dose was more effective than noradrenaline. The action of dopamine and of (−)-dopa was much more lasting than that of noradrenaline, as is shown in Figs. 3 and 4.

We were not successful with tyrosine; it was dissolved with difficulty, and a trial with the N-acetyl derivative of tyrosine was also ineffective. However, an infusion of 2 mg. meta-tyrosine (having the –OH in the *meta* position in the ring) was effective in increasing the pressor action of tyramine, though rather less so than 2 mg. (−)-dopa.

TABLE II

RATIO OF PRESSOR RESPONSE TO TYRAMINE BEFORE AND AFTER INFUSION IN THE PITHED RAT TREATED WITH RESERPINE

The response was taken as the area of the rise of pressure on the kymograph.

Substance Infused	Amount Infused	Ratio, Taking Response Before Infusion=1
Noradrenaline ..	5 μg.	9·5, 8·4, 4·9
	10 ,,	12·3, 24·7, 7·3
	20 ,,	12·4, 15·9
Dopamine	50 ,,	16·4
	100 ,,	24·6
	200 ,,	37·0
(−)-Dopa	1 mg.	8·8, 6·4
	2 ,,	8·6
(±)-*m*-Tyrosine ..	2 ,,	6·4
(−)-Phenylalanine	25 ,,	9·6
(−)-Adrenaline ..	20 μg.	0·3, 3·2
(−)-N-Acetyl Tyrosine ..	10 mg.	0·25
(−)-Tyrosine ..	1·6 ,,	0·9
	3·0 ,,	0·5

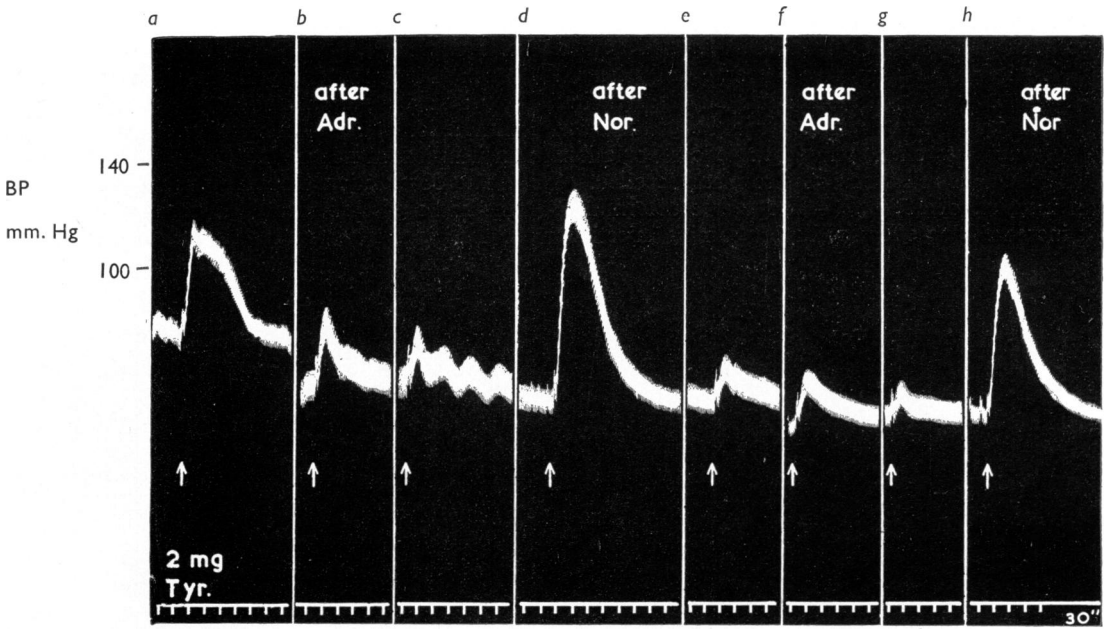

FIG. 1.—Blood pressure in spinal preparation of reserpine-treated cat. 2 mg. tyramine injected at each arrow. Between (*c*) and (*d*), and between (*g*) and (*h*), infusions of 0.5 mg. noradrenaline, after which the responses to tyramine were increased. Between (*a*) and (*b*), and between (*e*) and (*f*), infusions of 0.5 mg. adrenaline, which did not increase the responses.

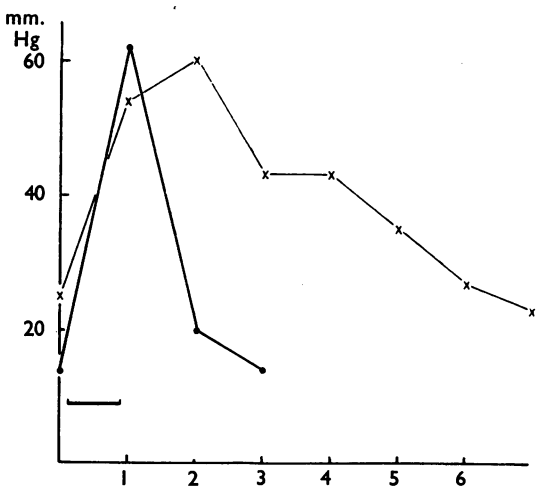

FIG. 2.—Mean rise of blood pressure in spinal preparation of reserpine-treated cat due to injection of 2 mg. tyramine. Black circles show increase after infusion (marked by horizontal line) of 0.5 mg noradrenaline in 8 cats. The increase was evident with the first injection of tyramine after the infusion, but had almost disappeared with the second injection. Crosses show increase after infusion of 10 mg. dopamine (2 cats). This increase persisted for several injections. Abscissae: successive injections of tyramine. Ordinates: rise in blood pressure (mm.Hg).

Finally we observed that an infusion of 25 mg. phenylalanine increased the pressor action of tyramine, having a prolonged effect.

Effects on Sympathetic Stimulation. — The observations on the effect of a noradrenaline infusion on sympathetic stimulation which have been made so far have been confined to vasoconstriction. We therefore turned to the iris of the cat's eye and to the nictitating membrane. An example of an experiment is given in Fig. 5. At the beginning, stimulation of the postganglionic fibres had no effect on the diameter of the pupil, and had a very small effect on the nictitating membrane. An intravenous infusion of 10 mg. dopamine was then given during a period of 15 min. About 7 min. after the end of the infusion the postganglionic fibres were stimulated again, using the same stimulus as before. The pupil dilated 5 mm. and the contraction of the nictitating membrane was 16 mm. on the drum. Stimulation at intervals during the next 40 min. continued to elicit similar large responses.

In one experiment (Fig. 6) a comparison was made between the effect of dopamine, noradrenaline, and (−)-dopa in restoring the response to sympathetic stimulation in the reserpine-treated cat. After the response of the iris had been restored by an infusion of 8 mg. dopamine,

injections of 4 mg. tyramine were given so that both the effect of sympathetic stimulation and of tyramine on the iris became very small. Noradrenaline was then infused during 20 min.; this increased the effect of tyramine greatly, but had much less effect on sympathetic stimulation. After further injections of tyramine the effect of an infusion of 30 mg. (−)-dopa was determined. It increased the effect of both tyramine injections and of sympathetic stimulation, though the effect of sympathetic stimulation was not increased as much as it had been by the infusion of dopamine.

We also observed that the effect of sympathetic stimulation on the iris was increased after giving an infusion of adrenaline.

The observation that an infusion of noradrenaline had a greater effect in increasing the response of the iris to tyramine than it had in increasing the

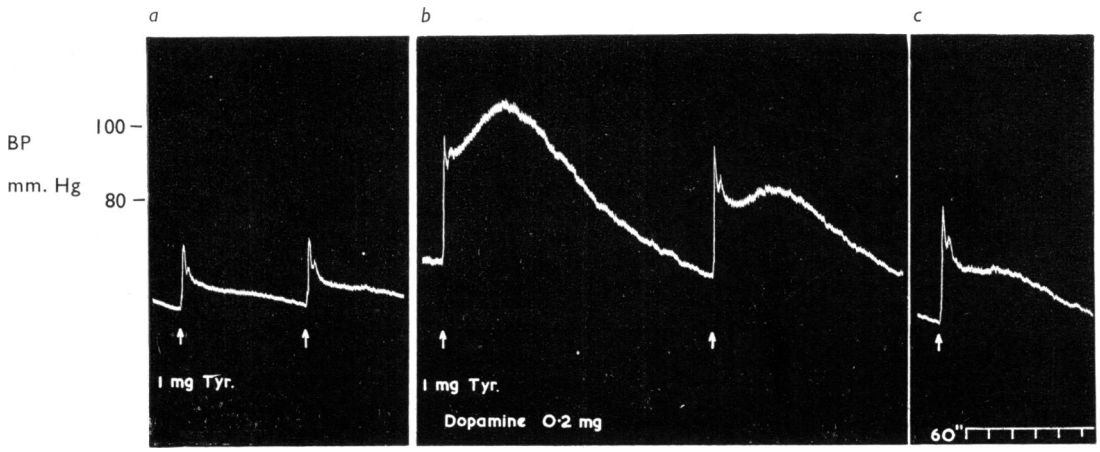

FIG. 3.—Blood pressure of pithed, reserpine-treated rat. Injections of 1 mg. tyramine into right jugular vein at each arrow. Between (*a*) and (*b*) 200 μg. dopamine was infused at 8 μg./min. into the left jugular vein. (*b*) The response to the 1st and 2nd injections of tyramine after the infusion of dopamine, and (*c*) the 3rd injection.

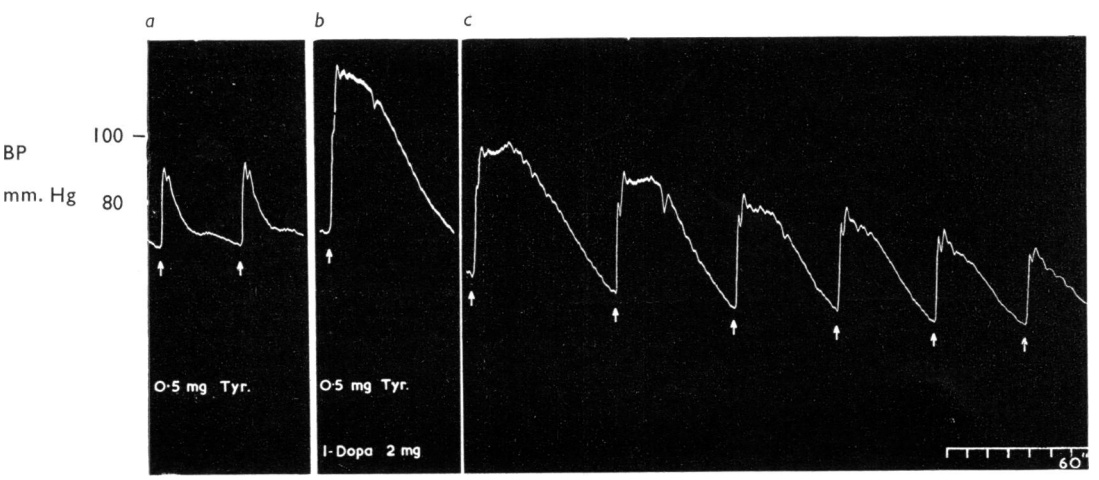

FIG. 4.—Responses to 0.5 mg. of tyramine in pithed, reserpine-treated rat (*a*). Between (*a*) and (*b*), 0.5 mg. of (−)-dopa was infused. The enhanced responses to tyramine after the infusion of (−)-dopa are shown in (*b*) and (*c*). Note that the response to the seventh injection of tyramine after the infusion of (−)-dopa was still greater than the response before the infusion.

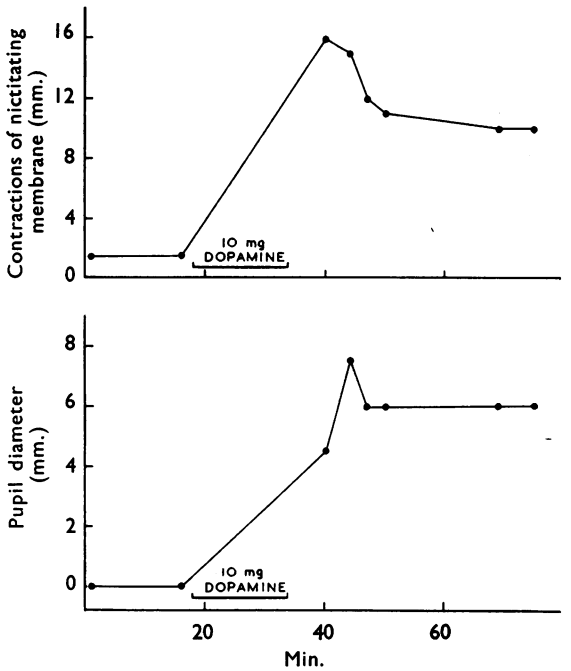

FIG. 5.—Reserpine-treated cat. Upper graph shows contractions of the nictitating membrane (mm. on kymograph record). The lower graph shows dilatation of the pupil measured directly. Stimulation of the postganglionic fibres leaving the superior cervical ganglion for 20 sec. with 5 mA. square wave pulses, 2 msec. duration, 25/sec. After the infusion of 10 mg. dopamine the responses were greater.

response to sympathetic stimulation was also made in experiments on the spleen. Before the infusion of noradrenaline was made, stimulation of the splenic nerves (in the presence of atropine) failed to cause any contraction of the spleen, and an injection of tyramine was also ineffective. After an infusion of 0.5 mg. noradrenaline, stimulation of the splenic nerves caused a very slight contraction of the spleen, while an injection of tyramine caused a large contraction.

Cat Uterus.—We made observations in which we compared the effect of an infusion of noradrenaline with that of an infusion of adrenaline on the inhibitory response to stimulation of the hypogastric nerves of the uterus of the virgin cat. An example of such a comparison is given in Fig. 7, in which the infusion of 100 μg. noradrenaline and the infusion of 100 μg. adrenaline increased the effect of sympathetic stimulation almost equally.

Effect of Infusion in Denervated Tissues.—Since noradrenaline is almost absent in denervated tissues (Euler and Purkhold, 1951; Burn and Rand, 1959), and since tyramine has no action in these tissues (Burn, 1932a), we carried out experiments to see if the infusion of noradrenaline would restore the response to tyramine. We denervated the right iris in two cats by removing the superior cervical ganglion, and after two weeks we determined the effect of an infusion of 1 mg. noradrenaline on the response of the iris to an intravenous injection of tyramine. In neither experiment did the infusion restore the response to tyramine, although the dose injected was as large as 4 mg. The pupil did not dilate at all, though the left pupil dilated maximally.

We made two experiments on the denervated vessels of the foreleg, after removal of the stellate ganglion of one side two weeks previously. The result in one of these is shown in Fig. 8, in which the changes in volume of the normally innervated foreleg, recorded by a plethysmograph, are seen at the top. The middle record is that of the denervated foreleg. The injection of 2 mg. tyramine caused constriction in the normal foreleg, and a slight passive dilatation in the denervated foreleg. These responses remained unchanged after the infusion of 1 mg. noradrenaline. The second experiment gave a similar result.

Infusion of Noradrenaline in the Rabbit Ear.—Finally experiments were made in the perfused rabbit ear in which nicotine normally has a constrictor effect. This constriction is absent if the ear is taken from a rabbit which has been treated with reserpine (Burn and Rand, 1958a), and we wished to know if the constrictor response could be restored by an infusion of noradrenaline. Fig. 9 is taken from an experiment of this kind. At the beginning the injection of 10 μg. nicotine acid tartrate into the fluid perfusing the ear caused only an increase in outflow. During the night noradrenaline was added to the fluid perfusing the ear in a concentration of 0.1 μg./ml. On the next morning a series of four injections of 10 μg. nicotine acid tartrate had a small but increasing constrictor effect as shown by the fall in outflow, and then the injection of 20 μg. caused a large constriction. Thereafter two injections to 10 μg. nicotine were each without effect. It appeared that the infusion of noradrenaline had restored some constrictor action of nicotine, but that after the larger dose of nicotine further doses were then ineffective.

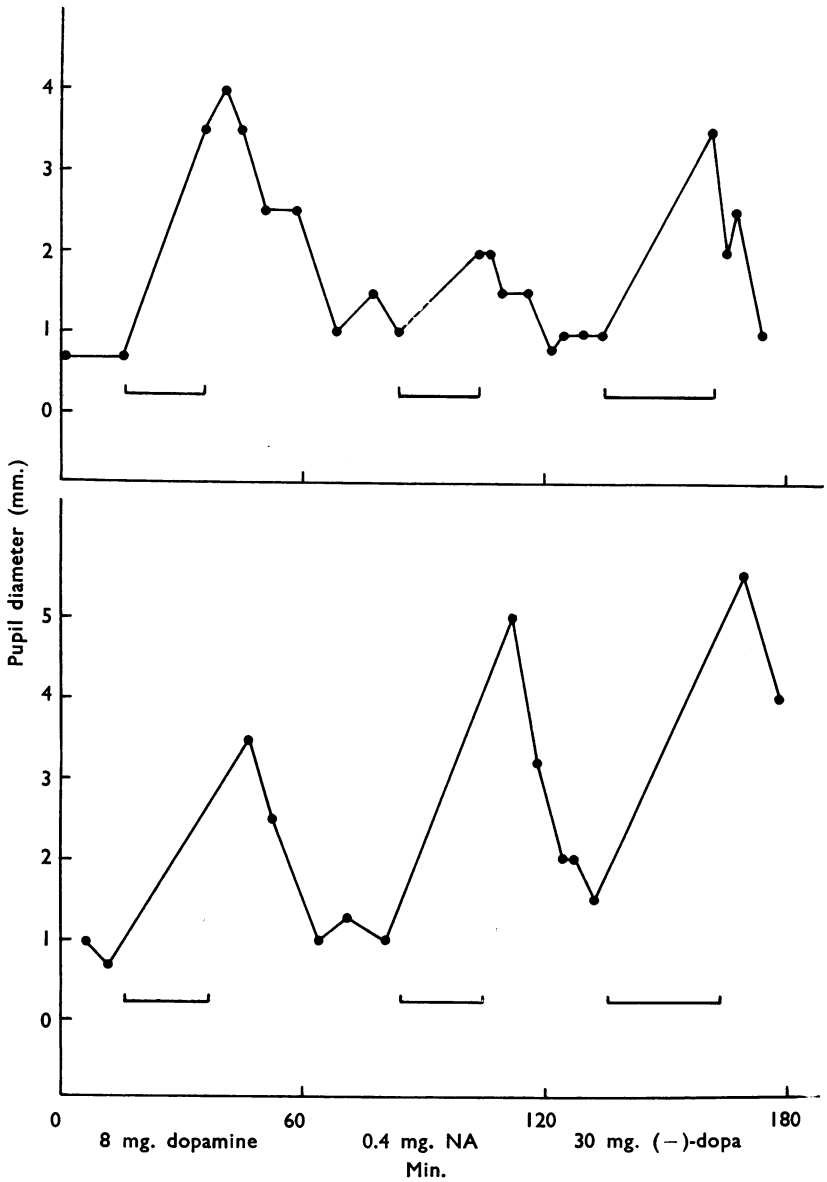

FIG. 6.—Reserpine-treated cat. Upper graph shows dilatation of the pupil in mm produced by postganglionic sympathetic stimulation. Lower graph shows dilatation produced by injection of tyramine (4 mg.). An infusion of noradrenaline (0.4 mg.) was less effective than an infusion of dopamine (8 mg.) or of (−)-dopa (30 mg.) in increasing the response to stimulation, but not less effective in restoring the response to tyramine.

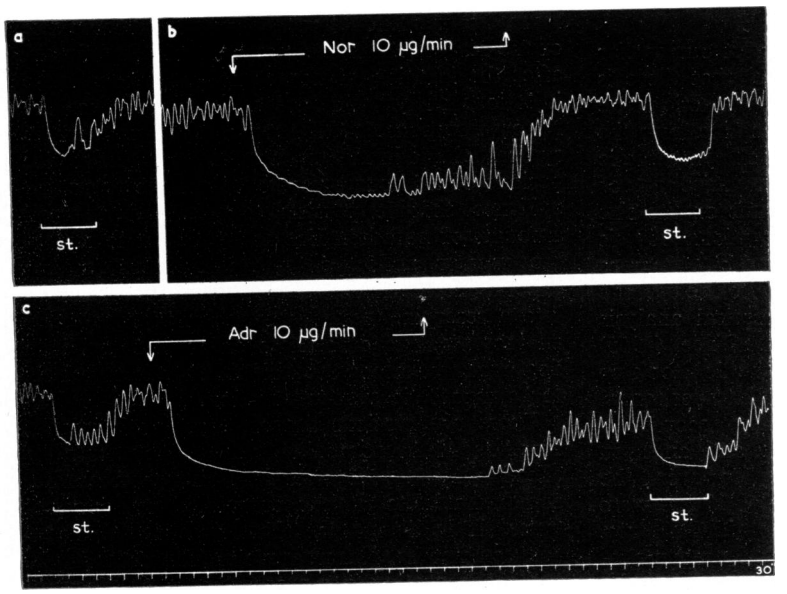

FIG. 7.—Right horn of uterus of virgin cat *in situ*. Right hypogastric nerve stimulated (St) for 2 min. with 20 mA. square wave pulses, 2 msec. duration, 25/sec. After an infusion of 100 µg. of noradrenaline, stimulation produced a greater and more complete inhibition than before. This increased response was only temporary, and about 30 min. later there was only a partial inhibition on stimulation. Then, after an infusion of 100 µg. adrenaline, stimulation was again more effective.

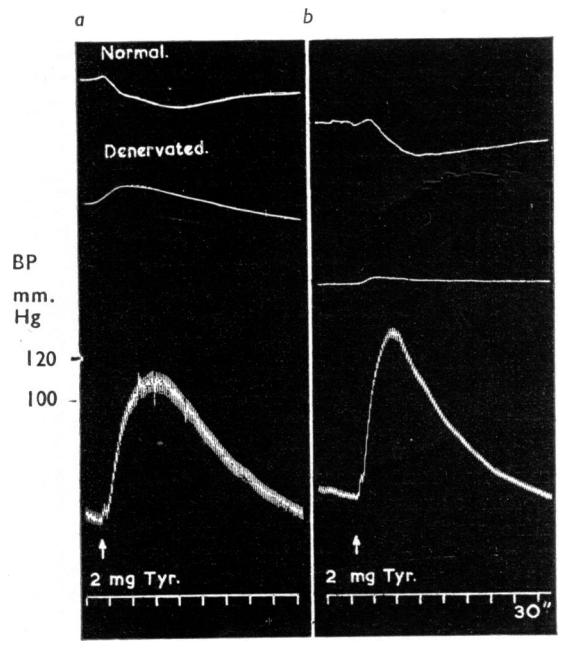

FIG. 8.—Normal (upper) and denervated (lower) foreleg plethysmograph records from cat in which right stellate ganglion had been removed 28 days previously. Spinal preparation. Tyramine (2 mg.) had no constrictor action on the vessels of the denervated foreleg (*a*) initially, or (*b*) after the infusion of 1 mg. noradrenaline.

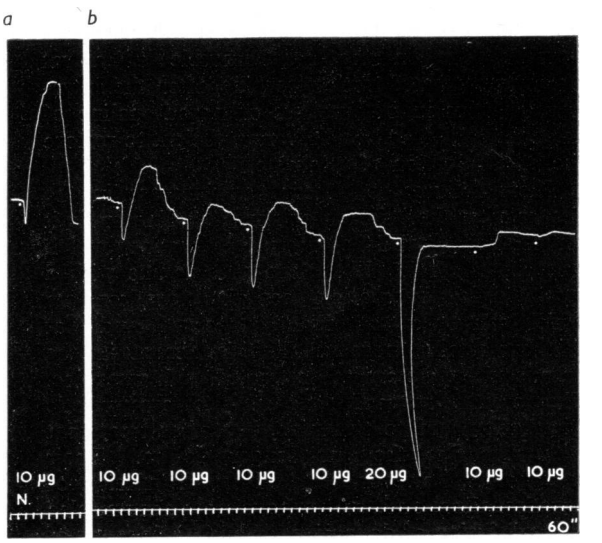

FIG. 9.—Perfused ear from reserpine-treated rabbit. In (*a*) 10 µg. nicotine acid tartrate produced dilatation. Noradrenaline (10^{-7}) was added to the perfusing solution overnight between (*a*) and (*b*). Then, during perfusion with fresh Locke solution, nicotine acid tartrate 10 µg. and 20 µg. produced constriction. After the injection of the larger dose the constrictor action of nicotine was lost.

Discussion

The observations described support our earlier suggestion (Burn and Rand, 1958b) that there is a store in the neighbourhood of sympathetic nerve endings which can take up noradrenaline from the blood stream and that the effect of sympathetic stimulation and the effect of an injection of tyramine and related amines depends on the size of the store. The earliest observations of this kind were made by Burn (1932a and b), but more recently efforts have been made to detect the uptake of catechol amines by chemical analysis of the tissues. Thus Raab and Gigee (1955) observed that the heart muscle of the dog took up noradrenaline and adrenaline after intraperitoneal injection of these substances; they recorded a 12-fold increase of noradrenaline and an even greater increase of adrenaline. However, the amounts they injected into a dog of 10 kg. were 38 mg. noradrenaline together with 75 mg. adrenaline. v. Euler (1956) infused amounts of noradrenaline between 2.5 and 7.8 μg./kg./min. into cats for over 30 min., and gave intraperitoneal injections of noradrenaline and adrenaline up to 2 mg./kg. He failed to observe any increase in the catechol amine content of the heart, the spleen, the liver, the kidney, and skeletal muscle. Recently Muscholl (private communication) has failed to observe any increase of noradrenaline in the heart muscle of a rat previously treated with reserpine after the intravenous infusion of amounts of noradrenaline of the order of 14 μg. which were sufficient to restore the pressor action of tyramine.

These failures to observe an increase in the tissues after the infusion of noradrenaline make it important to recall the precise observations which have been made. These are that organs with a sympathetic innervation contain noradrenaline which can be extracted and measured, and that this extractable noradrenaline is absent in the organs when the animal is treated with reserpine. The organs normally respond to sympathetic stimulation and to tyramine, but after treatment with reserpine they no longer do so. Their response is, however, restored by an infusion of noradrenaline, and, as we have now shown, also by an infusion of dopamine, or of (−)-dopa, or of meta-tyrosine or of phenylalanine. We have seen indications that dopamine is indeed more efficient in restoring the effect of sympathetic stimulation than is noradrenaline itself.

It is clear from studies of the restoration of the tyramine response that it is a very partial restoration. That is to say in the normal animal repeated injections of tyramine can be given for many hours without much diminution in the size of the response. In the reserpine-treated animals, after the infusion of noradrenaline, two or three injections of tyramine are sufficient to cause the restored response to disappear. The response to sympathetic stimulation appears to persist for periods of an hour or so, but we have as yet no evidence that it persists longer. Quite small amounts of noradrenaline are sufficient to restore the pressor action of tyramine; thus in one experiment the injection of 10 μg. noradrenaline into a reserpine-treated cat was enough to cause an increase, and amounts such as 0.25 mg. in a dog produced a considerable fall in the threshold for sympathetic vasoconstriction.

These observations suggest that much smaller amounts of noradrenaline can be shown to be effective by our physiological tests than can be demonstrated to have accumulated by chemical tests. It is likely that there are several stages between uptake of noradrenaline from the blood and incorporation into granules such as Blaschko and his colleagues have demonstrated in the adrenal medulla (Blaschko, 1959). Incorporation into granules of noradrenaline taken from the blood may indeed not occur at all, for what is present in the granules may be the result of synthesis only. But that the increase in the effect of tyramine or of sympathetic stimulation or of nicotine (in the rabbit ear vessels) as a result of an infusion of noradrenaline is due to retention of noradrenaline in some form and in some situation we think is most likely. In Fig. 9 an infusion of noradrenaline restored the constrictor action of nicotine in the rabbit ear vessels. When a larger dose of nicotine was given which produced a large constriction, the smaller doses then lost all effect. The observation is consistent with the constrictor action of nicotine being due to release of noradrenaline taken up by the vessels from the perfusing fluid. After the large dose constriction was no longer seen because all had been released.

We often observed that an infusion of noradrenaline restored the action of tyramine to a greater extent than it restored the effect of sympathetic stimulation. A difference exists between them since the effect of sympathetic stimulation is blocked by bretylium whereas the action of tyramine is not. Tyramine may compete by mass action for the sites to which noradrenaline is attached, while sympathetic impulses may act differently. If noradrenaline is taken up into a store, tyramine may release it before it has reached the sites from which it is released by sympathetic stimulation.

Do the precursors restore the action of tyramine themselves, or must they first be converted to noradrenaline ? It is difficult to see how phenylalanine can act directly, and some conversion in the direction of noradrenaline seems more likely. If so, it is evidence that the conversion can occur very quickly.

The evidence that noradrenaline restored the pressor action of tyramine much more effectively than adrenaline was clear ; however, an infusion of adrenaline was found to restore the action of sympathetic stimulation in causing pupil dilatation, and an infusion of adrenaline seemed to be as effective as one of noradrenaline in increasing the inhibitory action of hypogastric stimulation on the uterus. A sharp distinction between noradrenaline and adrenaline in relation to the store at sympathetic nerve endings therefore cannot be made.

We wish to thank Miss Roneen Hobbs for the experiments in which rabbit ears were perfused. We also wish to thank Dr. H. Blaschko for a supply of *m*-tyrosine. This work has been done during the tenure by one of us (M. J. R.) of a Fellowship from the Life Insurance Medical Research Fund of Australia and New Zealand.

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SYMPATHETIC POSTGANGLIONIC CHOLINERGIC FIBRES

BY

J. H. BURN AND M. J. RAND

From the Department of Pharmacology, University of Oxford

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When the postganglionic fibres to the nictitating membrane are stimulated in a cat treated with reserpine, the membrane contracts. The contraction is increased after the injection of eserine and is abolished with atropine. Thus the fibres stimulated appear to be cholinergic. When the splenic nerve is stimulated in a cat treated with reserpine there is similar evidence of the presence of cholinergic fibres; the spleen dilates, the dilatation is greater in the presence of eserine and is abolished by atropine. The hypogastric nerves when stimulated cause inhibition of the uterus of the virgin cat. When the cat is treated with reserpine, stimulation then causes contraction which is increased in the presence of eserine and abolished by atropine. There is also evidence of cholinergic fibres in the postganglionic supply to the vessels of the rabbit's ear. From this and other evidence it is suggested that a form of adrenergic mechanism may exist such that cholinergic fibres in the postganglionic sympathetic supply liberate acetylcholine; this in turn liberates noradrenaline from a store around the sympathetic nerve ending.

Many effects produced in the body by stimulation of sympathetic nerves are also produced by acetylcholine (after atropine) and by nicotine acting peripherally. Vasoconstriction in the vessels of the isolated and perfused rabbit ear is an example. Since these effects of acetylcholine and of nicotine disappear (a) after treatment of the animal with reserpine and (b) after degeneration of the sympathetic nerves, it seemed that the effects might actually be mediated through some part of the sympathetic mechanism. Cholinergic fibres have been shown to occur in various sympathetic nerves, the first demonstration of them having been made by Euler and Gaddum (1931).

We have therefore carried out an investigation to see if fibres liberating acetylcholine were present in postganglionic sympathetic nerves generally, and have studied the supply to the nictitating membrane, the spleen, the uterus of the non-pregnant cat and the perfused rabbit ear. Bacq and Fredericq (1935) had already given evidence for the presence of cholinergic fibres in the nictitating membrane. We thought that if sympathetic nerves in general contained cholinergic fibres, the acetylcholine liberated might then have the same effect as injected acetylcholine or nicotine.

METHODS

Our method has been to study the effect of stimulating postganglionic sympathetic fibres in cats or rabbits treated with reserpine. This treatment reduces or removes the store of noradrenaline present around the sympathetic nerve endings and makes it easier to see effects which might be produced by release of acetylcholine.

Reserpine was dissolved in a 20% solution of ascorbic acid, and usually a 1% solution was prepared which was injected into the peritoneal cavity on two successive days. The animal was used for the experiment on the third day. The amount injected was 1.5 mg./kg. on each day. The animals were kept in a warm room at a temperature of 27°.

For observations on the nictitating membrane, the cat was anaesthetized with chloralose. The superior cervical ganglion was exposed on one side and a pair of electrodes was applied so that one was in contact with the ganglion and the other just beyond. The contractions of the nictitating membrane were recorded by an isotonic lever.

For observations on the spleen the cat was partially eviscerated from the colon to the middle of the duodenum, the inferior and superior mesenteric arteries being divided. The fibres of the splenic nerve were identified near the origin of the splenic artery and a ligature was tied around them. The fibres were not cut. A pair of shielded electrodes could then be placed so as to stimulate them. An incision was made in the skin on the left side and the muscle layers were separated by retraction with two aneurysm needles so that the spleen could be brought out of the body cavity and put into a plethysmograph (Burn, 1952). The volume changes were recorded by a piston recorder.

For observations on the uterus, the cat was eviscerated and the ligament between the kidney and the ovary was divided on one side. A thread was attached to the ovary and passed over pulleys to a

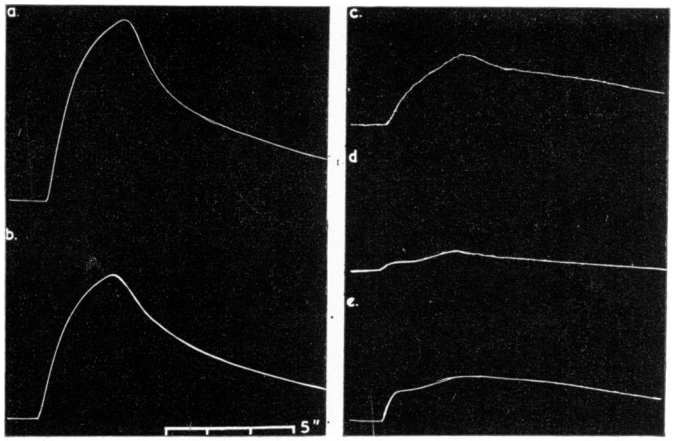

FIG. 1.—Contractions of nictitating membrane in response to postganglionic stimulation. (*a*) In normal cat, (*b*) in same cat after 4 mg./kg. atropine, (*c*) in cat treated with reserpine, (*d*) in same cat after 4 mg./kg. atropine, (*e*) in same cat after infusion of 0·5 mg. noradrenaline.

lever which recorded on the kymograph. The hypogastric nerves were identified and were passed through electrodes of a pattern recently described (Burn and Rand, 1960) which provided for a constant flow of oxygenated Krebs solution over the portion of nerve stimulated.

For observations on the perfused rabbit ear we used the preparation described by Gaddum and Kwiatkowski (1938) in which we applied electrodes to the fibres leaving the superior cervical ganglion. We also used the isolated ear, stimulating the mixed nerve at the base of the ear, using the perfusion system described by Burn (1952). For stimulation, the nerve was carefully dissected from its bed for about 7 mm. leaving both the central and the distal part undissected to keep the nerve in place. A pair of electrodes were placed around the dissected portion. Stimulation in all experiments was by square wave pulses usually of 2 msec. duration at a frequency of 25 per sec. The strength of stimulus was usually 1 mA. For observations on the pilomotor response to sympathetic stimulation in the cat's tail, cats were anaesthetized with chloralose and eviscerated to facilitate access to the lumbar sympathetic chain. We perfused the tail with Locke solution containing 4.5 g./l. sucrose at 35°, inserting the arterial cannula into the internal iliac artery of one side pointing towards the bifurcation; we tied the internal iliac of the other side and aorta just above the origin of the internal iliacs; thus the perfusing fluid passed into the caudal stump of the aorta. We stimulated the right lumbar sympathetic chain.

RESULTS

Nictitating Membrane.—In a previous paper (Burn and Rand, 1958b) we observed that when a cat had been treated with reserpine, although a large dose of tyramine failed to cause contraction of the nictitating membrane, stimulation of the postganglionic fibres was still able to do so. In Fig. 1 the effect of atropine on the contractions following sympathetic stimulation is shown. In a normal cat, the contraction was only slightly reduced by atropine even in the large dose of 4 mg./kg., but in a cat treated with reserpine it was very greatly reduced by this dose. In this cat the contraction increased after an intravenous infusion of 0.5 mg. noradrenaline.

Fig. 2 taken from an experiment in a cat treated with reserpine shows that eserine, given in two injections of 0.1 mg. each, augmented the contractions caused by sympathetic stimulation, especially those of weaker stimuli, and that atropine in a dose of 4 mg. (=1.5 mg./kg.) abolished the contractions. In two other experiments the effects of eserine and atropine were similar; in both, the effects of weaker stimuli were abolished by atropine and those of stronger stimuli greatly reduced. The threshold strength of stimulation was determined in three experiments and was found to be 0.39 mA., 0.075 mA.

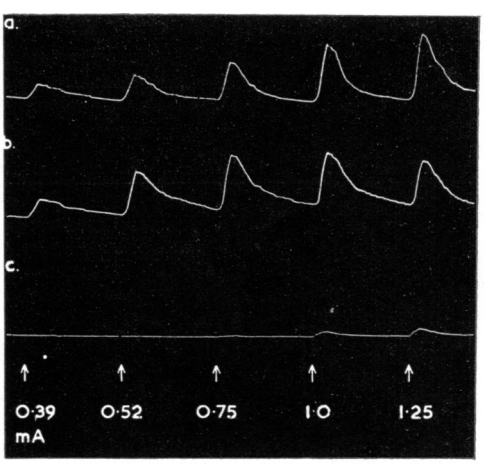

FIG. 2.—Contractions of nictitating membrane in response to postganglionic stimulation in cat treated with reserpine. (*a*) Responses to increasing strengths of stimulation as shown below, (*b*) increased responses after the injection of 0·2 mg. eserine, (*c*) responses abolished after the injection of 1·5 mg./kg. atropine.

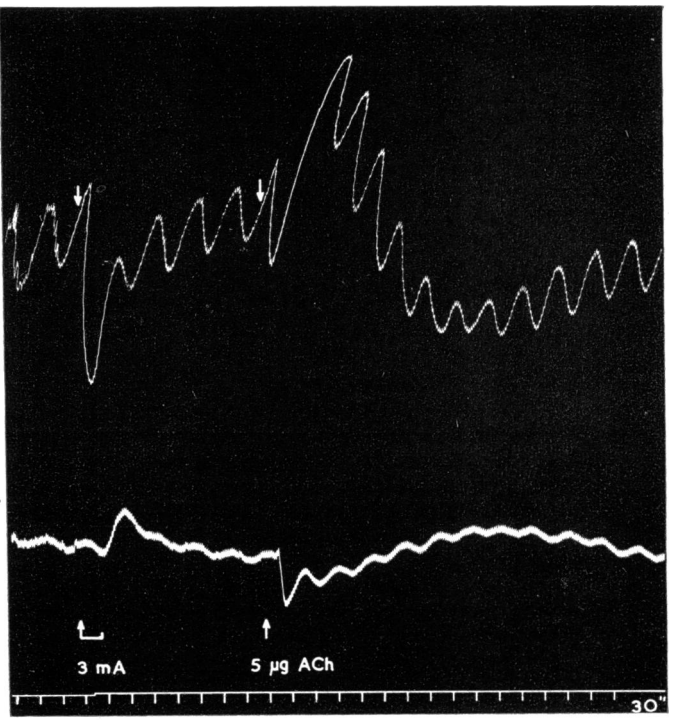

FIG. 3.—Spleen volume and blood pressure in cat treated with reserpine. Stimulation of splenic nerves for 30 sec. with strength 3 mA. caused small contraction of the spleen, and intravenous injection of 5 µg. acetylcholine caused dilatation followed by small slow contraction.

and 0.31 mA.; the threshold strength in one experiment on a normal cat was 0.25 mA.

Spleen.—Stimulation of the splenic nerve caused contraction of the spleen as shown in Fig. 3, in which the observation was made in a cat treated with reserpine. The effect was much smaller than that seen in a normal cat, but complete abolition of the contraction by reserpine treatment was rarely obtained. Fig. 3 also shows that in a cat treated with reserpine the intravenous injection of 5 µg. acetylcholine caused dilatation of the spleen, followed, as the blood pressure recovered, by a slight constriction. The stimulation of any cholinergic fibres which might be present in the splenic nerve would be expected to cause dilatation of the spleen, and would oppose the constrictor action of released noradrenaline.

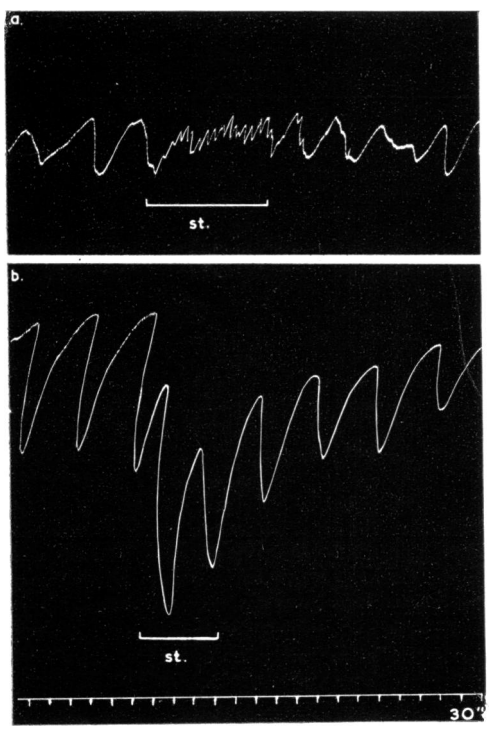

FIG. 4.—Spleen volume of cat treated with reserpine. (*a*) Stimulation of splenic nerves in presence of eserine caused a series of small waves, but had no general effect on the volume. (*b*) The same stimulation after the injection of 1 mg. atropine caused contraction of the spleen.

Such a balance of opposed effects would explain the observations recorded in Fig. 4, also made in a cat treated with reserpine; at first stimulation caused no appreciable volume change but altered the record during the period of application, so that there were many small waves. Then 1 mg. atropine was injected, and 4 min. later stimulation was repeated and a constrictor action was recorded, as if the injection of atropine had excluded the dilator effect of acetylcholine which initially masked the constrictor effect.

In one experiment recorded in Fig. 5, in a cat which was treated with reserpine, the effect of sympathetic stimulation was to produce dilatation of the spleen. An injection of eserine was then given and a repetition of the stimulation caused a

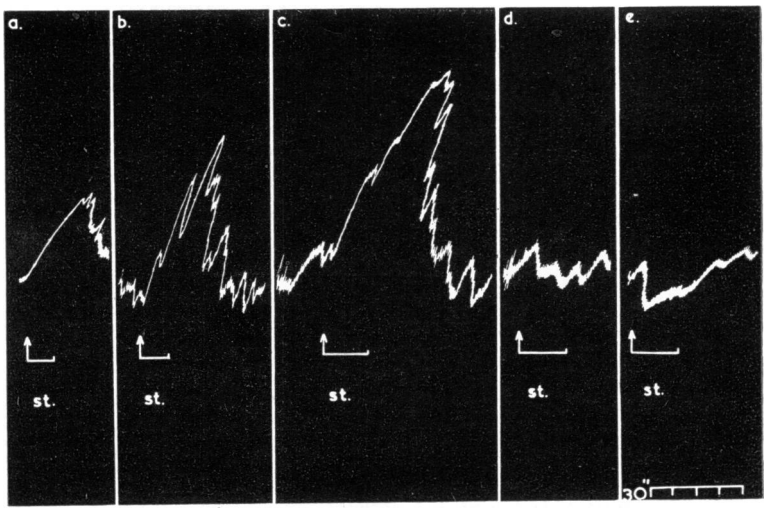

FIG. 5.—Spleen volume of cat treated with reserpine. (*a*) Stimulation of splenic nerves for 30 sec. caused dilatation of the spleen. (*b*) After the injection of 0·35 mg. eserine, stimulation for 30 sec. caused a greater dilatation. (*c*) Stimulation for 60 sec. caused still greater dilatation. (*d*) Stimulation for 60 sec. after 1·3 mg./kg. atropine had no effect. (*e*) Stimulation for 60 sec. after infusion of 0·25 mg. noradrenaline caused a very slight contraction.

rather larger dilatation. Stimulation was then applied for twice as long, causing a much larger dilatation. An injection of 4 mg. atropine was then given and 3 min. later stimulation had no effect. These were the principal observations, after which an infusion of 0.25 mg. noradrenaline was given. A further stimulation then caused a very slight constriction. An injection of tyramine which previously had no action caused a large constriction.

In other experiments of this kind in cats which were treated with reserpine, stimulation of the fibres of the splenic nerve caused a small constriction even in the presence of eserine ; it was, however, regularly observed that after the injection of 1 mg. atropine the constrictor response became 2 or 3 times greater than before.

Non-pregnant Cat Uterus. — Since the sympathetic innervation of the cat uterus when virgin or non-pregnant is inhibitory, observations were first made in normal cats and later in cats treated with reserpine. Fig. 6 is taken from an experiment in a normal virgin cat, and shows the inhibition due to stimulation of the hypogastric nerves. Eserine was then injected intravenously in three doses of 0.1 mg. at intervals of 5 min., and the stimulation was repeated. As a result the inhibition was diminished. Atropine was then injected in two doses each of 1 mg., and the stimulation was applied again ; the inhibitory effect was then greater.

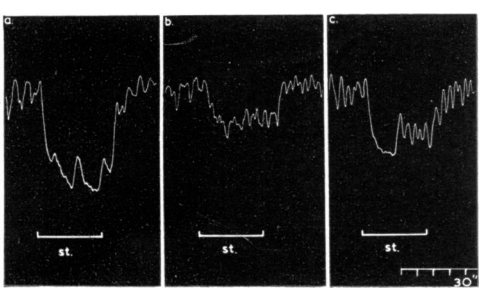

FIG. 6.—Uterus of virgin cat. (*a*) Stimulation of hypogastric nerves caused inhibition. (*b*) After injection of 0·3 mg. eserine the inhibition was reduced. (*c*) After the injection of 1 mg./kg. atropine the inhibition was increased.

In observations on virgin cats treated with reserpine, the inhibitory effect was absent as shown in Fig. 7. Stimulation of the hypogastric nerves caused a small contraction, which was augmented in the presence of eserine. Atropine was then injected, and stimulation no longer caused contraction, but a very slight relaxation. An intravenous injection of 0.5 μg. adrenaline caused relaxation. These results in the cat treated with reserpine were observed in 4 experiments.

An observation was also made on the isolated horn of the uterus of a normal virgin bitch ; it was found that acetylcholine caused contraction, but that in the presence of atropine it caused inhibition.

Perfused Rabbit Ear. — When perfusing the rabbit ear we did not find it easy to abolish the constrictor response to sympathetic stimulation by giving reserpine beforehand. Using the Gaddum-Kwiatkowski method we observed, however, that the effect of sympathetic stimulation was increased by adding eserine (5 μg./ml.) to the perfusing fluid. With Thorp's recorder it was possible to determine the volume of fluid (in drops) by which

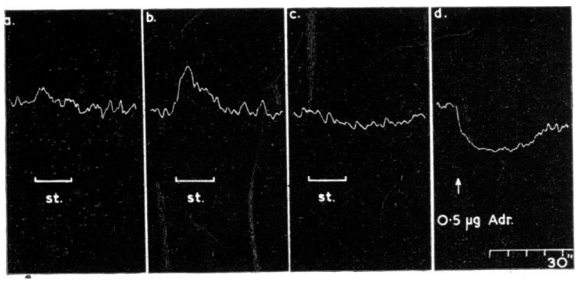

FIG. 7.—Uterus of virgin cat treated with reserpine. (*a*) Stimulation of hypogastric nerves caused slight contraction. (*b*) After injection of 0·3 mg. eserine, stimulation caused a greater contraction. (*c*) After the injection of 1 mg. atropine, stimulation caused a very slight relaxation. (*d*) Injection of 0·5 µg. adrenaline caused relaxation.

sympathetic stimulation, for a given time, reduced the outflow. Thus in one experiment the outflow was reduced by 330 drops in a first period of stimulation and by 356 drops in a second. Eserine was then added to the perfusion fluid and sympathetic stimulation then reduced the outflow by 486 drops. Similar observations were made in the isolated ear perfused through a cannula tied in the central artery at the base of the pinna. Fig. 8 shows the fall in outflow during stimulation of the mixed nerve, which was much greater after the addition of 3 µg./ml. eserine to the perfusion fluid.

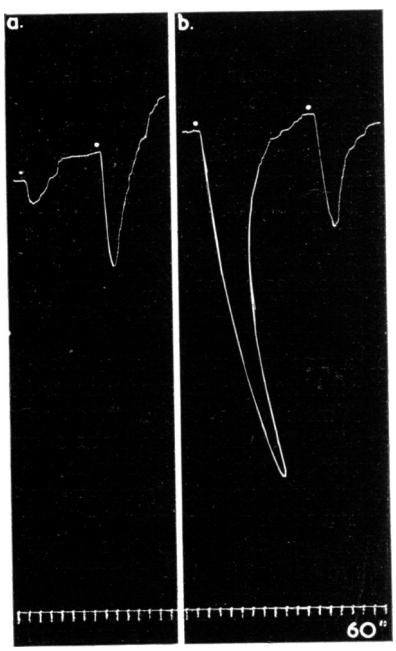

FIG. 8.—Record of outflow from rabbit ear during perfusion with Locke solution. (*a*) Stimulation of mixed nerve at the base of the ear for 10 sec. and then for 20 sec. caused slight reductions in outflow (5 mm. and 24 mm. on drum). (*b*) Stimulation 15 min. after eserine (2 µg./ml.) was added to the perfusion fluid, the duration of stimulation being respectively 20 sec. and 10 sec., caused larger reductions in outflow (75 mm. and 25 mm. on drum).

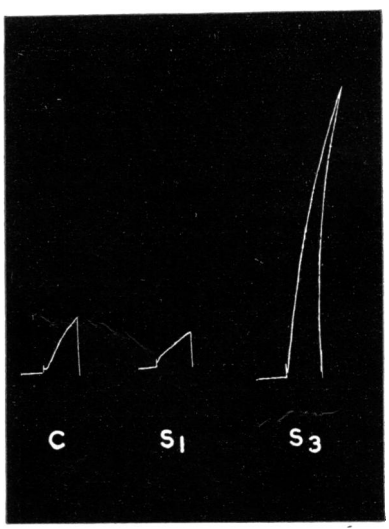

FIG. 9.—Tests of venous effluent from rabbit ear vessels on eserinized leech muscle. Perfusion with Locke solution containing eserine 5 µg./ml. At (C), effect of effluent before stimulation. At (S_1) effect of effluent during stimulation for 1 min. At (S_3) effect of effluent collected during the third min. after stimulation.

Using the Gaddum-Kwiatkowski perfusion we collected the venous effluent and tested it on the leech. Fig. 9 shows that samples collected after stimulation had a stimulant action on the leech muscle. Similar positive results were obtained in 5 experiments.

Block at Postganglionic Terminations. — We confirmed the observation of Brücke (1935) that, when a large amount of acetylcholine or of nicotine was injected into the skin at the base of a tuft of hair of the cat's tail, the effect of stimulation of the lumbar sympathetic chain was blocked, so that, while the adjacent tufts, respectively nearer to and further from the base of the tail, were erected during stimulation, the tuft at the site of injection remained unaffected. Fig. 10 gives an example of this action. A similar picture for nicotine is shown in Fig. 11.

These clear results were not obtained in all experiments, for the injection of nicotine or of acetylcholine not only blocked the effect of sympathetic stimulation, but partially blocked the effect of noradrenaline or adrenaline.

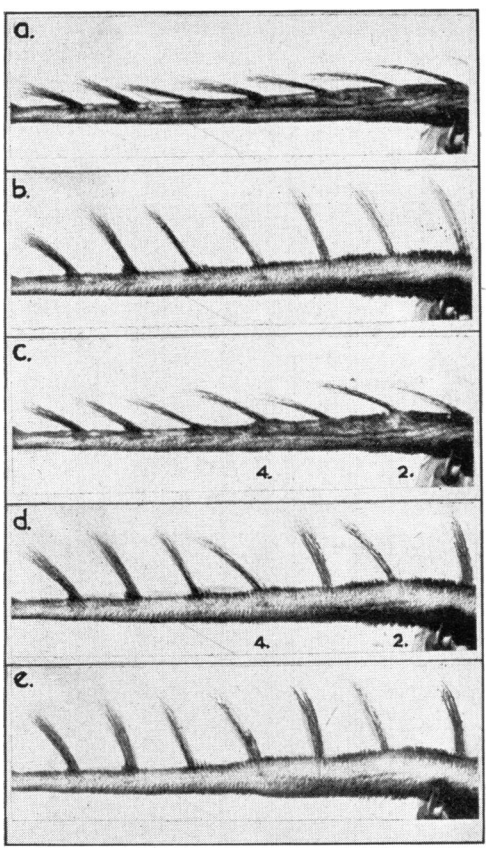

FIG. 10.—Pilomotor response in the tail of the cat (*a*) before stimulation, (*b*) during stimulation of right lumbar sympathetic chain, (*c*) after the intradermal injection of 0·2 mg. acetylcholine at the base of tufts 2 and 4, then (*d*) during stimulation tufts 2 and 4 were not fully erected, (*e*) after intravenous injection of 20 μg. noradrenaline all tufts were erected.

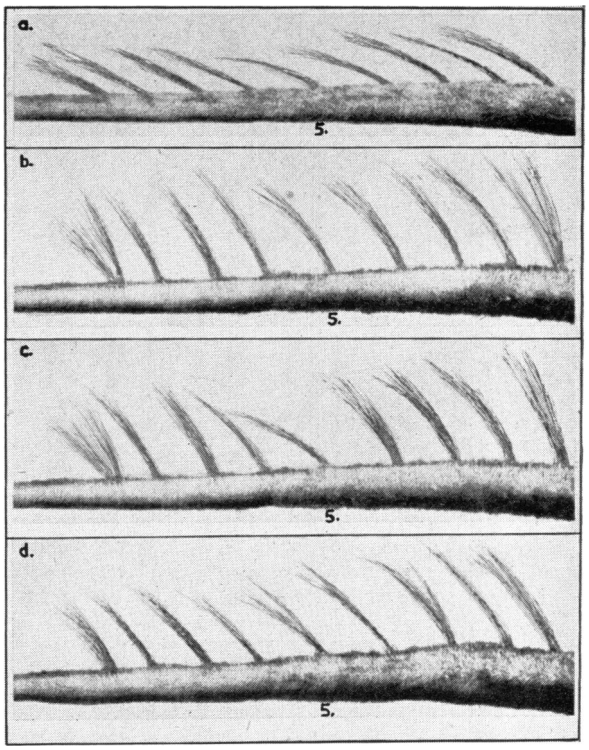

FIG. 11.—Pilomotor response in the tail of the cat. (*a*) Normal, (*b*) during sympathetic stimulation, (*c*) sympathetic stimulation, after intradermal injection of 0·2 mg. nicotine (base) at root of tuft 5, failed to erect this tuft, (*d*) intravenous injection of 30 μg. adrenaline caused erection of all tufts.

Other observations were made in the rabbit ear perfused with Locke solution by the method of Gaddum and Kwiatkowski (1938). We stimulated the postganglionic fibres and observed constriction. We observed that when acetylcholine was added to the Locke solution perfusing the ear, in concentrations from 2.5 to 20 μg./ml., the effect of sympathetic stimulation was blocked, but at the same time the constrictor effect of an injection of noradrenaline was reduced. Fig. 12 produces an illustration of this effect.

Observations with Choline 2,6-Xylyl Ether Bromide.—We had more success in blocking sympathetic effects with choline 2,6-xylyl ether bromide synthesized by Hey and Willey (1954) and investigated as a sympathetic blocking agent by Exley (1957). We tested its action by stimulation of the postganglionic fibres from the superior cervical ganglion both on the iris

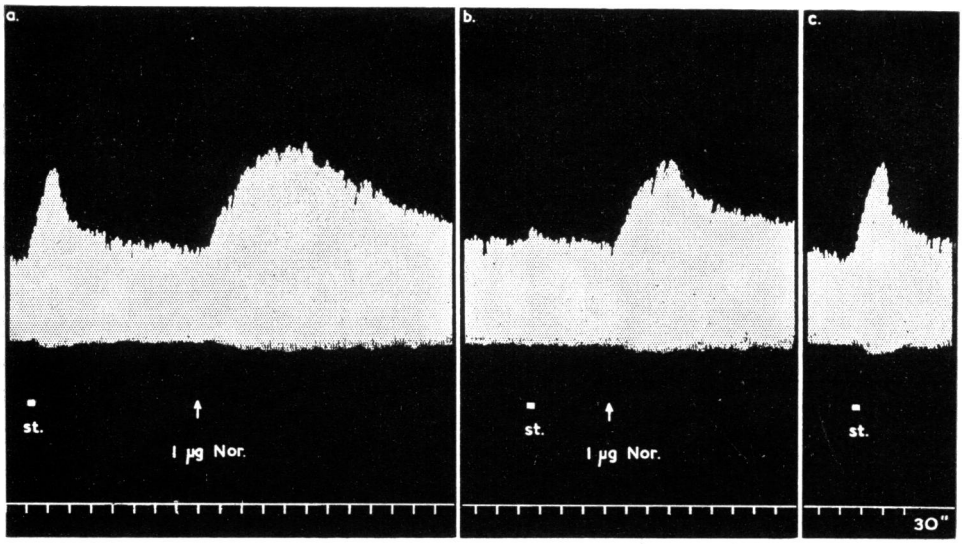

FIG. 12.—Gaddum drop-timer record of outflow from perfused rabbit ear. (*a*) At (st.): stimulation of postganglionic fibres caused constriction; at (1 µg. Nor): injection of noradrenaline. (*b*) Observations when 2·5 µg./ml. acetylcholine was added to perfusion fluid. At (st.): stimulation was blocked; at (1 µg. Nor): effect of injected noradrenaline was reduced. (*c*) At (st.): stimulation again effective when the ear was perfused without acetylcholine.

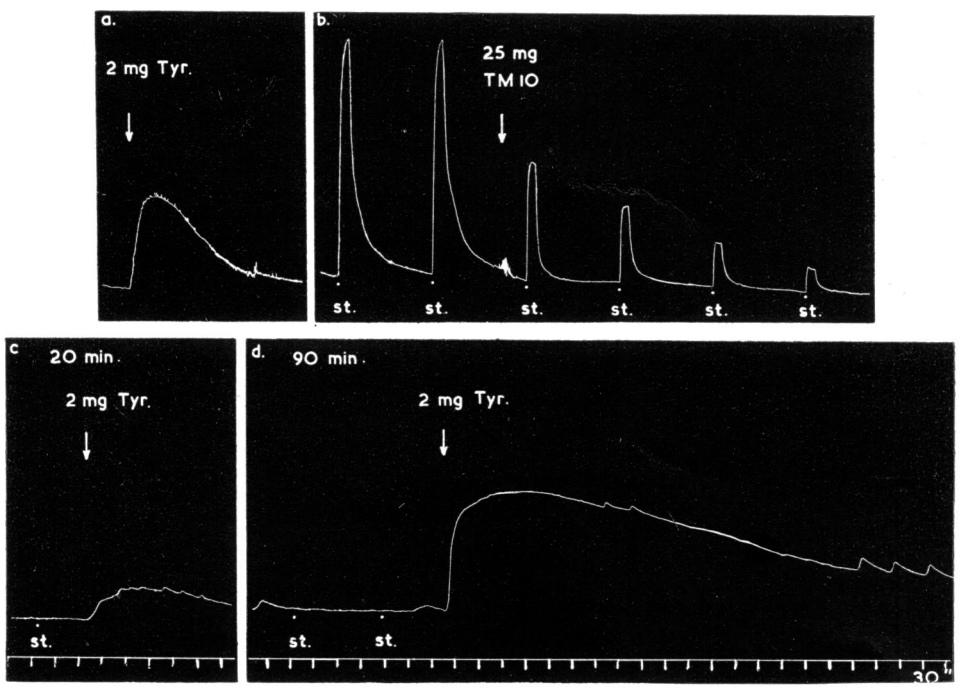

FIG. 13.—Nictitating membrane of spinal cat. (*a*) Intravenous injection of 2 mg. tyramine caused contraction, (*b*) stimulation of postganglionic fibres at 2 min. intervals. Injection of 25 mg. choline 2,6-xylyl ether bromide (TM10) reduced the response, (*c*) 20 min. after the injection of choline 2,6-xylyl ether bromide, the effect of stimulation was absent, and the effect of 2 mg. tyramine (Tyr) was reduced, (*d*) 90 min. after the choline 2,6-xylyl ether bromide, stimulation was ineffective even with a 6 times stronger stimulus, and 2 mg. tyramine had a greater effect than in (*a*).

and on the nictitating membrane. After the injection of 10 mg./kg. stimulation soon failed to act in both organs. Fig. 13 shows an experiment on the nictitating membrane in which the response to sympathetic stimulation was compared with that to tyramine. The immediate effect of choline 2,6-xylyl ether bromide was to depress the action of tyramine, but at the end of 90 min., when sympathetic stimulation with a strong stimulus was ineffective, the injection of tyramine produced a greater response than at first. Likewise tyramine still caused dilatation of the pupil. We also observed that the action of bretylium, N - *o* - bromobenzyl - N - ethyl - NN-dimethylammonium *p*-toluene-sulphonate (Boura, Green, McCoubrey, Laurence, Moulton, and Rosenheim, 1959), was essentially similar to choline 2,6-xylyl ether bromide in these respects, as shown in Fig. 14.

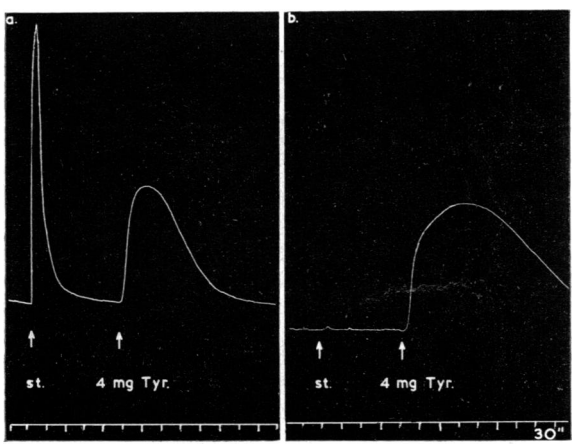

FIG. 14.—Nictitating membrane of spinal cat, 2·5 kg. (*a*) Contractions due to postganglionic stimulation (st.) and to 4 mg. tyramine (Tyr.), (*b*) 45 min. after injection of 25 mg. bretylium; stimulation ineffective, injection of 4 mg. tyramine had a greater effect than in (*a*).

Our colleague Huković also tested the action of choline 2,6-xylyl ether bromide and of bretylium on the constrictor action of acetylcholine observed in the presence of atropine in the perfused rabbit ear. He found that the constrictor response to acetylcholine and that to sympathetic stimulation was abolished, while that to tyramine was greater than before. Both substances also abolished the accelerator action of acetylcholine observed in the presence of atropine, and of sympathetic stimulation in the isolated atria, but augmented the action of tyramine.

DISCUSSION

Our previous observations (Burn and Rand, 1958b, 1960) have shown that the noradrenaline which can be extracted from tissues innervated by sympathetic fibres appears to play an important part in the action of tyramine and in the effect of sympathetic stimulation. The evidence was obtained from animals given reserpine and from normal animals. If the animal was treated with reserpine the extractable noradrenaline disappeared from the vessels, and both tyramine and sympathetic stimulation were then without effect. If an intravenous infusion of noradrenaline was given, both tyramine and sympathetic stimulation had an increased action, so that the effect of the infusion did not depend on the presence of reserpine. We have supposed that the infusion of noradrenaline partly refilled the store of extractable noradrenaline or in normal animals increased it. This is, however, a supposition, for attempts to demonstrate an increase of extractable noradrenaline have not succeeded as yet (Euler, 1956b).

Now acetylcholine and nicotine in the presence of atropine have been shown to exert a variety of actions which resemble those of stimulation of the sympathetic nerves. Since they lose these actions in animals treated with reserpine they may be acting by liberating noradrenaline from the store. These actions of acetylcholine and of nicotine, which are peripheral, include (a) vasoconstriction of the vessels of the rabbit ear (Burn and Rand, 1958a), (b) acceleration of the atria of the rabbit heart (Burn and Rand, 1958c), (c) pilomotor action in the cat's tail, (d) contraction of the nictitating membrane of the cat's eye (Burn, Leach, Rand and Thompson, 1959), and (e) inhibition of the isolated uterus of the virgin bitch. Moreover, de Burgh Daly and Scott (unpublished) have observed that acetylcholine injected into the splenic artery caused contraction of the spleen. We have found that intravenous injection of acetylcholine in the cat treated with reserpine caused dilatation of the spleen. There are in addition other actions which have not yet been demonstrated to be absent in the animal treated with reserpine. Ambache and Edwards (1951) observed that nicotine in the presence of atropine caused inhibition of the isolated ileum of the kitten.

The actions of acetylcholine and nicotine not only resemble those of the sympathetic nerves, but are in some way related to them, since the actions disappear after degeneration of the sympathetic

nerves. The constrictor action of nicotine in the perfused vessels of the rabbit ear, the pilomotor effect of nicotine and acetylcholine in the cat's tail, the contraction by nicotine of the nictitating membrane of the cat's eye, the contraction of the spleen by acetylcholine injected intra-arterially, are all lost after degeneration of the sympathetic nerves. This suggests that acetylcholine and nicotine act on part of the normal sympathetic mechanism, and it adds additional support to the suggestion that they release noradrenaline from the store near the nerve endings, since the store disappears when the sympathetic fibres degenerate (Euler and Purkhold, 1951 ; Burn and Rand, 1959).

If the store is part of the normal sympathetic mechanism and if acetylcholine can discharge noradrenaline from the store, the situation would become clarified if the postganglionic sympathetic fibres sending impulses to the store liberated acetylcholine.

Cholinergic fibres in sympathetic nerves were first demonstrated by v. Euler and Gaddum (1931) in the tongue of the dog. The classical case of a cholinergic innervation is, however, that in the sweat glands (Dale and Feldberg, 1935). Cholinergic fibres were then demonstrated in the sympathetic innervation of the vessels of the dog's hindleg (Bülbring and Burn, 1935) and in the innervation of the nictitating membrane (Bacq and Fredericq, 1935). Gaddum and Kwiatkowski (1939) observed that, in perfusions of the rabbit ear, stimulation of the great auricular and posterior auricular nerves liberated a substance in the venous effluent which caused contraction of the leech. However, they concluded that the liberation was not connected with sympathetic fibres, since the superior cervical ganglion had been removed two weeks before.

Recently Gillespie and Mackenna (1959) have found that, if rabbits were treated with reserpine, the isolated ileum was no longer inhibited by stimulation of the sympathetic fibres, but was caused to contract. The contraction was abolished by atropine, and Gillespie and Mackenna concluded that cholinergic fibres were present in the sympathetic supply. Recently also Huković (1959), working in this department, made a preparation of rabbit atria with sympathetic fibres attached. Stimulation of the fibres in the preparation from a normal rabbit caused acceleration of the atrial rate, but stimulation in a preparation from a rabbit treated with reserpine often caused inhibition, which was increased by eserine and abolished by atropine. He concluded that cholinergic fibres were present in the sympathetic supply.

In the present paper we have made similar observations in other tissues. Thus, in cats treated with reserpine, tyramine loses its action on the nictitating membrane altogether, but sympathetic stimulation does not ; it still causes a contraction. This contraction, however, we have found is abolished by atropine. When it has been abolished or greatly reduced by atropine it can be augmented again by an infusion of noradrenaline. This suggests that the fibres which are active in stimulating the nictitating membrane in the reserpine-treated animal are cholinergic. Similarly cholinergic fibres may be present in the splenic nerve, for, in some experiments from reserpine-treated animals, stimulation of these fibres caused dilatation of the spleen, like the injection of acetylcholine. In the nictitating membrane the effects of acetylcholine released from cholinergic fibres and of noradrenaline would appear to be additive. In the spleen the effects seem to be opposed, so that in reserpine-treated animals in which stimulation of the splenic nerve still caused contraction, the contraction was greatly increased after the administration of atropine, presumably because the dilator action of acetylcholine was abolished. Cholinergic fibres also seem to be present in the sympathetic supply to the virgin cat uterus. Here again the effects of acetylcholine and those of noradrenaline (or adrenaline) appeared to be opposed ; acetylcholine causes contraction of the virgin uterus, and stimulation of the hypogastric nerves in the reserpine-treated cat was found to cause contraction also, the contraction being increased by eserine and abolished by atropine. Thus a normally inhibitory sympathetic nerve supply seems to contain cholinergic fibres. Koelle (1955) from histochemical studies found that " there is probably some representation of typical cholinergic fibres in practically all predominantly adrenergic and sensory nerves." He went on to ask whether the terms cholinergic and adrenergic might refer to the predominant rather than to the exclusive types of transmitting agents of the nerve fibres.

Three observations respecting the postganglionic mechanism may now be considered together. First that the effect of sympathetic stimulation can be increased by infusing noradrenaline into the blood. Second that acetylcholine (after atropine), by a nicotine-like action exerted at the periphery, can reproduce sympathetic effects, and that it does so by causing a release of noradrenaline from the store at the nerve endings since it no longer acts when the sympathetic fibres have degenerated, or in the animal treated with reserpine. Third that

cholinergic fibres seem to be present wherever they are looked for in sympathetic fibres.

These observations at once suggest a function for the cholinergic fibres present in the sympathetic supply to organs like the spleen. The cholinergic fibres are probably adrenergic *in effect,* since the acetylcholine they discharge will act on the store of noradrenaline and liberate noradrenaline from it. This idea serves to explain an observation previously made by one of us (Burn, 1932) and illustrated in *J. Physiol.* (vol. 75, p. 150). There has hitherto been no explanation for it. When the lumbar sympathetic chain was stimulated during perfusion of the hindleg of a dog, a stimulus of 3 sec. duration caused a fall in arterial resistance. When the same stimulus was applied for 30 sec. the main effect was a rise in arterial resistance. It appeared that in stimulating for 3 sec. vasodilator fibres were excited, while in stimulating for 30 sec. vasoconstrictor fibres were excited. It may now be suggested that the 3 sec. stimulation resulted in a release of acetylcholine and a fall of arterial resistance due to the direct action of this substance; the 30 sec. stimulation resulted in a more prolonged release of acetylcholine, which was enough to liberate noradrenaline from the store. The usual conception of adrenergic fibres is that they are fibres directly liberating noradrenaline. We now have a second conception, namely, that fibres liberating acetylcholine may, by doing so, discharge noradrenaline from the store.

The cholinergic fibres may be blocked by excessive amounts of acetylcholine, as Brücke (1935) first showed in the pilomotor muscles of the cat's tail, and as we have seen in the vessels of the rabbit ear. The action of the blocking agents choline 2,6-xylyl ether bromide and of bretylium may be similar. These substances block sympathetic impulses completely, but do not prevent the action of tyramine. Indeed, 1 hr. after giving choline 2,6-xylyl ether bromide or bretylium, the action of tyramine is increased. Choline 2,6-xylyl ether bromide and bretylium, which block not only sympathetic stimulation but also the action of acetylcholine in producing sympathetic effects, seem well suited from their structure to prevent the action of sympathetic fibres from releasing noradrenaline from the store.

At the present time we have nothing more than hints on the site of the store and we mention them only to indicate directions in which further research might be made. v. Euler (1956) has expressed the view that the noradrenaline present in the spleen is probably within the fine terminations of the nerves since there is no evidence that it is located outside the nervous tissue itself. He calculated that the amount present would be from 3 to 30 mg./g., though the amount in the nerves before they reach the spleen is (in the ox) about 15 μg./g. If the store is located as v. Euler suggests, then the fine nerve terminals must be able to take up noradrenaline from the circulating blood. The fact that the store disappears when the nerves degenerate is consistent with the view that the store is within the nerves. On the other hand the fact that acetylcholine injected into the splenic artery can release noradrenaline from the store might indicate that the store is outside the nerves. It is conceivable that the store is held in chromaffin cells which have been described by Nordenstam and Adams-Ray (1957) and confirmed by Burch and Phillips (1958) in human skin, and have been observed by Leach (see Burn and Rand, 1958a) in the skin of the rabbit ear. They have also been observed in the cat's nictitating membrane and in the skin of the cat's tail (Burn, Leach, Rand, and Thompson, 1959). These cells disappeared in animals treated with reserpine, and in the nictitating membrane they also disappeared after degeneration of the sympathetic fibres. Much further work is, however, required to connect them with the store.

Our observations raise questions about the postganglionic sympathetic fibres, though they cannot be answered at present. Since the evidence for purely adrenergic fibres is very strong [obtained from histochemical examination of sympathetic ganglia (Koelle and Koelle, 1959) as well as from chemical identification of noradrenaline, dopamine and dopa decarboxylase in the fibres (Schümann, 1958)], there appear to be two mechanisms by which noradrenaline is released. The first is by simple release from the adrenergic fibres, and the second by the action of cholinergic fibres releasing noradrenaline from the store. These mechanisms seem fundamentally different, and it is, therefore, surprising that they should exist side by side; it is further surprising that both should be blocked by choline 2,6-xylyl ether bromide and by bretyllium.

Another question concerns the cholinergic fibres. Do some of these liberate acetylcholine to act directly, and others liberate it to set free noradrenaline from a store? The sweat glands are stimulated by the release of acetylcholine, but the cholinergic fibres to the muscle vessels of the dog's hindleg may certainly prove to be fibres whose main function is to release noradrenaline from a store, since in many dogs vasodilator

effects of stimulation are not seen unless special steps (such as the use of eserine) are taken to unmask them.

A conception which might unify the observations is that sympathetic postganglionic fibres were, like the other peripheral efferent fibres, originally cholinergic. In the course of evolution, for the purpose of developing an adrenergic mechanism, they came to innervate a store of noradrenaline, perhaps present in chromaffin tissue, so that the innervation in some respects resembled that of the adrenal medulla. In further development, the fibres were modified so that the noradrenaline in the chromaffin tissue was developed in the fibre itself, the true adrenergic fibres thus being formed. The blocking action of bretylium may, however, indicate that even these still possess some kind of cholinergic background. Thus postganglionic fibres may contain representatives of the three stages of development, the proportion varying according to the tissue innervated and perhaps according to the species.

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ANOXIA AND VENTRICULAR FIBRILLATION; WITH A SUMMARY OF EVIDENCE ON THE CAUSE OF FIBRILLATION

BY

J. H. BURN AND S. HUKOVIĆ

From the Department of Pharmacology, University of Oxford

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Experiments are described showing that ventricular fibrillation is more readily produced in isolated rabbit hearts by electrical stimulation when the oxygen supply to the heart is reduced. This evidence completes investigations which have been made into factors affecting the production of fibrillation. These investigations have shown that factors which shorten the duration of the action potential, particularly those which cause the "plateau" to disappear, facilitate fibrillation, and those which lengthen the duration of the action potential prevent fibrillation. The reason for the length of the cardiac action potential may therefore be to prevent fibrillation. When the action potential is of normal length the fact that two adjacent fibres are out of phase does not matter; the one which is first to contract is not re-excited by the one which is second to contract, because at that moment the first is inexcitable. If the action potential is short, then the first may be already repolarized and may be excited by spread of excitation from the second. Factors which inhibit metabolism shorten the action potential.

When electrodes are attached to the ventricle of the isolated rabbit heart perfused through the aorta, a high rate of stimulation can then be applied for a given time. This causes the ventricles to fibrillate, as may be seen when the stimulation stops by using the electrodes as leads to an ECG. Ordinarily the fibrillation induced in this way continues only for 1 to 2 min. and a normal rhythm returns. However, under various conditions, such as in the presence of a low concentration of potassium or a high concentration of calcium, or in the presence of metabolic inhibitors, the fibrillation has been found to persist. The observations now to be described have been made to test the effect of a partial lack of oxygen.

METHODS

Rabbits were stunned and bled. The hearts were removed and attached to the apparatus for perfusion as already described (Armitage, Burn, and Gunning, 1957). The perfusion fluid contained (in mM.) NaCl 132, KCl 5.6, $CaCl_2$ 2.2, $NaHCO_3$ 2.5, $NaH_2PO_4.2H_2O$ 0.92, dextrose 11.1, sucrose 13.1. The solution was saturated with 95% oxygen+5% CO_2 for the control observations, and with 47.5% oxygen, 47.5% nitrogen and 5% CO_2 for the rest. The temperature of the perfusing solution was 32° and was maintained within ±0.5°, using the device of Saxby (1956). Hearts were perfused at a pressure of 50 cm. water. Platinum fish-hook electrodes were inserted in the wall of the left ventricle, one at the apex and the other near the base. The stimuli were 1–2 mA. in strength, 2 msec. in duration and applied at a frequency of 20/sec. for 3 min. When stimulation was not being applied, the electrodes were used as leads to a Cossor electrocardiograph (model 1314); a record of the rhythm was obtained every 5 min. during fibrillation. The solutions saturated with the two gas mixtures were kept in separate flasks and perfused from separate Marriotte bottles.

When perfusion with any solution was begun, it was continued for at least 15 min. before stimulation was applied. When after stopping stimulation a normal rhythm was restored within 5 min., the result was recorded as "no fibrillation." When fibrillation persisted for more than 5 min., the result was recorded as "fibrillation."

RESULTS

Effect of Anoxia.—The following is an example of an experiment to determine the effect of reducing the oxygen supply. An isolated rabbit heart was perfused with a solution saturated with 95% oxygen for 15 min. and was then stimulated electrically for 3 min. When the stimulation stopped the normal rhythm was resumed at once. The perfusion fluid was then changed to one

saturated with 47.5% oxygen and 47.5% nitrogen, and after allowing this solution to perfuse for 20 min., stimulation was applied again. When the stimulation stopped, the heart was fibrillating and continued to do so for the next 15 min. Ice-cold saline was then poured over the heart and a normal rhythm returned. The perfusion fluid was changed back to the solution saturated with 95% oxygen. Stimulation was applied for 3 min. and when it was stopped a normal rhythm returned at once. The perfusion fluid was again changed to one saturated with 47.5% oxygen and 47.5% nitrogen, and after 20 min. the stimulation was applied for 3 min. Again the heart fibrillated when the stimulation was turned off, and continued to do so for the next 15 min.

This straightforward result was not obtained in all experiments, for in some the heart did not fibrillate when the solution contained only 47.5% oxygen. Use was then made of the observation (Armitage, Burn, and Gunning, 1957) that, when the potassium concentration was lowered, the proportion of hearts fibrillating was increased. The following experiment is an example.

The heart was first perfused with the usual solution containing 5.6 mM. potassium. When the perfusion fluid was saturated with 95% oxygen, stimulation did not cause fibrillation. When the perfusion fluid was changed to one saturated with 47.5% oxygen, stimulation caused fibrillation, but this continued for 2 min. only and then a normal rhythm returned. The heart was next perfused with a solution containing a lower potassium concentration, 4.5 mM., and saturated with 95% oxygen. Stimulation caused fibrillation which continued for 1 min. only before a normal rhythm returned. The heart was then perfused with a solution also containing 4.5 mM. potassium but saturated with 47.5% oxygen. Stimulation then caused fibrillation which continued for more than 15 min.

In all, 14 experiments were carried out, and, in 9, evidence was obtained that the lower oxygen saturation caused the heart to fibrillate more readily. In 4 experiments the results were similar to those in the first experiment described, and were the more convincing because, after observing that when a heart was perfused with a solution saturated with 47.5% oxygen, stimulation caused persistent fibrillation, it was possible to return to the solution saturated with 95% oxygen and show that stimulation caused only transient fibrillation. The results of the 9 experiments are set out in Table I.

In Expt. No. 1 of Table I the potassium concentration was 4.2 mM., and the result of the first trial of stimulation, when the perfusion fluid was saturated with 95% oxygen, was that there was no fibrillation. The result of the second trial when the fluid was saturated with 47.5% oxygen was that persistent fibrillation developed. The result of the third trial was the same as that of the first.

TABLE I

EXPERIMENTS INDICATING THAT REDUCTION OF OXYGEN PERCENTAGE FAVOURED VENTRICULAR FIBRILLATION

The % oxygen represents the percentage of oxygen in the gas mixture with which the perfusion fluid was saturated.

Expt. No.	KCl (mM.)	No Fibrillation		Fibrillation	
		Trial	% Oxygen	Trial	% Oxygen
1	4·2	1	95	2	47·5
		3	95		
2	5·6	1	95	2	47·5
		3	95	4	47·5
3	5·6	1	95	2	47·5
		3	95		
4	5·6	1	95	2	47·5
		3	95		
5	5·6	1	95	2	47·5
6	5·6	1	95		
	5·6	2	47·5		
	4·5	3	95	4	47·5
7	5·6	1	95	2	47·5
8	5·6	1	95	2	47·5
				3	95·0
9	5·6	1	95	2	47·5
				3	95·0

There were 5 inconclusive experiments of which the following example is given. The heart was perfused with a solution containing 5.6 mM. potassium and was saturated with 95% oxygen. After stimulation fibrillation was transient. The heart was then perfused with the same solution saturated with 47.5% oxygen. When stimulation was applied, fibrillation was again transient. Perfusion was then continued with a solution containing a lower potassium concentration (4.4 mM.) and saturated with 95% oxygen. After stimulation this time, fibrillation was persistent. At the end of 15 min., fibrillation was stopped by pouring ice-cold saline over the heart. This result suggested that the reduction in potassium concentration had been too great, and a higher concentration (5.0 mM.) was tried. However, the heart fibrillated persistently when perfused with this solution saturated with 95% oxygen. Even

when a return was made to the original concentration of 5.6 mM. potassium, the heart perfused with the solution saturated with 95% oxygen fibrillated persistently after stimulation. Finally the heart was perfused with a solution containing 7.0 mM. potassium, but the heart did not fibrillate persistently after stimulation whether the solution was saturated with 95% or with 47.5% oxygen. With the lower saturation, however, it fibrillated for 4 min. There were 4 other similar inconclusive experiments which appeared to be due to an increasing tendency towards fibrillation as the duration of an experiment increased.

Discussion

The observations described show that when the perfusion fluid was saturated with a gas mixture containing 47.5% oxygen instead of 95% oxygen, electrical stimulation of the ventricles more readily produced persistent fibrillation. Now oxygen lack has been shown to shorten the duration of the cardiac action potential. Trautwein, Gottstein, and Dudel (1954) found that, in cat papillary muscle stimulated at 140/min., the duration of the action potential was diminished by oxygen lack, the plateau being lost. This effect was reversed when oxygen was supplied. The work was confirmed in a later paper (Trautwein and Dudel, 1956). Webb and Hollander (1956) showed that the action potential of the rat atrium was diminished by anoxia ; after 4 min. the diminution was 13%, after 10 min. it was 47% and after 17 min. it was 81%.

The evidence that the factors which facilitate fibrillation are those which shorten the action potential, and conversely that those which prevent fibrillation are those which lengthen it, is now considerable. Acetylcholine shortens the atrial action potential (Hoffman and Suckling, 1953) and facilitates atrial fibrillation in the dog (Burn, Vaughan Williams, and Walker, 1955). Acetylcholine has practically no effect on the ventricular action potential (Hoffman and Suckling, 1953) and has no effect on ventricular fibrillation (Armitage, Burn, and Gunning, 1957). Adrenaline lengthens the atrial action potential in the rat (Webb and Hollander, 1956), and prevents acetylcholine from causing atrial fibrillation (Burn, Gunning, and Walker, 1957).

Various metabolic inhibitors have been shown to shorten the action potential. Thus Macfarlane and Meares (1955) have shown that dinitrophenol reduces the plateau of depolarization in the atrium and ventricle of the frog to about one-third of its normal length, and Kuschinsky, Lüllmann and Muscholl (1958) have shown that it reduces the duration of the action potential in the atrial muscle of the rat. Dinitrophenol was found to facilitate fibrillation in the rabbit ventricle (Armitage, Burn, and Gunning, 1957), and this effect was reversible. Another metabolic inhibitor is sodium azide, which Macfarlane (1956) found would reduce the duration of the action potential in the frog ventricle, and in keeping with this Goodford (1958) found that, in the presence of sodium azide, rapid stimulation of the ventricles led to persistent fibrillation as in the presence of dinitrophenol. The effect of sodium azide was reversible.

A third metabolic inhibitor is sodium monoiodoacetate. Lüllmann (1959) found that in its presence the duration of the action potential in the atria of the rat was shortened, and Goodford (1958) found that in its presence rapid stimulation of the ventricles caused fibrillation. This effect was not reversible.

A lack of glucose has also been shown to shorten the action potential in the rat atrium, the shortening becoming greater, the longer the time of glucose deprivation (Webb and Hollander, 1956). Goodford (1958) found that after perfusing rabbit hearts with glucose-free solution they continued to beat normally for periods of 2 hr. or more. By the end of that time some hearts began to fibrillate spontaneously, and, when the perfusion fluid was changed to one containing glucose, the fibrillation was arrested.

A factor which works in the opposite direction is a fall of temperature. The effect of lowering the temperature on the duration of the action potential was observed by Trautwein, Gottstein, and Dudel (1954) in cat papillary muscle. They found that in the range 38°–19°, the action potential, particularly the plateau, became longer as the temperature fell. The same observation was also made by Hollander and Webb (1955). Marshall (1957) observed the same change in the transmembrane potential of single fibres of the rabbit atrium ; the duration of the action potential rose from 240 msec. to 400 msec. as the temperature fell from 27° to 18°. Now fibrillation can be arrested by pouring ice-cold saline over an isolated heart (Dirken, Gevers, Heemstra and Huizing, 1955). Beaulnes and Day (1957) working on isolated atria found that fibrillation produced at 37° was arrested when the temperature was lowered to 29° and began again when the temperature was raised. Similar observations were made by Goodford (1958) on the ventricles ; he found that fibrillation

was arrested when the temperature was lowered from 37° to 32°.

Changes in calcium concentration affect the duration of the action potential. Hoffman and Suckling (1956) have found that the plateau was prolonged and the action potential was longer when the calcium concentration was low. When the calcium concentration was raised, repolarization occurred at a faster rate. Milton (1959) has shown that the proportion of hearts fibrillating in response to electrical stimulation is directly proportional to the calcium concentration in the perfusing fluid.

Changes in potassium concentration finally have a special interest, since there are two effects. Weidmann (1956) showed that large amounts of potassium shortened the action potential of the turtle ventricle. Vaughan Williams (1959) also found that in rabbit atria the action potential was shorter at 10 mM. concentration than at 5 mM. However, below 5 mM. the action potential also shortened as the concentration was reduced. Now, Milton (1959) found that when the potassium concentration was decreased below 5.6 mM. the proportion of hearts fibrillating increased. On the other hand, high concentrations of potassium are known to have a fibrillatory action. For example, Grumbach (1956) found that the injection of 1 ml. 0.77 M. potassium chloride into the fluid perfusing an isolated heart caused fibrillation if the heart had been exposed to adrenaline earlier.

Having thus seen several examples which point to a connexion between the duration of the action potential and fibrillation, we may ask what the connexion is. When the action potential in a fibre is short the fibre becomes re-excitable very soon, and, if some nearby fibres are contracting, a spread of the excitation from them may cause the fibre to contract again.

We ordinarily suppose that an impulse from a pacemaker arrives at distant fibres simultaneously. But, when the impulses come quickly, the rate of conduction from the pacemaker to two distant but adjacent points may not be identical, and the impulse may not arrive simultaneously at the two fibres. One will then contract before the other contracts. Provided the action potential is long, the first fibre will be inexcitable when the second contracts. But if the action potential is short, and the first fibre is rapidly repolarized, then this fibre will be stimulated to contract again by spread of excitation from the second fibre. There will be a local extrasystole.

Thus fibrillation could result from the spread of excitation from one fibre to another, when the fibres are out of phase and when the action potential is very short. The purpose of the long action potential of cardiac muscle seems to be to prevent this spread of excitation and so to prevent fibrillation. It is clear that the maintenance of the long action potential depends on a process which requires energy, since it is not maintained when there is lack of oxygen or glucose, or when metabolic inhibitors are present.

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THE NEUROMUSCULAR BLOCKING PROPERTIES OF A SERIES OF BIS-QUATERNARY TROPEÏNES

BY

C. G. HAINING, R. G. JOHNSTON AND J. M. SMITH

From the Duncan, Flockhart Research Laboratories, Wheatfield Road, Edinburgh

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Linkage of two tropine esters through their nitrogen atoms by the chain $-[CH_2]_m-O-CO-[CH_2]_n-CO-O-[CH_2]_m-$, in which m was 2 or 3 and n varied from 0 to 6, gave compounds which produced neuromuscular block without depolarization. Reversibility by neostigmine was confirmed for a few compounds. Potency was found to depend upon the tropine ester employed and upon the values of n and m. Short duration and hypotensive properties were favoured by the higher values of n. The duration of action of the compound based on the phenylacetic acid ester of tropine, in which $n=4$ and $m=2$, varied considerably in different species. Epimerization, in which the relative positions of the methyl group and the linking chain on the quaternary tropane nitrogen atom were reversed, did not produce substances having more favourable properties than those possessed by the unepimerized compounds.

Bis-onium compounds in which the quaternary nitrogen atoms are present as part of substituted tropane nuclei have been examined for neuromuscular blocking properties by Gyermek and Nádor (1957). Some were considerably more active than tubocurarine in laboratory animals, but so far none has shown sufficient advantages over muscle relaxants currently in use to justify its introduction into clinical practice.

Linking of the tropane nuclei in such compounds has been effected in three ways; by means of an alkyl or aralkyl chain between the nitrogen atoms of the tropane nuclei (Kimura and Unna, 1950; Gyermek and Nádor, 1952; Eckfield, 1959), through the tropic acid hydroxyl groups of two atropine molecules with the formation of ether linkages (Kimura, Unna and Pfeiffer, 1949), or through the 3-hydroxyl groups of quaternized tropine molecules to form di-esters of dicarboxylic acids such as isatropic (Hotovy, Jacobi and Kuessner, 1956; Just, 1953), succinic or phthalic acid (Gyermek and Nádor, 1953).

The esterifying acid, the quaternizing group used, and the chain length are all known to be of importance in determining potency and side actions (Gyermek and Nádor, 1957; Haining, Johnston and Smith, unpublished observations), but the effect of chain structure on duration of action has received little attention. In $\alpha\omega$-dicarboxylic esters of choline the presence of ester groups at suitable points within the chain shortens duration of action (Brücke, 1956). However, potent members of this series, such as suxamethonium, have disadvantages (Churchill-Davidson, 1958), one of the most important being that, since they are depolarizing agents, paralysis due to them cannot be reversed by neostigmine. On the other hand, bis-quaternary tropeïne neuromuscular blocking compounds tend to exert a competitive type of action (Haining *et al.*, unpublished observations).

The compounds described here were prepared in the hope that, by linking two quaternary tropeïne moieties by a chain susceptible to attack by esterases, it would be possible to obtain competitive neuromuscular blocking agents of short duration.

METHODS

Neuromuscular Block in Cats, Dogs and Rabbits.—The neuromuscular blocking activity of compounds was investigated in cats anaesthetized with a mixture of chloralose 50 mg./kg. and urethane 500 mg./kg. given by intraperitoneal injection, or in dogs which had received pentobarbitone sodium intraperitoneally. One hind leg was fixed rigidly in a vertical position by means of a pin through the lower end of the femur. Shielded silver electrodes were placed on the sciatic nerve which was then crushed proximal to the electrodes. The gastrocnemius muscle was attached to a flat spring myograph and the contractions recorded on smoked paper. Muscle twitches were elicited by supramaximal rectangular pulses of less than 1 msec. duration.

The standard used throughout these experiments was *NN'*-4,9-dioxo-3,10-dioxadodecamethylenebis(3-phenylacetoxytropanium bromide) (DF596). The relative potency of this compound in different species has been reported previously (Haining, Johnston and Smith, 1959). It was approximately half as potent as tubocurarine in the cat, and the recovery rate was approximately equal to that of suxamethonium (Fig. 1). DF596 has the advantage over compounds such as gallamine triethiodide or tubocurarine commonly used as standards in that it has little or no cumulative properties. This allowed considerably more estimates to be made in any given period of time. The quantities in the text refer to chloride unless otherwise stated. The potency and duration of action of compounds and their effects on blood pressure were determined by comparison with DF596 in the same animal. Whenever possible doses of each compound were chosen which would reduce the height of the gastrocnemius twitch by approximately 25% and 75%. The percentage reduction in twitch height and fall in blood pressure resulting from each dose were plotted against the logarithm of the dose. Since the slopes of the regression lines varied with the rate of recovery, potencies were compared at doses estimated to give a 50% reduction of twitch, and effects on blood pressure were compared at these doses. Estimates of duration of action were made by visual comparison of tracings. Values obtained for long-acting compounds were least reliable since, with compounds of the same duration as gallamine triethiodide, cumulative effects made comparisons difficult.

Blood pressure was recorded from the carotid artery with a mercury manometer. Animals were usually maintained with artificial respiration. Drugs were dissolved in physiological saline and were injected into the femoral vein.

In order to determine the rate of recovery in rabbits, drug solutions were infused into the marginal ear vein by means of a continuous slow injector (Palmer). Head drop was judged to be complete when the rabbit was unable to raise its head if tapped gently on the nose with a finger. The infusion was then stopped. Times required for the following stages in recovery were recorded: (1) The animal was able to raise its head in response to a light tap on the nose. (2) The animal was able to right itself immediately when quickly placed on its back. (3) With the hind limbs lifted clear of the ground, the animal was able to support itself and walk on its forelimbs. The time for complete recovery, when the animal was able to walk without assistance, was also determined. This, however, could not be obtained so precisely as with the other measurements, since animals after recovering from paralysis usually showed little inclination to move. During infusions, animals were allowed to sit on a rubber mat, and were restrained slightly with the hand. They usually remained quite still as long as they were able to get a good grip with their feet.

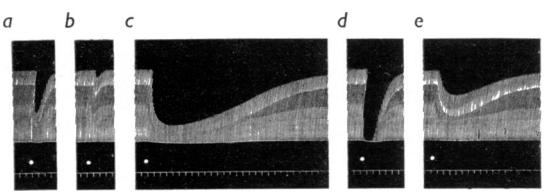


FIG. 1.—Cat. Maximal twitches of the gastrocnemius muscle elicited by indirect stimulation. Time, min. All drugs were given intravenously. (*a*) At time 0 min., 0.5 mg./kg. of DF596 was given. (*b*) At 7 min., 0.3 mg./kg. of DF596. (*c*) At 14 min. 0.3 mg./kg. of tubocurarine chloride. (*d*) At 67 min., 0.8 mg./kg. of DF596. (*e*) At 78 min., 0.15 mg./kg. of tubocurarine chloride.

The effects of large intravenous doses in conscious rabbits were investigated in animals maintained with the aid of a Drinker-type respirator. The rabbit was placed in a Perspex box with head protruding and chin resting on a support. A good seal at the neck was obtained with a collar of tambour rubber. Partial vacuum within the box was obtained by an Edwards two-stage vacuum pump 2SC50, and was broken at intervals by a simple spring-loaded flap valve. An adjustable leak consisting of a plate sliding over a slit in the box was provided to allow for various sizes of rabbits. Initially the valve was operated by a relay and timing circuit (Austin, 1954), but, as this was extremely noisy and gave jerky respiratory movements, it was found more convenient to operate the valve by means of a cam fitted on the shaft of a windscreen wiper motor (Lucas type C.W.1).

The Isolated Frog Rectus Abdominis Muscle Preparation.—Muscle strips were suspended in frog Ringer solution in a 10 ml. bath. Regular contractures to acetylcholine were obtained at 5 min. intervals. Antagonists were added to the bath 1 min. before the agonist, which was allowed to act for 1 min. When compounds were tested for ability to produce a contracture they were allowed to act for 5 min.

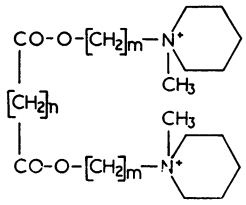
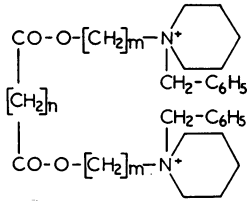
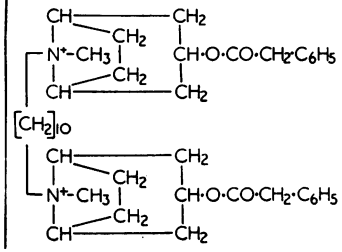
Action in Chicks.—To determine mode of action, compounds were administered subcutaneously to day-old chicks which were then observed until they died (Buttle and Zaimis, 1949). Drugs known to have competitive or depolarizing action were used at the same time for purposes of comparison.

The Isolated Human Foetal Phrenic Nerve-Diaphragm Preparation.—These preparations were obtained from foetuses of between 16 and 24 weeks. Sectors of diaphragm with nerve and ribs attached were removed from the foetus within 1 or 2 hr. of delivery, placed in Tyrode solution (NaCl, 0.9%; KCl, 0.042%; $CaCl_2$, 0.024%; $NaHCO_3$, 0.05%; dextrose, 0.1%) for transport to the laboratory. On arrival they were set up in an organ bath in oxygenated Tyrode solution at 36° to 37° within a further 30 min. Maximal contractions were obtained by stimulating the muscle indirectly with rectangular pulses of less than 1 msec. duration at a rate of about 5/min.

TABLE I

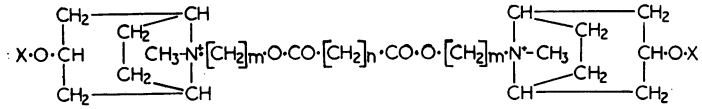
RELATIVE POTENCY, DURATION OF ACTION AND TYPE OF ACTION SHOWN BY BIS-QUATERNARY TROPEÏNE COMPOUNDS IN VARIOUS TESTS

DF596 was used as the standard for comparisons of potency and duration except for DF41. Col. 1, Relative potency in the cat. Number of estimates in parentheses. Col. 2, Relative duration of action in the cat. Col. 3, Degree of hypotension produced in the cat. Shown as $<$, $\equiv$ (equivalent to), $>$ that due to DF596. Col. 4, Anti-acetylcholine activity on the isolated guinea-pig ileum. Atropine sulphate = 1·0. Col. 5, Action on the frog rectus abdominis muscle. C, compound produced a contracture. A, compound antagonized the action of acetylcholine. Col. 6, Type of paralysis produced by injection into chicks. F, flaccid. S, spastic. † Indicates that the compound was also tested in rabbits (Table II). * Indicates that neuromuscular block was reversed by neostigmine. ‡ Comparison with tubocurarine.

Series	DF No.	Name of Compound	Structure	Anion	*m*	*n*	1	2	3	4	5	6	
		Suxamethonium chloride						++					†
		Tubocurarine chloride						+++++					†
		Gallamine triethiodide						++++ to +++++					†
	616	*NN'*-4,9-Dioxo-3,10-dioxadodecamethylenebis(1-methylpiperidinium iodide)		2 I^-	2	4	0·6 (2)	+ to ++	$<$		C	S	
	628	*NN'*-4,9-Dioxo-3,10-dioxadodecamethylenebis(1-benzylpiperidinium bromide)		2Br^-	2	4	$<$0·2 (1)				A	S F	
	41	*NN'*-Decamethylenebis(3-phenylacetoxytropanium bromide)		2Br^-			0·8 (2) ‡	++++ to +++++ ‡		0·02		F	† *

(Continued overleaf)

TABLE I—*continued*



			X	Anion	*m*	*n*	1	2	3	4	5	6	
I	623	*NN'*-4,7-Dioxo-3,8-dioxadecamethylenebis(3-acetoxytropanium bromide)	-CO-CH_3	$2Br^-$	2	2	0·2 (1)	++++	<		A		
	632	*NN'*-4,9-Dioxo-3,10-dioxadodecamethylenebis(3-acetoxytropanium bromide)		$2Br^-$	2	4	0·25 (2)	+++	<		A		
	631	*NN'*-4,11-Dioxo-3,12-dioxatetradecamethylenebis(3-acetoxytropanium bromide)		$2Br^-$	2	6	0·4 (2)	++	≡		A		
II	619	*NN'*-4,6-Dioxo-3,7-dioxanonamethylenebis(3-benzoyloxytropanium bromide)	-CO-C_6H_5	$2Br^-$	2	1	0·5 (1)	++++			A	F	
	618	*NN'*-4,7-Dioxo-3,8-dioxadecamethylenebis(3-benzoyloxytropanium bromide)		$2Br^-$	2	2	0·7 (1)	++++	≡		A	F	†
	620	*NN'*-4,9-Dioxo-3,10-dioxadodecamethylenebis(3-benzoyloxytropanium bromide)		$2Br^-$	2	4	1·2 (2)	+++	<		A	F	†
	622	*NN'*-4,10-Dioxo-3,11-dioxatridecamethylenebis(3-benzoyloxytropanium bromide)		$2Br^-$	2	5	1·3 (3)	++	>		A		†
	634	*NN'*-4,11-Dioxo-3,12-dioxatetradecamethylenebis(3-benzoyloxytropanium bromide)		$2Br^-$	2	6	0·2 (2)	+	≻		A		
III	604	*NN'*-4,5-Dioxo-3,6-dioxaoctamethylenebis(3-phenylacetoxytropanium bromide)	-CO-CH_2-C_6H_5	$2Br^-$	2	0	<0·2 (2)		>	0·01	A	F	
	595	*NN'*-4,6-Dioxo-3,7-dioxononamethylenebis(3-phenylacetoxytropanium bromide)		$2Br^-$	2	1	1·2 (3)	+++ to ++++	<	0·01	A	F	† *
	593	*NN'*-4,7-Dioxo-3,8-dioxadecamethylenebis(3-phenylacetoxytropanium		$2Br^-$	2	2	1·0 (2)	+++ to ++++	<	0·01	A	F	†

		enebis(3-phenylacetoxytropanium bromide)						(1)						
	596	*NN'*-4,9-Dioxo-3,10-dioxadodecamethylenebis(3-phenylacetoxytropanium bromide)		$2Br^-$	2	4	1·0	++		0·1	A	F	† *
	621	*NN'*-4,10-Dioxo-3,11-dioxatridecamethylenebis(3-phenylacetoxytropanium bromide)		$2Br^-$	2	5	0·4 (2)	+	>		A	F	
	635	*NN'*-4,11-Dioxo-3,12-dioxatetradecamethylenebis(3-phenylacetoxytropanium bromide)		$2Br^-$	2	6	<0·1 (3)	+	>		A		
	696	*NN'*-4,9-Dioxo-3,10-dioxadodecamethylenebis(3-β-phenylpropionyloxytropanium bromide)	$-CO-CH_2-CH_2-C_6H_5$	$2Br^-$	2	4	0·9 (2)	++	< ≡		A		†
	767	*NN'*-5,7-Dioxo-4,8-dioxaundecamethylenebis(3-phenylacetoxytropanium bromide)		$2Br^-$	3	1	1·6 (1)	+++	<		A		
	625	*NN'*-5,8-Dioxo-4,9-dioxadodecamethylenebis(3-phenylacetoxytropanium iodide)		$2I^-$	3	2	1·8 (3)	+++	<		A		† *
IV	653	*NN'*-5,9-Dioxo-4,10-dioxatridecamethylenebis(3-phenylacetoxytropanium iodide)	$-CO-CH_2-C_6H_5$	$2I^-$	3	3	1·2 (2)	++	<		A		
	646	*NN'*-5,10-Dioxo-4,11-dioxatetradecamethylenebis(3-phenylacetoxytropanium iodide)		$2I^-$	3	4	0·6 (2)	+	≡		A		
	649	*NN'*-5,12-Dioxo-4,13-dioxahexamethylenebis(3-phenylacetoxytropanium iodide)		$2I^-$	3	6	0·1 (2)	+	>		A		
	723	*NN'*-6,7-Dioxo-5,8-dioxadodecamethylenebis(3-phenylacetoxytropanium bromide)	$-CO-CH_2-C_6H_5$	$2Br^-$	4	0	0·2 (1)	+++ to ++++	>		A		†
	686	Epimer of DF593		$2Br^-$	2	2	0·5 (1)	+++ to ++++	<		A		†
V	668	Epimer of DF596	$-CO-CH_2-C_6H_5$	$2Br^-$	2	4	0·8 (1)	+++			A		†
	719	Epimer of DF635		$2Br^-$	2	6	0·2 (2)	+	>		A		

Anti-acetylcholine Activity.—This was determined on the isolated guinea-pig ileum preparation by the method of superfusion (Adam, Hardwick and Spencer, 1954). Regular submaximal contractions were obtained with acetylcholine at 90 sec. intervals. Antagonists were dissolved in Tyrode solution and applied to the gut for a period of 75 sec. before the application of acetylcholine. The effects of several concentrations of antagonist were compared with those of several of atropine sulphate. The percentage reduction in the height of contraction was plotted against the logarithm of the concentration. Since regression lines were not always parallel, comparisons of concentrations required to reduce the height of contractions by 50% were made.

Results

Table I shows the structure of compounds investigated and the results of tests carried out in cats in which their potency, duration and effect on blood pressure were compared with those of DF596. Relative activity in antagonizing the action of acetylcholine on the isolated guinea-pig ileum, the effect produced on frog rectus abdominis muscle, and the type of paralysis resulting from injection into chicks are also shown.

Table II gives the mean doses of the compounds necessary to cause head drop in rabbits, and the mean recovery times with standard deviations, in each case. The head-drop doses apply only to the particular drug concentrations and infusion rates employed, since with the short-acting compounds variations in either parameter resulted in a marked alteration of estimated potency.

Of the possible variations to the basic structure shown in Table I, the effects due to the following were investigated; changing the tropine ester, reversing the conformation of the methyl group and the linking chain about the nitrogen atom of the tropane nucleus, and altering the values of *n* and *m*.

Derivatives of Phenylacetyltropeïne

Compounds derived from phenylacetyltropeïne in which *m* was 2 (Series III) were investigated in greatest detail. To obtain neuromuscular blocking potency of the same order as DF596, the value of *n* required was not critical in the range 1 to 4. If the chain was lengthened or shortened, there was a drop in potency. In cats, the oxalic acid ester [DF604, *NN'*-4,5-dioxo-3,6-dioxaoctamethylenebis(3-phenylacetoxytropanium bromide)] had less than one sixth the activity of the malonate [DF595, *NN'*-4,6-dioxo-3,7-dioxanonamethylenebis(3-phenylacetoxytropanium bromide)] whilst the suberic acid ester [DF635, *NN'*-4,11-dioxo-3,12-dioxatetradecamethylenebis(3-phenylacetoxytropanium bromide)] was less than one tenth as effective as the adipate (DF596).

Table II

POTENCY AND DURATION OF ACTION OF BIS-QUATERNARY TROPEÏNE COMPOUNDS ESTIMATED BY THE HEAD DROP TEST IN RABBITS

Compound	Infusion Rate (ml./min.)	Conc. (mg./ml.)	Mean Head Drop Dose (mg./kg. with S.D.)	Revovery Time in Min. with S.D.			
				Head Drop	Righting Reflex	Fore Limbs	Walking Unaided
Suxamethonium chloride	1·0	0·3	0·32 (0·07)	2·7 (0·7)	3·9	7·2	7·5 (1·4)
Tubocurarine ,,	0·5	0·2	0·18 (0·04)	8·2 (3·3)	9·9	14·3	17·7 (4·0)
Gallamine triethiodide ..	0·5	0·5	0·43 (0·08)	5·5 (1·3)	7·4	10·4	12·2 (3·4)
DF41	0·5	0·2	0·24 (0·06)	3·6 (2·0)	4·1	4·9	5·4 (1·9)
DF618	0·5	0·3	0·33 (0·07)	1·7 (0·6)	2·3	3·0	3·4 (2·2)
DF620	0·5	0·3	0·21 (0·07)	1·9 (0·7)	2·3	3·2	3·9 (0·9)
DF622	1·0	0·3	0·23 (0·03)	2·9 (1·0)	3·6	5·0	5·3 (1·1)
DF595	0·5	0·3	0·29 (0·05)	2·1 (0·5)	2·2	3·0	3·4 (0·9)
DF593	0·5	0·3	0·32 (0·10)	2·0 (0·5)	2·0	3·2	3·6 (1·1)
DF769	0·5	0·3	0·15 (0·03)	2·5 (1·3)	2·8	3·9	4·6 (1·0)
DF596	1·0	0·3	0·30 (0·13)	1·5 (0·6)	2·2	2·7	3·6 (1·0)
DF696	1·0	0·3	0·33 (0·10)	2·1 (1·2)	2·5	3·7	4·1 (1·3)
DF625	0·5	0·3	0·23 (0·02)	2·5 (1·0)	2·7	4·5	5·7 (1·3)
DF723	1·0	2·0	1·53 (0·26)	1·0 (0·5)	1·1	2·7	3·0 (1·0)
DF686	1·0	0·3	0·33 (0·07)	4·6 (3·6)	5·0	6·9	7·8 (3·1)
DF668	0·5	0·3	0·17 (0·02)	1·4 (0·4)	1·9	2·4	4·6 (1·7)

When the interquaternary distance was altered, by giving n values from 1 to 6, the duration of paralysis was affected. With relatively long chains in which n was 5 (DF621) or 6 (DF635), a duration of action even more transient than that of suxamethonium was obtained as judged by tests in the cat, whilst with short chains as in the malonic (DF595) and succinic (DF593) acid esters the paralysis was intermediate in duration between that of suxamethonium and gallamine triethiodide. In rabbits there was virtually no gradation in recovery time with compounds where n varied between 1 and 4; other values of n were not examined. There was little difference in the times required for recovery from head drop after DF596 ($n=4$) and DF595 ($n=1$) although in the cat the duration of action of DF595 was considerably longer (Fig. 2). The rate of recovery

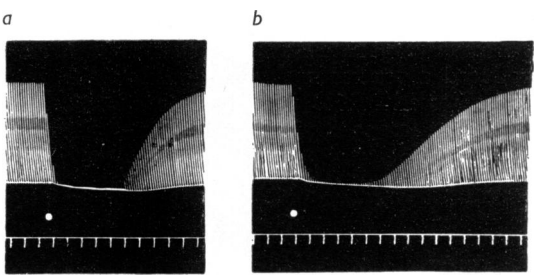


FIG. 2.—Cat. Maximal twitches of the gastrocnemius muscle elicited by indirect stimulation. Time, min. (*a*) 1 mg./kg. of DF596 was given intravenously. (*b*) 0.6 mg./kg. of DF595 intravenously.

following DF596 was comparable with that obtained with suxamethonium in the cat and dog, but this was not so in the rabbit, in which recovery after DF596 took place in about half the time needed after suxamethonium. As with suxamethonium, it was possible to produce a steady neuromuscular block by slow intravenous infusion of DF596 into cats and dogs, and recovery on stopping the infusion was quite rapid (Fig. 3).

In cats, a similar gradation of the effects of compounds upon blood pressure was also observed as the value of n was varied. Least hypotension was observed with the malonic acid ester DF595 ($n=1$), but as the number of methylene groups increased so also did the degree of hypotension produced. DF596 had a considerable hypotensive action in cats; weight for weight approximately 0.05 that of phenactropinium chloride (Robertson, Gillies and Spencer, 1957). In dogs, effects on blood pressure were much less marked. The oxalic acid ester (DF604) behaved anomalously, it was of very low activity in causing paralysis

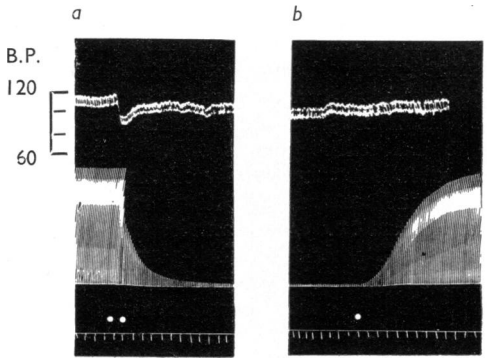


FIG. 3.—Dog anaesthetized with pentobarbitone. Upper tracing, carotid blood pressure in mm. Hg. Lower tracing, maximal twitches of the gastrocnemius muscle elicited by indirect stimulation. Time, min. (*a*) 0.1 mg./kg. of DF596 was injected at first dot. At second dot, an intravenous infusion of 0.015 mg./kg./min. of DF596 was started. (*b*) 45 min. later, the infusion of DF596 was stopped.

and gave a much greater fall in blood pressure than the malonic acid ester (DF595).

Derivatives of Other Esters

With compounds derived from acetyltropeïne (Series I) or benzoyltropeïne (Series II) in which m was 2, changes in duration of neuromuscular blocking action and effect on blood pressure were in the same direction as with the corresponding members of series derived from phenylacetyltropeïne (Series III) as n was increased from 2 to 6.

The most active benzoyltropeïne derivatives were the adipic [DF620, *NN'*-4,9-dioxo-3,10-dioxadodecamethylenebis(3-benzoyloxytropanium bromide)] and pimelic [DF622, *NN'*-4,10-dioxo-3,11-dioxatridecamethylenebis(3-benzoyloxytropanium bromide)] acid esters. These were approximately equal in potency to the most active members of the phenylacetyltropeïne series. In general, however, benzoyltropeïnes were slightly longer acting than their phenylacetyl analogues. If Series II and III are compared, it can be seen that the most active benzoyltropeïnes have higher values of n than in the phenylacetyltropeïnes.

The three acetyltropeïne analogues prepared had low activity in the cat.

The adipic acid ester derived from β-phenylpropionyltropeïne [DF696, *NN'*-4,9-dioxo-3,10-dioxadodecamethylenebis(3-β-phenylpropionyloxytropanium bromide)] closely resembled the corresponding ester derived from phenylacetyltropeïne in potency, duration and effect on blood pressure.

The Effect of Altering the Value of m

The effect of increasing the number of methylene groups in the chain between the ester groups and the quaternary nitrogen atoms from 2 to 3 was investigated only for the phenylacetyltropeïne derivatives (Series IV). This change tended to enhance the activity of the most potent members and at the same time to shorten the duration of action. For example, *NN'*-5,8-dioxo-4,9-dioxadodecamethylenebis(3-phenylacetoxytropanium iodide) (DF625) was considerably more potent and of shorter duration than *NN'*-4,7-dioxo-3,8-dioxadecamethylenebis(3-phenylacetoxytropanium bromide) (DF593). The potency of compounds did not depend only upon interquaternary distance. The positions of the ester groups were of importance in this respect. Compounds DF723 ($n=0$; $m=4$), DF625 ($n=2$; $m=3$) and DF596 ($n=4$; $m=2$), which all contained ten carbon atoms in the chain but differed in the positions of their ester groups, varied greatly in both potency and duration.

The Effects of Alterations at the Quaternary Nitrogen Atoms

When the relative positions of the methyl group and the linking chain on the quaternary tropane nitrogen were reversed (Series V), the properties of phenylacetyltropeïne derivatives were not much altered.

Miscellaneous Compounds

The possibility of substituting simpler groups for tropine esters in these compounds was investigated. Both *NN'*-4,9-dioxo-3,10-dioxadodecamethylenebis(1-methylpiperidinium bromide) (DF616) and *NN'*-4,9-dioxo-3,10-dioxadodecamethylenebis(1-benzylpiperidinium bromide) (DF628), in which the tropeïne moiety was replaced by a piperidine ring, were less potent than DF596, and the mode of action of the compounds differed from that of other members of the series.

NN'-Decamethylenebis(3-phenylacetoxytropanium bromide) (DF41) was included because it enabled a comparison to be made between a bis-quaternary tropeïne compound with only methylene groups in the chain and another (DF593) of about the same interquaternary distance with ester groups also present in the chain.

The Mode of Action of Compounds

The neuromuscular block obtained with several of the more active compounds could be reversed by a subsequent injection of neostigmine or edrophonium, both in the anaesthetized cat and in the isolated mammalian nerve-muscle preparation. Compounds tested in this way are indicated in Table I.

All members of the series described here in which each quaternary nitrogen atom was present in a tropane nucleus produced a flaccid paralysis when injected into chicks. No contracture was observed when one of these compounds was added to the fluid bathing an isolated frog rectus abdominis muscle, but contractures due to acetylcholine were reduced or abolished.

Both DF616 and DF628 containing a piperidine ring in place of the tropeïne moiety showed some of the properties characteristic of depolarizing agents. DF616 gave a spastic paralysis in the chick apparently identical with that given by suxamethonium, tested at the same time. This differed from the paralysis due to DF628, which was spastic initially but subsequently gave way to flaccidity. On the frog rectus muscle, DF616 produced a contracture when added to the bath fluid whereas DF628 itself had no effect on the muscle but antagonized the action of acetylcholine.

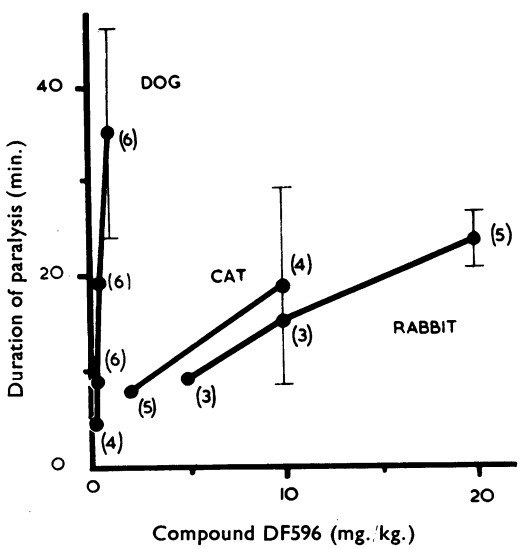


FIG. 4.—Duration of paralysis after an intravenous injection of DF596. Rabbits were maintained in a Drinker type respirator. The time for the first voluntary movement was measured. Dogs were anaesthetized with pentobarbitone and cats with a mixture of chloralose and urethane. Time for recovery of the indirectly-elicited gastrocnemius twitch to 50% of its pre-injection height was measured. Standard deviations are shown by vertical bars. Numbers of animals used are shown in brackets.

Duration of Action of Compounds in Different Species

Different species varied in their ability to inactivate DF596. Fig. 4 shows that rabbits can deal with about 15 mg./kg. and dogs about 0.5 mg./kg. in 20 min. Duration of action of DF596 was compared with that of tubocurarine and suxamethonium in mice by estimating the time elapsing after recovery of the righting reflex until animals were able to retain their hold on an almost vertical wire mesh screen. Recovery after DF596 was more rapid than after suxamethonium or tubocurarine (Table III). Confirmation of these results was obtained by determining the ratio of subcutaneous ED50 (paralysis) to the intravenous ED50 (paralysis) in mice. A high ratio would be expected if considerable metabolism of the compound had taken place before onset of paralysis. Table IV shows that the ratios for the relatively long-acting relaxants tubocurarine and gallamine triethiodide were 2.1 and 1.6 respectively whereas for the much shorter acting suxamethonium chloride the value was 9.7. The values of 32 and 29 obtained for DF595 and DF596 suggest that these compounds are even more rapidly inactivated than suxamethonium.

TABLE III

THE DURATION OF ACTION OF NEUROMUSCULAR BLOCKING AGENTS IN MICE

The values in col. 2 give the time after recovery of righting reflex until the animal was able to support itself on a near-vertical wire mesh screen after an intravenous infusion of the drug in sec. with s.d.

Compound (1)	(2)
Suxamethonium	108±18
Tubocurarine	285±190
DF596	16±18

TABLE IV

RATIO OF SUBCUTANEOUS ED50 TO INTRAVENOUS ED50 OF NEUROMUSCULAR BLOCKING AGENTS IN MICE

ED50, the quantity for paralysis in mice with 95% limits.

Compound	ED50 (mg./kg.)		Subcut. ED50 / I.V. ED50
	Subcutaneous	Intravenous	
Tubocurarine	0·094 (0·087–0·101)	0·045 (0·037–0·056)	2·1
Gallamine triethiodide	1·85 (1·56–2·20)	1·16 (1·02–1·31)	1·6
Suxamethonium	1·60 (1·46–1·74)	0·165 (0·132–0·207)	9·7
DF595	14·3 (13·4–15·3)	0·45 (0·38–0·53)	32
DF596	8·0 (7·1–9·0)	0·28 (0·26–0·31)	29

Effects of Compounds on the Isolated Human Foetal Phrenic Nerve-Diaphragm

This preparation was used to assess the likely potency of compounds in man. It was appreciated that the preparation might lead to erroneous conclusions for compounds destroyed *in vivo* only and that it was unlikely to be a suitable preparation for comparing compounds

TABLE V

RELATIVE POTENCY AND DURATION OF ACTION OF NEUROMUSCULAR BLOCKING AGENTS ON THE ISOLATED HUMAN FOETAL DIAPHRAGM

DF596 was used as the standard of comparison for potency. An asterisk indicates that the compound was antagonized by neostigmine. The relative duration of action is measured arbitrarily by the number of + : the greater the number the greater the duration of action.

Compound	No. of Estimates	Relative Potency	Relative Duration of Action	Approx. Conc. Giving 50% Reduction of Twitch in 5 Min. (μg./ml.)
Suxamethonium	3	>5	++	2–10
Tubocurarine	2		+++++	1–5
Gallamine triethiodide	1	1	++++	
DF595 ..	2	3*	++ to +++	10–20
DF596 ..	4	1*	+ to ++	20–80
DF668 ..	1	<1		

acting competitively with those exerting a depolarizing action. Different preparations were found to vary greatly in their recovery rates after the same compound. Our observations confirmed those of Buller and Young (1949), who found that, generally, recovery was slow so that potency was estimated by bracketing or matching responses. Values shown in Table V are all relative to DF596 compared on the same preparation. DF596 was considerably less potent than suxamethonium on this preparation, and duration of action was also somewhat less. DF595 was approximately 3 times as potent as DF596 and was of longer duration than suxamethonium.

The epimeric form of DF596 (DF668) did not appear to offer any advantages.

Discussion

The results clearly indicate that symmetrical bis-quaternary tropeïne compounds, in which two tropine esters were linked through their nitrogen atoms by a polymethylene chain containing two ester linkages, exhibited a neuromuscular blocking action of short duration which is reversible by neostigmine.

The phenylacetyltropeïne derivatives (Series III) in which n varies between 0 and 5 resembled the dicholine esters of $\alpha\omega$-dicarboxylic acids (I) prepared and tested by Bovet, Bovet-Nitti, Guarino, Longo and Fusco (1951) with regard to the influence which alterations in the value of

$$(CH_3)_3\overset{+}{N}\text{-}[CH_2]_2\text{-O-CO-}[CH_2]_n\text{-CO-O-}[CH_2]_2\text{-}\overset{+}{N}(CH_3)_3$$

I

n have on potency. The two series differed, however, in mode of action since the choline derivatives exerted a depolarizing action while the tropine compounds resembled tubocurarine in their behaviour. The side-effects of the members of the two series were also opposite in nature; increasing the value of n in the choline series increased hypertensive properties whereas similar changes in the tropine series increased hypotension.

Competitive action in neuromuscular blocking agents is usually associated with bulky terminal groups. All symmetrical bis-quaternary tropeïnes tested by us have behaved like competitive agents as judged by tests in chicks and on frog muscle. None has been reported by other workers to exert a depolarizing action. The swing from depolarizing towards a competitive action seen on replacing the *N*-methyl group in the piperidine analogue of DF596 (DF616) by a benzyl group (DF628) supports the idea that the bulk of the terminal cationic head is of importance.

From consideration of structure alone it was expected that the tropeïne derivatives would behave like tubocurarine. Comparison of results obtained by other workers for gallamine triethiodide, benzoquinonium, tubocurarine and laudexium suggests that with competitive neuromuscular blocking agents, the equivalent dose for man on a mg./kg. basis might be intermediate between those required for cat and mouse. With such agents the order of decreasing sensitivity is rabbit>cat>man>mouse. In laboratory animals, DF596 conforms to this pattern. The duration of action of DF596 relative to that of suxamethonium varied considerably in different species; it was approximately the same in cats, slightly shorter in dogs, much shorter in rabbits and mice and also somewhat shorter on the human foetal diaphragm. On this last preparation it was much less potent than suxamethonium, and, assuming that the preparation gives a valid estimate of activity in man, the potency of DF596 would be expected to be less than one fifth that of suxamethonium chloride; possibly only a tenth that of tubocurarine chloride.

It is clear that, with compounds of the type investigated here, marked species differences exist not only in potency, as is known to be so with most neuromuscular blocking agents, but also in duration of action. With DF596, the ratio of doses needed to paralyse in the most and least sensitive species examined was actually less than that found for tubocurarine in the same test, but duration of action in different species varied considerably.

The mechanism controlling the duration of action of members of this series was not investigated, but, by analogy with suxamethonium and other dicholine esters of $\alpha\omega$-dicarboxylic acids, it is reasonable to suppose that splitting of the chain by enzymatic hydrolysis may occur. The possibility of other mechanisms being involved, however, is shown by the failure of Hidalgo, Wilken and Seeberg (1959) to demonstrate hydrolysis of relatively short-acting hypotensive agents under conditions effective with acetylcholine. The relatively brief action of members of Series III in rabbits and the lack of gradation in this effect shown with different values of n leads to the suspicion that esterase activity at the chain is not the sole factor governing duration. DF41, which has only terminal ester links, was long acting in the cat but relatively short acting in rabbits. It is possible that the esterases capable of attacking terminal ester groups in addition to those effective in the chain play a part in the breakdown of these tropine esters; in rabbits this is quite likely

since the presence of an esterase in serum capable of splitting atropine and homatropine is well known (Ambache, 1955).

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INHIBITION OF MAST CELL DISRUPTION AND HISTAMINE RELEASE IN RAT ANAPHYLAXIS *IN VITRO*. COMPARISON WITH COMPOUND 48/80

BY

I. MOTA AND TISUE ISHII

From the Department of Histology and Embryology, Faculty of Medicine, São Paulo, Brazil

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In vitro anaphylactic reaction causes mast cell damage and histamine release from rat tissue. Histamine release is correlated with mast cell damage and both phenomena are simultaneously inhibited by various metabolic inhibitors, antipyretics, calcium lack and previous heating of the tissue at 45°. The mast cell damage produced by antigen in sensitized rat tissues is morphologically similar to that caused by compound 48/80, both agents causing extrusion of granules. Mast cell damage and histamine release induced by antigen or by compound 48/80 are inhibited alike by several substances and conditions. It is suggested that in rats the histamine-releasing mechanism of the antigen-antibody reaction in anaphylaxis is very similar to that of compound 48/80.

Liberation of histamine by antigen *in vitro* has been demonstrated in sensitized tissues of several species. Although histamine could be detected in relatively high amounts in plasma of shocked rats (Mota, 1957, 1958a) there are no data on histamine release by antigen from isolated sensitized tissues in this species. It is known that in guinea-pig anaphylaxis, histamine liberation is correlated quite well with mast cell damage, both these processes being inhibited in the same way by several agents (Mota, 1958b, 1959; Humphrey and Mota, 1959). Thus it seemed interesting to find out if isolated sensitized rat tissues would release histamine when in contact with antigen, whether this release was correlated with mast cell damage and how far the inhibitors of guinea-pig anaphylaxis (Mongar and Schild, 1957a, 1957b, 1958) would also affect rat anaphylaxis. Furthermore a comparative study of the effect of these same inhibitors on histamine release and mast cell disruption induced by compound 48/80 was also performed.

METHODS

Wistar rats of either sex, body weight 150 to 250 g., were used in all experiments. Crystalline bovine serum albumin and normal horse serum were used as antigens. Horse serum was dialysed against cold saline for 48 hr., filtered and stored under aseptic conditions. Four schedules of sensitization were used: (*a*) animals intraperitoneally injected with 1 ml. of alum-precipitated horse serum at 2-day intervals for three injections; (*b*) animals intraperitoneally injected with 1 ml. of horse serum plus 1 ml. of *Haemophilus pertussis* vaccine containing 20,000m. phase I organisms; (*c*) animals injected with 0.2 ml. of horse serum emulsified in Freund's adjuvant according to the technique of Lipton, Stone, and Freund (1956); (*d*) animals injected intraperitoneally with 1 ml. of a 1% solution of bovine serum albumin plus 1 ml. of *Haemophilus pertussis* vaccine containing 20,000m. phase I organisms.

The animals were used between the tenth and the twentieth day after sensitization. They were bled under ether anaesthesia and their skin and mesentery removed.

Removal and Treatment of Skin.—The abdominal skin was shaved with an electrical clipper, cut in pieces of about 1 sq. cm., carefully removed from the abdominal wall and kept in cold Tyrode solution. Each piece of skin was used as a sample. The samples were then shaken in 6 ml. stoppered flasks at 37° for 15 min. with 3.6 ml. Tyrode solution or Tyrode containing the required concentration of inhibitor. After this treatment either bovine serum albumin, horse serum, or compound 48/80 was added. Bovine serum albumin was added to give a final concentration of 1 mg./ml., horse serum to give a 10% solution, and compound 48/80 to give 0.1 mg./ml.

The tissue was then incubated for a further period of 15 min. after which the skin was transferred to N hydrochloric acid, cut into very small pieces, boiled for 2 or 3 min., and kept at 4° until assayed. The incubation solution was acidified with one drop of concentrated hydrochloric acid, boiled and kept

at 4°. As a control to each experiment samples of sensitized skin were incubated in Tyrode solution and samples of non-sensitized skin were incubated with the same concentration of each antigen.

Removal and Treatment of Mesentery.—The mesentery was dissected away from the small intestine and cut into several small pieces which were dropped in cold Tyrode solution. One or two of these pieces were used as a sample. The samples were incubated in the conditions already described for the skin samples and then transferred to the fixative or to N hydrochloric acid. The incubation solution was acidified with one drop of concentrated hydrochloric acid, boiled and kept at 4°.

Histamine Assay.—The extracts and incubation solutions were neutralized with sodium hydroxide, and when necessary diluted with Tyrode solution to the required volume. The histamine content was estimated on the atropinized guinea-pig ileum using histamine acid phosphate as a standard. In order to exclude any non-histamine component in the solution they were retested after inhibition of histamine by mepyramine 10^{-8}. Histamine release in controls was subtracted from that released by antigen or compound 48/80. Histamine release was expressed as a percentage of the total tissue histamine.

Mast Cell Observation.—The skin was fixed by immersion in 50% aqueous ethanol containing 10% formaldehyde and 5% acetic acid. Fixation was allowed to proceed overnight and frozen sections 50 μ thick were stained by 1% toluidine blue. The mesentery was fixed and stained in 10% formaldehyde containing 1% toluidine blue and 0.1% acetic acid, and examined as a whole-amount preparation. The number of disrupted mast cells in the mesentery was counted and calculated as a percentage of the total (500 cells being counted).

Inhibitor Solutions.—All inhibitors were dissolved in Tyrode solution and when necessary sodium hydroxide was used to help solution. The *p*H was then determined electrometrically and adjusted with hydrochloric acid to 7.8 in all solutions.

Results

Histamine Release from Rat Skin by Antigen.—Samples of sensitized skin were incubated with antigen and the percentage of histamine released was determined. The results are shown in Tables I and II. It can be seen that antigen released histamine from sensitized rat skin and that this release varied from rat to rat but was very constant in samples of abdominal skin taken from the same animal. Results of control pieces show that the spontaneous histamine release was small and that none of the antigens released histamine from non-sensitized rat skin.

Histamine Release from Rat Mesentery by Antigen.—Histamine release from mesentery is

TABLE I

HISTAMINE RELEASE BY ANTIGEN FROM SIX SAMPLES OF SKIN FROM A SENSITIZED RAT

Control Release μg.	Antigen Release μg.	Residue μg.	% Total Tissue Histamine Released	
			Control	Antigen
0·03	2·0	11	0·2	15
0·16	2·7	14	0·9	16
0·20	1·8	12	1·4	13
0·20	1·9	13	1·3	12
0·05	2·2	13	0·3	14
0·15	2·1	14	0·9	13

TABLE II

HISTAMINE RELEASE BY ANTIGEN FROM SENSITIZED RAT SKIN *IN VITRO*

The controls represent the percentage release from a sensitized piece of rat skin incubated with Tyrode solution (A) and from a non-sensitized piece of rat skin incubated with the antigen (B).

Rat	Group	Days After Sensitization	% Total Tissue Histamine Released		
			Antigen	Controls	
				A	B
1	A	20	18	1	0·8
2	A	20	9	1	0·7
3	A	18	6	1	2
4	A	16	9	3	1
5	A	16	11	1	1
6	A	19	10	1	1
7	A	19	7	1	2
8	B	12	13	2	2
9	B	12	25	1	0·9
10	B	12	14	2	3
11	B	15	16	1	1
12	B	15	11	1	2
13	B	12	23	2	1·8
14	B	12	20	2	2
15	B	11	16	1	0·9
16	B	13	8	0·7	0·6
17	C	13	10	0·5	1
18	C	10	14	2	2·1
19	C	15	18	1	1
20	C	15	15	0·9	0·8
21	D	13	20	3	2
22	D	13	11	4	3
23	D	13	23	1	2
24	D	13	18	2	1
25	D	14	24	3	3

TABLE III

HISTAMINE RELEASE AND MAST CELL DISRUPTION BY ANTIGEN IN RAT MESENTERY *IN VITRO*

Each figure refers to one animal.

Method of Sensitization. (See Methods)	Histamine Release %		Mast Cell Disruption %	
		Mean		Mean
a	22, 18, 28, 30	25	10, 11, 12, 15, 16	13
b	37, 40, 35, 36, 29, 31	35	10, 68, 51, 28, 48, 21	36
c	18, 28, 17	31	23, 10, 8, 46	22
d	17, 28, 29, 26, 21	19	32, 21, 29, 44, 61	37

shown in Table III. It can be seen that the percentage of histamine released was greater than that released from the skin possibly due to the thinness of this tissue, allowing an almost immediate contact of antigen with the cells. The amount of the spontaneously released histamine from mesentery was usually too small to be detected.

Mast Cell Disruption.—Microscopic examination of skin and mesentery after *in vitro* contact with antigen showed constant and definite alterations of mast cells, very similar to those previously described in the intact animal (Mota, 1953, 1957); both conditions were characterized by extrusion of granule similar to that caused by compound 48/80 (Mota, Beraldo, and Junqueira, 1953). It must be emphasized that this kind of alteration was present only in sensitized tissues after contact with antigen, and was absent from non-sensitized tissues incubated with antigen; it was easily distinguishable from artificial disruption. The percentage of mesenteric mast cells showing extrusion of granules after contact with antigen is shown in Table III. It can be seen that a variable number of mast cells were disrupted and that some of them preserved their usual morphology.

Effect of Metabolic Inhibitors on Histamine Release by Antigen or Compound 48/80.—Samples of sensitized skin were used in these experiments. The effects of sodium iodoacetate, sodium *p*-chloromercuribenzoate, sodium *o*-iodosobenzoate, *N*-ethylmaleimide and sodium cyanide were investigated. The results of these experiments are shown in Table IV and summarized in Fig. 1. It can be seen that all metabolic inhibitors

TABLE IV

EFFECT OF VARIOUS METABOLIC INHIBITORS ON HISTAMINE RELEASE BY ANTIGEN AND COMPOUND 48/80 FROM RAT SKIN

Releaser	Inhibitor	Molar Concentration	% Total Tissue Histamine Released by		% Inhibition
			Releaser Alone	Releaser + Inhibitor	
Antigen	Sodium iodoacetate	0·001	11	1	
			16	5	
			12	1	80
			15	4	
			23	2	
Compound 48/80	,, ,,	0·001	19	2	
			35	4	
			14	1	90
			16	2	
			11	1	
Antigen	Sodium *p*-chloromercuribenzoate	0·001	11	3	
			16	2	
			12	3	87
			15	1	
			23	2	
Compound 48/80	,, ,,	0·001	28	2	
			19	2	92
			14	1	
			35	3	
Antigen	*N*-Ethylmaleimide	0·001	11	1	
			16	1	
			12	2	93
			15	1	
			23	1	
			9	—	
Compound 48/80	,, ,,	0·001	25	3	
			21	4	
			27	1	96
			35	1	
			19	2	
Antigen	Sodium *o*-iodosobenzoate	0·005	11	1	
			16	3	
			12	2	
			15	1	90
			23	1	
			9	1	
			20	1	
Compound 48/80	,, ,,	0·005	28	2	
			19	2	
			14	1	96
			35	1	
			19	1	
Antigen	Sodium cyanide	0·005	11	2	
			16	2	93
			12	1	
Compound 48/80	,, ,,	0·005	21	5	
			25	3	86
			22	1	

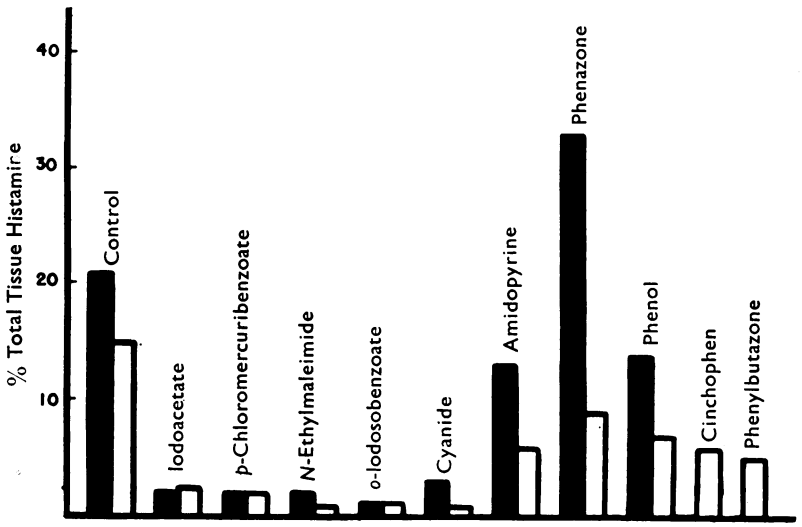

FIG. 1.—Effect of metabolic inhibitors and antipyretics on histamine release by antigen (white columns) or compound 48/80 (black columns) from rat skin.

used abolished almost completely the histamine release either by antigen or compound 48/80.

Effect of Metabolic Inhibitors on Mast Cell Damage by Antigen or Compound 48/80.—When pieces of sensitized mesentery were previously incubated with the various metabolic inhibitors, addition of antigen or compound 48/80 caused practically no mast cell damage as can be seen in Table V.

TABLE V

EFFECT OF METABOLIC INHIBITORS ON MAST CELL DISRUPTION *IN VITRO* CAUSED BY ANTIGEN OR COMPOUND 48/80, IN RAT MESENTERY

The values represent % mast cell disruption caused by antigen or compound 48/80 alone, and in the presence of inhibitor.

Inhibitor/ Releaser	Sodium Iodo-acetate	Sodium *o*-Iodoso-ben-zoate	Sodium *p*-Chloro-mercuri-benzoate	*N*-Ethyl-male-imide	Sodium Cyanide
Antigen 21	0	0	0	0	0
,, 38	0	1	2	0	11
,, 67	0	0	5	0	12
,, 18	0	0	0	0	0
,, 10	0	0	0	0	0
,, 68	0	0	2	0	3
Com-pound 48/80 62	0	2	2	0	5
,, 73	6	1	1	0	3
,, 56	0	0	0	0	2
,, 45	0	2	0	0	0
,, 28	0	0	0	0	0

Effect of Antipyretics on Histamine Release by Antigen or Compound 48/80.—Samples of sensitized skin were used in these experiments. The effects of amidopyrine, phenazone, cinchophen, and phenylbutazone were investigated. It was observed that the addition of compound 48/80 to a solution of cinchophen or phenylbutazone caused a precipitate and these antipyretics were not tried with 48/80. The results of these experiments are shown in Table VI and summarized in Fig. 1. The antipyretics caused an inhibition of histamine release by both antigen and compound 48/80 with the exception of phenazone, which although reducing histamine release by antigen increased histamine release by compound 48/80.

Effect of Calcium Lack on Histamine Release and Mast Cell Damage by Antigen or Compound 48/80.—Pieces of sensitized mesentery were incubated in calcium-free Tyrode solution containing 0.01% sodium dihydrogen edetate (EDTA) for 15 min. and then antigen (bovine serum albumin) or compound 48/80 was added. As can be seen in Fig. 2, both histamine release and mast cell damage were prevented by calcium lack.

Effect of Previous Heating of the Tissue to 45°.—Pieces of sensitized mesentery were kept at 45° for 5 min. and restored to 37°. Subsequent incubation of the mesentery with antigen (bovine serum albumin) or compound 48/80 showed that this treatment inhibited both histamine release and

TABLE VI
EFFECT OF VARIOUS ANTIPYRETICS ON HISTAMINE RELEASE BY ANTIGEN OR COMPOUND 48/80 FROM RAT SKIN

Releaser	Inhibitor	Molar Concentration	% Total Tissue Histamine Released by		% Inhibition
			Releaser Alone	Releaser + Inhibitor	
Antigen	Amidopyrine	0·01	18	10	
			5	2	
			9	4	45
			9	5	
			10	6	
			16	9	
Compound 48/80	,,	0·01	25	18	
			21	10	38
			27	15	
			11	11	
Antigen	Phenazone	0·01	25	25	
			15	5	
			9	6	29
			9	7	
			10	8	
			16	8	
Compound 48/80	,,	0·01	25	21	
			21	45	0
			27	34	(Histamine
			11	22	release
			24	35	increased
			17	45	from
					21% to
					34%)
Antigen	Phenol	0·01	11	5	
			16	9	
			12	4	50
			15	6	
			11	5	
			23	12	
Compound 48/80	,,	0·01	25	14	
			21	23	
			27	13	36
			11	5	
			24	15	
Antigen	Cinchophen	0·01	18	10	
			5	3	
			9	9	45
			9	3	
			10	4	
			16	7	
,,	Phenylbutazone	0·005	18	8	
			5	3	
			9	4	45
			9	6	
			10	6	
			16	8	

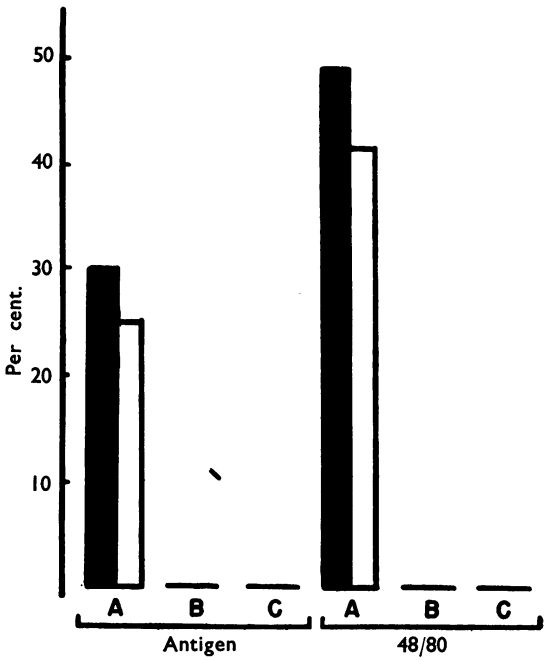

FIG. 2.—Effect of Tyrode solution (A), calcium lack (B) or previous heating of the tissue at 45° (C) on histamine release (black columns) and mast cell disruption (white columns) induced by antigen or compound 48/80 in sensitized mesentery.

mast cell damage by antigen or compound 48/80. The results of these experiments are shown in Fig. 2.

DISCUSSION

It can be seen from these results that antigen releases histamine from sensitized isolated rat tissue as it does in other species and that histamine liberation is well correlated with mast cell damage in rat anaphylaxis.

The present paper also shows that the several substances and conditions known to inhibit histamine release and mast cell damage in guinea-pig anaphylaxis are equally effective in the rat. Furthermore it is interesting to observe that in rats the mast cell damage induced by antigen or compound 48/80 is morphologically similar and that the histamine-releasing effect of both is inhibited by various agents. Thus there seems to be a close parallelism between the histamine-releasing mechanism of antigen and compound 48/80 in the rat. This fact suggests that in this species compound 48/80 may provide a good model for studying histamine release in anaphylaxis although the results of the experi-

ments with phenazone seem to suggest that the two reactions are not identical.

It is known that in the guinea-pig previous heating of the tissue at 45° causes a persistent inactivation of the histamine-releasing mechanism by antigen but does not interfere with the histamine-releasing mechanism of chemical histamine liberators (Mongar and Schild, 1957b). However, the present results show that in the rat this effect is not specific for anaphylaxis and that the tissue component inactivated at 45° is also required in the reaction leading to histamine liberation by compound 48/80. Furthermore the experiments with calcium lack suggest that in this species calcium ions are necessary for histamine release either by antigen or by compound 48/80.

The finding that various sulphydryl blocking agents inhibit mast cell damage by compound 48/80 had already been observed by Junqueira and Beiguelman (1955), and the present observations confirm their results. The inhibitory effects of *p*-chloromercuribenzoate, *o*-iodosobenzoate, iodoacetate, and *N*-ethylmaleimide suggest that free sulphydryl groups are implicated in the chain reaction leading to histamine release by antigen-antibody reaction as well as by compound 48/80 in the rat.

It is known that there is some analogy between the effects of the histamine releasers and the effects of antigen-antibody reaction in anaphylaxis. Furthermore it was shown that the histamine released by the antigen-antibody reaction and that which can be released by chemical histamine liberators have at least one common site of origin, both agents causing mast cell damage (Mota, 1958b, 1959). On the other hand there are also differences between the histamine releasing mechanism of these two agents. Thus while histamine liberators can release histamine from mast cell granules, the antigen-antibody reaction releases histamine only from intact cells (Copenhaver, Nagler, and Goth, 1953; Mongar and Schild, 1956; MacIntosh, 1956). Furthermore, several agents which will inhibit mast cell damage and histamine release by antigen will not inhibit mast cell damage and histamine release by chemical histamine liberators in guinea-pigs (Mongar and Schild, 1957a, 1957b, 1958; Mota, 1958b, 1959). These results led to the conclusion that the histamine release mechanism of the antigen-antibody reaction and that of chemical releasers was quite different. However, this conclusion does not seem to apply to all species since at least in the rat there is a very close similarity between the histamine release mechanism of the antigen-antibody reaction and that of compound 48/80. This does not mean that the histamine release mechanism of these two agents is necessarily identical. For instance, if all inhibitors acted on one step of the histamine release mechanism common to antigen-antibody reaction and compound 48/80 both these agents could be inhibited in the same way, and yet other stages in their mechanism of action could be quite different. It is possible that this common step is the activation of a cellular enzyme system as suggested by Rocha e Silva (1959). In regard to this possibility it is interesting to consider Benditt's findings (1956) showing that rat mast cells are rich in a chymotrypsin-like enzyme, and the more recent observations of Braun-Falco and Salfed (1959) showing that rat mast cells are rich in leucine-aminopeptidase and that rupture of these cells is simultaneous with release of this enzyme.

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THE MODE OF NEUROMUSCULAR BLOCKING ACTION OF CHLORPROMAZINE

BY

C. SU AND C. Y. LEE

From the Pharmacological Institute, College of Medicine, National Taiwan University, Taipei, Formosa

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The inhibitory action of chlorpromazine on skeletal muscle has been studied with isolated preparations. In the nerve-muscle preparations of the frog sartorius and the rat diaphragm, the twitch responses to indirect stimulation are much more strongly depressed by chlorpromazine than those to direct stimulation. The conductivity of the nerve trunk is unaffected. The contractures of the frog rectus abdominis muscle caused by acetylcholine are depressed by chlorpromazine,but the contractures due to KCl are not influenced. Larger doses of chlorpromazine cause contracture by themselves, and this cannot be prevented by tubocurarine. In the sartorius muscle of the toad, the depolarization due to acetylcholine is reduced by chlorpromazine. The paralysing action of chlorpromazine adds to that of tubocurarine, and is antagonized to some extent by eserine or neostigmine. Muscles treated with chlorpromazine do not completely recover on washing. High concentrations of chlorpromazine depress the release of acetylcholine by motor-nerve stimulation, although they do not affect the enzymic synthesis of acetylcholine by acetone-dried powder of guinea-pig brain. The differences between the neuromuscular block produced by chlorpromazine and that by tubocurarine are discussed.

Several papers have been published concerning the action of chlorpromazine on striated muscle. Huidobro (1954) observed that chlorpromazine did not modify the twitch of cat quadriceps muscle stimulated through its nerve. Dobkin, Gilbert, and Lamoureux (1954) reported that chlorpromazine did not block the neuromuscular junction in either frog or cat sciatic-gastrocnemius preparation.

Contrarily, many authors state that chlorpromazine inhibits striated muscle. Kopera and Armitage (1954) observed that chlorpromazine depressed the response of the gastrocnemius muscle of cats when stimulated either directly or indirectly through the sciatic nerve. They, as well as Burn (1954), considered that the action of chlorpromazine was not curare-like but was a direct action on the muscle. On the other hand, Ryall (1956) demonstrated that chlorpromazine could inhibit neuromuscular transmission in the isolated phrenic nerve-diaphragm preparation of rabbits, and Wislicki (1958) obtained the same result with the sciatic-gastrocnemius preparation of cats. Ryall (1956) suspected that chlorpromazine paralysed the muscle by decreasing acetylcholine synthesis at the motor nerve endings, but this idea has been rejected recently by Otubo (1959). The latter author stated that chlorpromazine at first stimulated the endplate membranes and later inhibited them.

In view of these conflicting results, we decided to perform experiments to throw some light upon the mode of action of chlorpromazine on striated muscle.

METHODS

Isolated preparations of striated muscle were used throughout so as to avoid interfering factors such as the actions of chlorpromazine on the central nervous or the cardiovascular systems.

Sartorious Muscle of the Frog.—The sartorius nerve muscle preparation of *Rana tigerina* was dissected out and set up in an organ bath containing 50 ml. of frog Ringer solution kept at room temperature and constantly oxygenated. Indirect and direct single electric shocks (rectangular in shape, supramaximal and of 0.5 and 5 msec. durations respectively) were applied once every 10 sec. The stimulator was a Model 751 by Arthur H. Thomas Co. The isotonic twitches of the muscle were recorded on smoked paper.

Diaphragm of the Rat.—The phrenic nerve-diaphragm preparation of the rat (Bülbring, 1946) was suspended in 50 ml. Tyrode solution, kept at 37° to 38° and constantly oxygenated with 95% O_2 and 5%

CO_2. Contractions were caused and recorded in the manner described for the sartorius muscle of the frog.

Rectus Abdominis Muscle of the Frog.—The rectus abdominis muscle of *R. tigerina* was excised and mounted in a 5 ml. bath containing frog Ringer solution at room temperature. Dose-response curves were obtained by applying acetylcholine in concentrations of 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} g./ml. and recording the contractures on smoked paper. The contracture produced by 10^{-3} g./ml. acetylcholine was considered as the maximum response. The dose-response curves to KCl were produced similarly.

Depolarization of the Toad Sartorius Muscle.—A method similar to that of Fatt (1950) was used. The sartorius muscle of the toad (*Bufo melanostictus*) was mounted in a glass chamber containing 50 ml. of frog Ringer solution. An electrode was placed at the pelvic end and another near the bottom of the chamber in contact with the solution. In order to determine the position of maximum density of endplates, a preliminary record was obtained of the potential differences along the surface of each muscle preparation in the following manner: the level of the Ringer solution in the bath was lowered stepwise by draining 1 ml. away each time, and the potentials were measured by means of a potentiometer. With part of the muscle immersed in solution, the potential difference recorded was that between the upper end and a point on the muscle at the surface of the bathing solution. This procedure was repeated after substituting a solution of acetylcholine (8×10^{-6} g./ml.) for the Ringer. The point on the muscle surface which gave the maximum potential difference before and after using acetylcholine represented the focus of maximum endplate density. The depolarization by acetylcholine at the region of maximum endplate density was measured again, and the value was taken as 100%. The same procedure was repeated at 15 min. intervals after the application of chlorpromazine.

Estimation of Acetylcholine Release from the Rat Diaphragm.—A sector of the diaphragm, with phrenic nerve attached but without ribs, was suspended in a 3 ml. bath in Krebs bicarbonate solution containing 5×10^{-6} g./ml. neostigmine at 37°. After tetanizing at 25 shocks/sec. for 20 min. the fluid was withdrawn and assayed for acetylcholine by its depressor effect on the blood pressure of the anaesthetized rat (Straughan, 1958). Since the resting output of acetylcholine was negligible, no correction for this was made.

Acetylcholine Synthesis by Guinea-pig Brain.—The acetone-dried powder from guinea-pig brain was prepared and incubated at 37° with the reaction mixture of Feldberg and Hebb (1947). The acetylcholine produced was assayed on the frog's rectus abdominis muscle according to the method of Feldberg and Mann (1945).

The drugs used were chlorpromazine hydrochloride (Wintermin, Shionogi), tubocurarine chloride (Intocostrin-T, Squibb), acetylcholine chloride (Ovisot, Daiichi), neostigmine methylsulphate (Vagostigmine, Shionogi), and eserine salicylate (Merck). Concentrations of drugs are expressed as weights of salts, or as μM. or mM.

RESULTS

Sartorius Muscle of the Frog

The muscle contractions elicited by indirect stimulation were depressed markedly by chlorpromazine in concentrations of 30 to 50 μM. (11 to 18 μg./ml. of chlorpromazine hydrochloride) after an initial slight augmentation. The responses to direct stimulation also suffered inhibition which was, however, much smaller (Fig. 1). When a segment of the nerve trunk between the muscle and the electrodes was soaked in 10 to 100 μM. solutions of chlorpromazine, no change in the amplitude of contraction occurred for more than 1 hr. Similar results were obtained in a few experiments with sciatic nerve-gastrocnemius preparations of the frog.

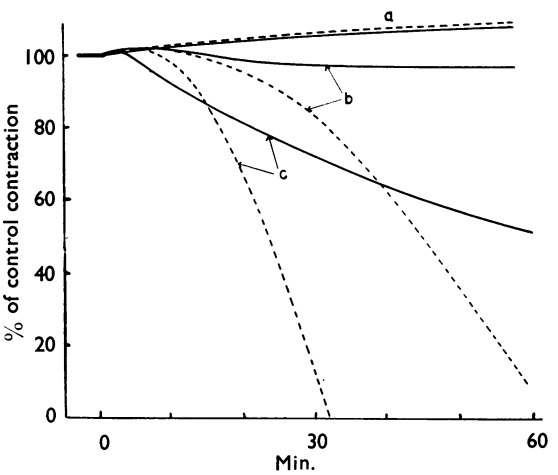

FIG. 1.—Frog, motor nerve-sartorius muscle. Amplitudes of single twitches in response to supramaximal stimulation. Solid line, direct stimulation; broken line, indirect stimulation. **a**, 10 μM.; **b**, 30 μM.; and **c**, 50 μM. of chlorpromazine given at zero time.

These results indicate that chlorpromazine exerts primarily a neuromuscular blocking action, plus a smaller action on the muscle fibres.

The slight augmentation of the contractions by 10 μM. of chlorpromazine and the initial transient augmentation after 30 to 50 μM. might have been due to the mild anticholinesterase activity of this drug (Erdös, Baart, Shanor, and Foldes, 1958).

The diminished contractions of this preparation could usually be restored by repeated washing with

Ringer's solution, even after a complete block had occurred. When the blocking action was fully developed the tetanic contraction caused by repetitive stimulation was no longer sustained, and no post-tetanic potentiation was found (Fig. 2).

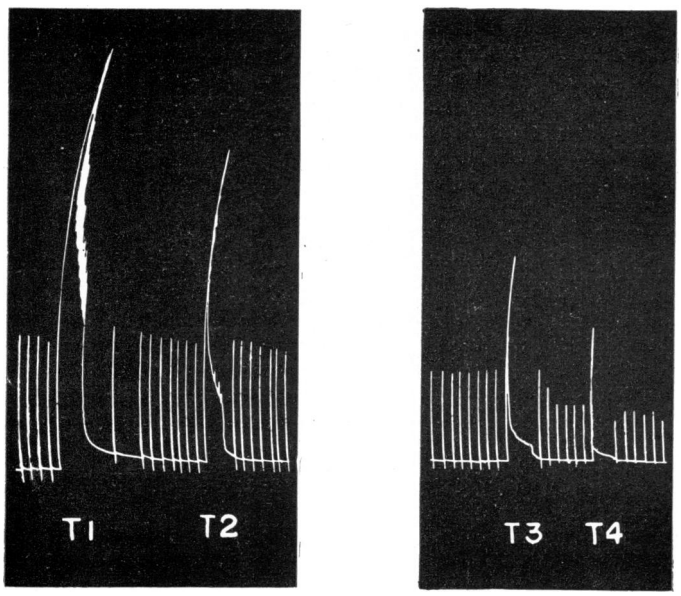

FIG. 2.—Frog, motor nerve-sartorius muscle. Tetanic stimulations 15 min. (left) and 20 min. (right) after application of 50 μM. chlorpromazine. Pulse frequency: 60/sec. (**T1** and **T3**) and 120/sec. (**T2** and **T4**).

Neostigmine and eserine (2×10^{-7} to 5×10^{-6} g./ml.) exerted a slight antagonistic effect. Adrenaline (5×10^{-7} to 5×10^{-6} g./ml.) was totally ineffective. The paralytic potencies of a dose of chlorpromazine, of an equivalent dose of tubocurarine and of the mixture of half-doses of the two agents were approximately equal, judging from the time required for complete paralysis.

Phrenic Nerve-Diaphragm Preparation of the Rat

Chlorpromazine in concentrations of 10 to 30 μM. reduced the muscle responses to indirect stimulation, after a brief augmentation. With a 40 μM. solution, a complete neuromuscular block was observed within 30 min., while the muscle still responded well to direct stimulation. Repetitive stimulation usually caused a well-sustained tetanic contraction (Fig. 3).

Washing sometimes retarded the blocking process, but the amplitude of twitches never recovered appreciably. Larger doses of chlorpromazine tended to provoke contracture of the diaphragm. Our results are in agreement with those of Ryall (1956). Eserine and neostigmine were only feebly effective unless applied early, when they generally enhanced the twitches (Fig. 4).

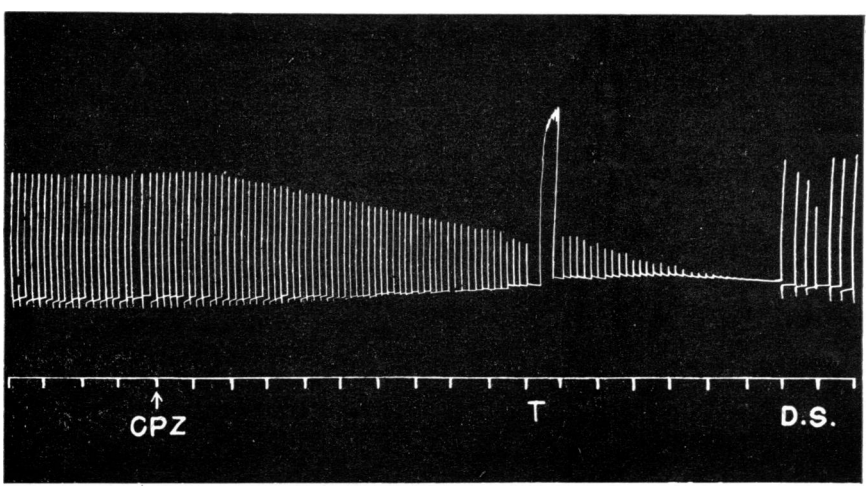

FIG. 3.—Rat, phrenic nerve-diaphragm. Supramaximal indirect stimulation. **CPZ**, 40 μM. chlorpromazine, tetanic stimulation at **T**. Direct stimulation at **D.S.** Time in min.

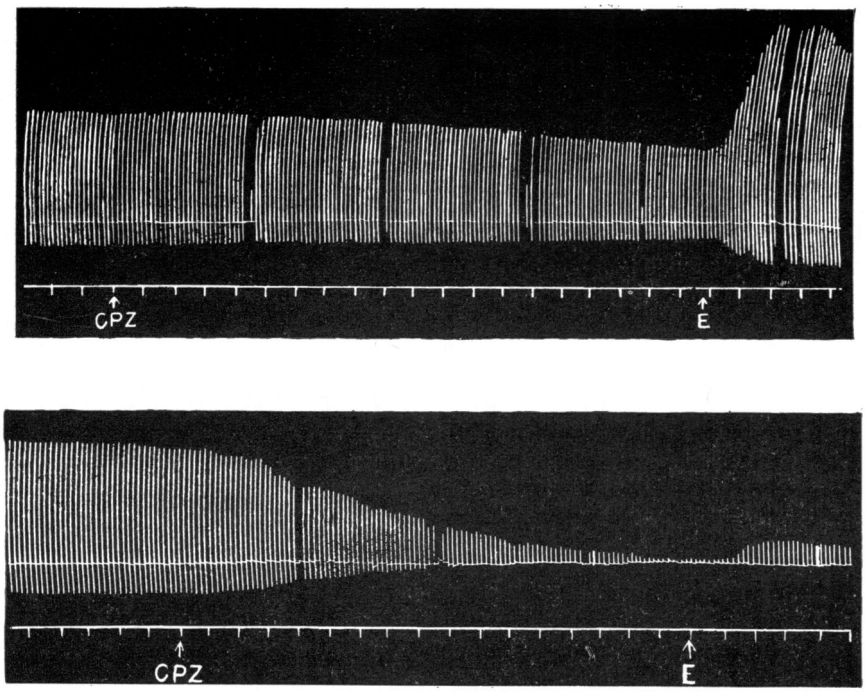

FIG. 4.—Rat, phrenic nerve-diaphragm. **CPZ**, chlorpromazine 30 μM. (above) and 50 μM. (below). Eserine 10^{-6} g./ml. at E. Time in min.

Rectus Abdominis Muscle of the Frog

The amplitudes of contracture caused by acetylcholine decreased after treatment of the muscle with solutions of chlorpromazine in concentrations higher than 0.1 μM. This effect was slower and weaker, on the molar basis, than that of tubocurarine. The action proceeded gradually and approached completion in 60 min.

The higher the concentration of acetylcholine, the more the response to it was depressed, and repeated washing did not lessen the depression appreciably (Fig. 5). Thus the action of chlorpromazine is at least partly irreversible.

On the other hand, chlorpromazine showed an approximately additive effect when used in combination with tubocurarine (Fig. 6). The addition of eserine or neostigmine completely antagonized chlorpromazine as far as the responses to low concentrations of acetylcholine were concerned, whereas the responses to higher concentrations of acetylcholine were depressed (Fig. 5). This suggests a mode of action different from that of tubocurarine.

Responses to KCl were not depressed by chlorpromazine (Fig. 7), since Ringer's solution alone after 1 hr. flattened the dose-response curve as much as chlorpromazine did. This was confirmed by the observation that, at the end of the tests with acetylcholine, the addition of an excess of KCl (300 mM.) still produced a maximum contracture.

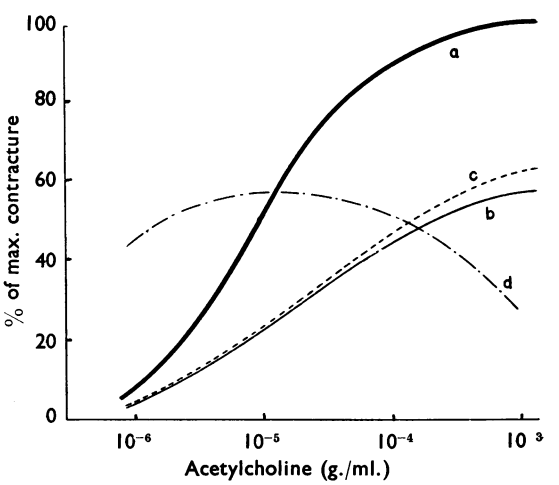

FIG. 5.—Frog, rectus abdominis. Responses to acetylcholine. **a**, Control. **b**, 2 μM. chlorpromazine for 60 min.. **c**, 2 μM. chlorpromazine for 60 min. followed by washing for 60 min. **d**, 2 μM. chlorpromazine and 10^{-6} g./ml. neostigmine for 60 min.

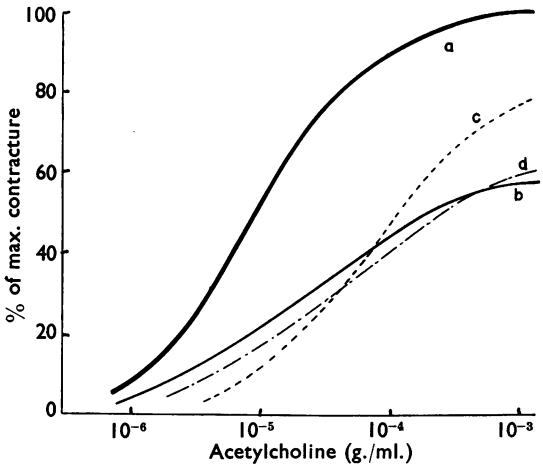

FIG. 6.—Frog, rectus abdominis. Responses to acetylcholine. **a**, Control. **b**, 2 μM. chlorpromazine for 60 min. **c**, 0.5 μM. tubocurarine for 60 min. **d**, 1 μM. chlorpromazine and 0.25 μM. tubocurarine for 60 min.

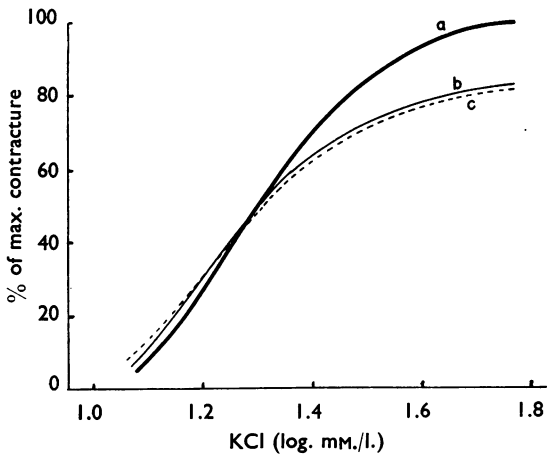

FIG. 7.—Frog, rectus abdominis. Response to KCl. **a**, Control. **b**, 2 μM. chlorpromazine for 60 min. **c**, Frog-Ringer for 60 min.

High concentrations of chlorpromazine caused contracture of the rectus muscle, which relaxed only very slowly on washing (Fig. 8). Previous treatment of the muscle with tubocurarine (4×10^{-5} g./ml. for 30 min.) had no influence on it, as Ryall (1956) observed for the diaphragm of the rabbit. The contracture produced by chlorpromazine, therefore, does not demonstrate an endplate depolarizing action of this agent, but seems to require some other explanation.

Depolarization in the Toad Sartorius Muscle by Acetylcholine

Chlorpromazine in 5 to 50 μM. solutions did not itself cause depolarization, but greatly diminished the magnitude of the depolarization caused by acetylcholine. The effect could be eliminated very slowly by washing several times unless large doses were used. Neostigmine reversed the inhibition effectively (Fig. 9). This experiment provided further evidence that chlorpromazine exerted an important inhibitory action on the motor endplates.

Acetylcholine Release by Motor-nerve Stimulation

As shown in Table I, the release of acetylcholine by motor-nerve stimulation was greatly reduced by 50 μM. of chlorpromazine. In one experiment (Expt. No. 3), it was completely abolished 1 hr. after the application of chlorpromazine. When 10 μM. of chlorpromazine was applied, however, no constant results were obtained; in one experiment

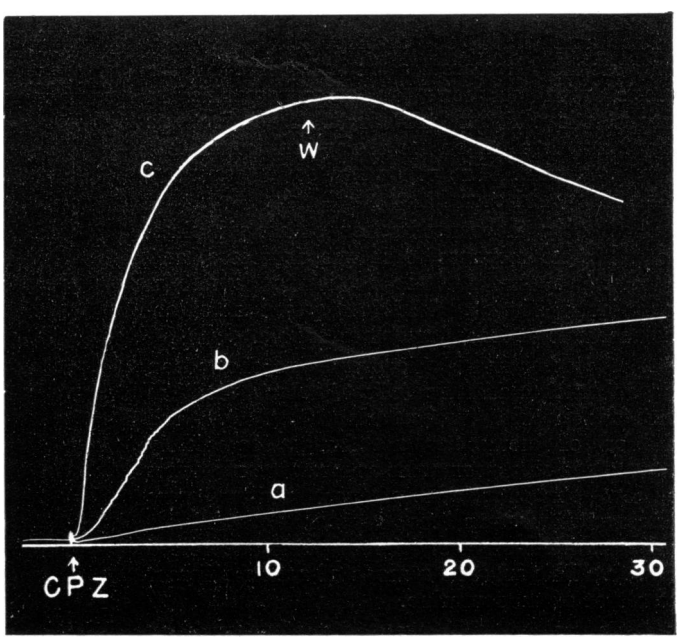

FIG. 8.—Frog, rectus abdominis. Contracture caused by chlorpromazine (CPZ), **a**, 10 μM., **b**, 100 μM., **c**, 1 mM. Washing at W. Time in min.

TABLE I

ACETYLCHOLINE OUTPUT OF THE RAT EXCISED DIAPHRAGM PREPARATION BEFORE AND AFTER THE ACTION OF CHLORPROMAZINE

There was 20 min. between the first and second periods of each control experiment.

Expt. No.	Acetylcholine Output (ng./20 min.)				Concentration of Chlorpromazine (μM.)
	Before Chlorpromazine		After Chlorpromazine		
	1st Period	2nd Period	30 Min.	60 Min.	
1	39	36	27	10	50
2	41	45	23	10	50
3	37	41	13	0	50
4	49	45	45	49	10
5	27	30	23	18	10

no reduction of acetylcholine released was found whereas in another experiment the amount of acetylcholine released was reduced by about 40% 60 min. after the application of chlorpromazine.

Synthesis of Acetylcholine by the Guinea-pig Brain

The results of a typical test are shown in Table II. It will be seen that chlorpromazine, even in concentrations as high as 100 μM., did not affect appreciably the synthesis of acetylcholine.

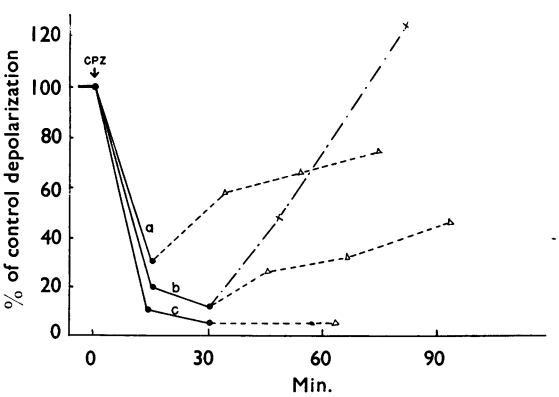

FIG. 9.—Toad, sartorius muscle. Magnitudes of depolarization caused by 8×10^{-6} g./ml. acetylcholine. **a**, 5 μM., **b**, 10 μM., **c**, 50 μM. chlorpromazine (●——●) given at zero time. Recovery in Ringer solution (Δ - - - Δ), and in chlorpromazine + neostigmine 10^{-6} g./ml. (X—.—X).

TABLE II

SYNTHESIS OF ACETYLCHOLINE BY THE GUINEA-PIG BRAIN

Figures refer to μg. of acetylcholine produced per mg. of acetone-dried powder.

Chlorpromazine Concentration (μM.)	Acetylcholine after Incubation Time of	
	1 hr.	2 hr.
0 (control)	0·18	0·34
1	0·18	0·32
10	0·19	0·33
100	0·18	0·32

DISCUSSION

Although Kopera and Armitage (1954), as well as Burn (1954), reported that the paralytic action of chlorpromazine in cats and rats was due to its direct action on muscle fibres, Ryall (1956) demonstrated that chlorpromazine inhibited neuromuscular transmission, with little effect on direct muscle stimulation, in the isolated phrenic nerve-diaphragm of rabbits. In our experiments using the nerve-muscle preparations of the frog sartorius and the rat diaphragm, chlorpromazine depressed the twitch responses to indirect stimulation much more markedly than those to direct stimulation. In addition, chlorpromazine decreased the size of the contractures of the frog rectus muscle due to acetylcholine while leaving those due to KCl unaffected. These facts indicate that the direct action of low concentrations of chlorpromazine on the muscle fibres is of minor importance. Since the conductivity of the nerve trunk was not affected by chlorpromazine, the main site of paralysis is probably the neuromuscular junction.

The blocking action is not of depolarizing type because the drug did not depolarize the endplates, did not cause fasciculation and did not antagonize the action of tubocurarine.

On the contrary, chlorpromazine reduced the endplate depolarization as well as the contracture caused by acetylcholine, and it was partly antagonized by anticholinesterases. In addition, it has been reported by many authors that chlorpromazine enhances the paralytic action of tubocurarine and its analogues (Wislicki, 1958). Our results show that the combined effect of chlorpromazine and tubocurarine should be classified as summation rather than as potentiation (" supra-additive synergism ") in the sense defined by Barstad (1956). It is therefore concluded

that the neuromuscular blocking action of chlorpromazine resembles that of tubocurarine in many respects.

There is evidence, however, that the action of chlorpromazine is not exactly like that of tubocurarine. First, it inhibits the muscle fibres directly and causes contracture when somewhat larger doses are applied: the mechanism of the latter action was not studied in the present work. Secondly, the anticurare agents exert only a slight antagonistic effect when the muscle is stimulated indirectly. Thirdly, post-tetanic potentiation of the muscle twitches is absent, and tetanus is well-sustained in the rat's diaphragm preparation. Fourthly, the dose response curves to acetylcholine on the frog's rectus abdominis muscle before and after treatment with chlorpromazine are not parallel, and they become bell-shaped after addition of anticurare agents.

Besides, whereas the enzymic synthesis of acetylcholine is unaffected, the release of acetylcholine by motor-nerve stimulation is inhibited by high concentrations of chlorpromazine. This inhibition may also constitute a part of the cause of neuromuscular block, indicating the multiplicity of the mode of action of chlorpromazine.

Moreover, the paralysis due to chlorpromazine could not be removed by repeated washing of the muscle preparations, except for the sartorius muscles of frogs and toads which recovered slowly. The irreversible nature of the paralysis has also been encountered in muscles of cats (Kopera and Armitage, 1954 ; Wislicki, 1958). Thus, the motor endplates are blocked by chlorpromazine with an affinity much stronger than that of tubocurarine, although the degree of irreversibility may vary largely among different kinds of muscle and different animal species.

Finally, although our results indicate that chlorpromazine has a direct influence on neuromuscular transmission, they do not exclude the possible participation of a central action of the drug on muscle tone in the intact animal. On the contrary, the neuromuscular junction seems to be less sensitive than the central nervous system to the drug, judging from the relatively high concentration required for neuromuscular block *in vitro*. The same conclusion may be drawn from the observations of several authors (Ryall, 1956 ; Wislicki, 1958 ; Otubo, 1959) that inhibition of striated muscle was consistently obtained only after intra-arterial injections ; either negative or inconsistent results were obtained after intravenous injections of doses sufficient to act on the central nervous system.

We should like to thank Professor G. Brownlee and Dr. D. W. Straughan, of the Department of Pharmacology, King's College, London, for their kindness in showing the method for the estimation of acetylcholine release from the rat diaphragm preparation to one of us (C. Y. L.). We are also indebted to Dr. E. Bülbring, of the Department of Pharmacology, Oxford, for correcting our English manuscript.

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THE QUANTITATIVE ASSAY OF CORTICOTROPHIN USING RATS TREATED WITH HYDROCORTISONE ACETATE

BY

J. B. DEKANSKI AND MARGARET I. HARVIE

From the Department of Pharmacology, Organon Laboratories, Newhouse, Scotland

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An attempt has been made to modify the Sayers method for the assay of corticotrophin by substituting for hypophysectomy an injection of sufficient hydrocortisone acetate to suppress the release of endogenous pituitary corticotrophin in intact rats over the period of the assay. The results of forty such assays of subcutaneous corticotrophin seem to indicate that this procedure is about 1½ to 2 times as efficient as the original method using hypophysectomized rats. For a 2 + 2 point assay it was necessary to use a minimum of twenty-four to thirty-two rats.

For some years attempts have been made to modify the technique involving adrenal ascorbic acid depletion for the assay of corticotrophin (ACTH) (Sayers, Sayers and Woodbury, 1948) by substituting hypophysectomized rats with animals in which the release of endogenous corticotrophin is sufficiently blocked by the administration of corticosteroids prior to the assay.

The investigations of Ingle and Kendall (1937), Ingle, Higgins and Kendall (1938), Sayers and Sayers (1947), and Gray and Munson (1951) showed that several corticosteroids inhibit the release of pituitary corticotrophin as judged by changes in ascorbic acid content and weight of the adrenal glands. At the time there was some confusion concerning the relative inhibitory effects of cortisone and deoxycortone. Sayers and Sayers (1947) were the first to suggest that several crystalline cortical steroids, including cortisone, hydrocortisone and deoxycortone, were most effective in blocking release of corticotrophin from the pituitary. Casentini, De Poli, Hukovic and Martini (1957), in a comparative test of the relative potencies of various corticosteroid preparations, found that deoxycortone had about the same activity as hydrocortisone acetate in a test on unilaterally adrenalectomized rats (Abelson and Baron, 1952; Porter and Jones, 1956).

On the other hand, Fortier, Yrarrazaval and Selye (1951) showed that both cortisone and cortisone acetate were completely ineffective in preventing the release of pituitary corticotrophin in response to stress, and Moya and Selye (1948), Gershberg, Fry, Brobeck and Long (1950) and Hall, Finerty, Hall and Hess (1951) were unable to confirm that deoxycortone acetate had an inhibitory effect under similar conditions. Hodges (1953, 1954) found that rats were far more suitable for a quantitative assay after treatment with deoxycortone acetate than after cortisone acetate, but even larger doses of deoxycortone failed to give a satisfactory preparation. Recently Hodges and Vernikos (1958) found that prednisolone and hydrocortisone were far more effective in inhibiting corticotrophin release and suggested the use of these preparations instead of deoxycortone acetate (Hodges, 1955) for a modified ascorbic acid depletion technique not involving hypophysectomy.

For over two years intact rats pretreated with hydrocortisone acetate have been used in this laboratory for subcutaneous and intravenous Sayers (Munson modification) assays of various corticotrophin preparations with satisfactory results, and an analysis of forty such subcutaneous bio-assays is reported in this paper. It is essential that the total dose of hydrocortisone acetate should be sufficient to suppress the release of endogenous pituitary corticotrophin over the period of the assay and that the subsequent injection of the corticotrophin preparation should give an ascorbic acid depletion similar to that in hypophysectomized rats. The blocking effect of hydrocortisone acetate was investigated in some detail in intact rats with different doses and routes of administration, in particular studying the intensity and duration of effect and the optimum time interval between injection of hydrocortisone and corticotrophin. From the results of these preliminary experiments, which will be reported separately, a standard procedure has been adopted.

METHODS

Male albino rats, weighing about 150 g. from Wistar stock, were kept in a room at constant temperature between 24 and 25° for 2 to 3 weeks before the test and were fed on Aberdeen standard diet. Hydrocortisone acetate was injected intraperitoneally into groups of eight rats in a total dose of 12 mg./100 g. body weight given in two equal doses over two days, at 2 p.m. on the day before the assay and at 8 a.m. on the following morning. The corticotrophin standard and test preparations were injected subcutaneously 4 hr. later. The total dose was dissolved in 0.6 ml. of gelatin per 150 g. body weight and given in three equal doses at 12, 1 and 2 p.m. About 10 to 15 min. after the last injection of corticotrophin the rats were anaesthetized with urethane injected subcutaneously; at 3 p.m. (45 to 50 min. later) 3 hr. after the first injection of corticotrophin each pair of adrenals was dissected out, weighed on a torsion balance to the nearest 0.1 mg. and the ascorbic acid content determined, the final response being expressed as μg. of ascorbic acid per 100 mg. of adrenals.

Determination of Ascorbic Acid.—Each weighed pair of adrenals was homogenized in a tube containing 0.5 ml. of 2.5% metaphosphoric acid, freshly prepared. An extra 9.5 ml. of the acid was then added and the tubes allowed to stand at least 30 min. It was possible to keep the homogenized suspension in the refrigerator overnight providing that freshly prepared standard solutions of pure ascorbic acid were kept under similar conditions. The tubes were centrifuged at about 2,500 revs./min. for 5 min., and an 8 ml. aliquot of the clear supernatant was added to a test tube containing 7 ml. of 4.53% sodium acetate (adjusted to *p*H 7.0 with acetic acid) and 1 ml. of 0.03% 2,6-dichlorophenolindophenol. The resultant pink colour was measured within 30 sec. of mixing with a Spekker absorptiometer, using a 2-cm. cell and Ilford filter No. 604 giving maximum transmission at 520 mμ. A calibration graph was prepared for ascorbic acid in concentrations up to 200 μg. per 10 ml. of 2.5% metaphosphoric acid and the unknown values for test solutions interpolated.

Drugs, Solutions and Media

Intraperitoneally.—Hydrocortisone acetate suspension (Hydro-Adreson, Organon; 25 mg./ml.).

Subcutaneously.—Corticotrophin: the weighed quantities of standard corticotrophin and various test preparations were dissolved in minimum volumes of 0.01 N HCl and 17% gelatin was added to give the

TABLE I

INDIVIDUAL RESULTS OF A SUBCUTANEOUS 3+3 POINT ASSAY

Purified corticotrophin was used in doses of 5, 10 and 20 μg. against 0.15, 0.3 and 0.6 i.u. of purified Home Standard (60 i.u./mg.), dissolved in 17% gelatin.

Total Dose	Adrenal Weight (mg.)	Ascorbic Acid per Two Adrenals (μg.)	Ascorbic Acid per 100 mg. Adrenals (μg.)	Total Dose	Adrenal Weight (mg.)	Ascorbic Acid per Two Adrenals (μg.)	Ascorbic Acid per 100 mg. Adrenals (μg.)	Total Dose	Adrenal Weight (mg.)	Ascorbic Acid per Two Adrenals (μg.)	Ascorbic Acid per 100 mg. Adrenals (μg.)
0·15 i.u. Standard	24·8	91·5	369	0·3 i.u. Standard	24·4	83·5	342	0·6 i.u. Standard	25·5	69·5	273
	26·3	98·0	373		30·9	107·5	348		27·3	79·5	291
	28·6	135·0	472		26·2	98·5	376		29·4	77·5	264
	29·9	100·0	334		32·4	102·0	315		31·7	101·5	320
	28·1	129·5	461		26·8	97·0	362		26·9	94·5	351
	21·2	84·5	399		31·1	119·0	383		27·2	92·0	338
	26·0	90·5	348		30·5	97·0	318		30·3	96·0	317
	23·9	97·5	408		26·0	76·5	294		28·0	81·5	291
Mean	26·1	103·3	395·5		28·5	97·6	342·3		28·3	86·5	305·6
5 μg. Test	25·4	91·5	360	10 μg. Test	26·9	80·0	297	20 μg. Test	26·6	92·5	348
	22·5	91·0	404		21·7	75·5	348		21·2	64·0	302
	24·2	95·5	395		29·2	98·0	336		34·2	103·0	301
	33·0	142·0	430		25·1	97·0	386		25·0	84·0	336
	31·4	112·5	358		23·5	82·5	351		29·2	85·5	293
	29·4	118·0	401		36·9	101·5	275		28·0	93·5	334
	30·4	121·0	398		23·9	90·5	379		33·3	70·0	210
	24·0	86·0	358		25·0	96·0	384		26·3	82·5	314
Mean	27·5	107·2	388·0		26·5	90·1	344·5		28·0	84·4	304·8

highest required dose in 0.6 ml. Dilutions with gelatin were made to give half the concentration of the higher dose for a 2+2 point assay and half and one quarter for a 3+3 point assay. Suitable doses were found in the range 0.15, 0.3, 0.6 and 1.2 units per 0.6 ml./150 g. of body weight. Solutions of gelatin (Type A, American Agricultural Chemical Co.) were made according to U.S.P., XV, p. 1094.

Anaesthetic.—Urethane (British Drug Houses, Ltd., 25% in distilled water, 0.65 ml./100 g. of body weight).

RESULTS

Table I gives the results and Table II the analysis of variance of a typical corticotrophin assay using hydrocortisone acetate blocked rats. From these results it was calculated that the test material contained 31.0 U/mg. with fiducial limits (P=0.05) 21.7–44.2 U/mg. The root error mean square, s=38.0, the slope, b=−143.8, and the index of precision, $\lambda=0.264$.

TABLE II
THE ANALYSIS OF VARIANCE

Source of Variation	Sum of Squares	d.f.	Mean Square
Difference between standard and test	50	1	50
Regression	59,944	1	59,944
Departure from parallelism	88	1	88
Curvature	277	1	277
Difference of curvature ..	110	1	110
Doses	60,469	5	
Error	60,641	42	1,443·8
Total ..	121,110	47	

Fig. 1 shows the frequency distributions of the root error mean square, the slope, and the index of precision $\lambda\left(=\frac{s}{b}\right)$ of the forty assays. The mean values were found to be $s=36.7\pm0.9$, $b=162.7\pm7.5$, and $\lambda=0.244\pm0.013$.

From the accumulated data of the forty assays, the values of the combined variance and combined slope were calculated and found to be $s^2=1{,}389.2$ and $b=153.1$. Using these values in the formula

$$V(M)=\frac{s^2}{b^2}\left[\frac{1}{N_S}+\frac{1}{N_T}+\frac{(M-\bar{x}_S+\bar{x}_T)^2}{\Sigma(x-\bar{x})^2}\right]$$

from which approximate fiducial limits may be calculated, Table III was constructed showing the effect of varying the number of rats per assay on the errors which might be expected in a 2+2 point and a 3+3 point symmetrical assay ($N_S=N_T=\frac{1}{2}N$). The minimum variance would occur when the doses of test have been so chosen that they match exactly the doses of standard, that is, when $\bar{y}_T=\bar{y}_S$ In this instance

$$M-\bar{x}_S+\bar{x}_T=0 \text{ and } V(M)=\frac{s^2}{b^2}\left[\frac{1}{N_S}+\frac{1}{N_T}\right].$$

For the purpose of presentation of the results an arbitrarily chosen "maximal" error was also calculated if the doses of standard and test had been badly matched but overlapping, that is, if (1) S1=T2, in a 2+2 point assay with doses S1, S2, T1, T2 and if (2) S1=T2 and S2=T3, in a 3+3 point assay with doses S1, S2, S3, T1, T2, T3. In both these examples $M-\bar{x}_S+\bar{x}_T\left(=\frac{\bar{y}_T-\bar{y}_S}{b}\right)=0.3010$ and $V(M)=\frac{s^2}{b^2}\left[\frac{1}{N_S}+\frac{1}{N_T}+\frac{0{\cdot}3010^2}{\Sigma(x-\bar{x})^2}\right]$.

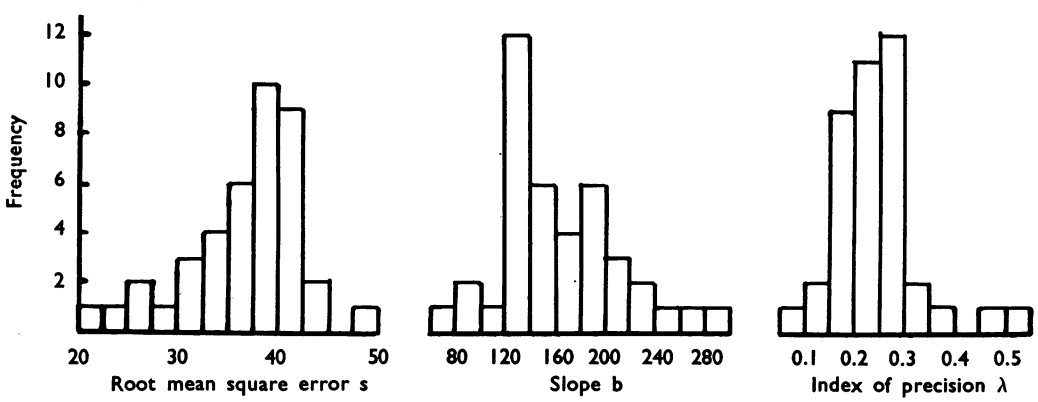

FIG. 1.—Histograms for root mean square error s (mean=36.7, median 38.1); slope b (mean=162.7, median=151.8) and index of precision λ (mean=0.244, median=0.228); n=40.

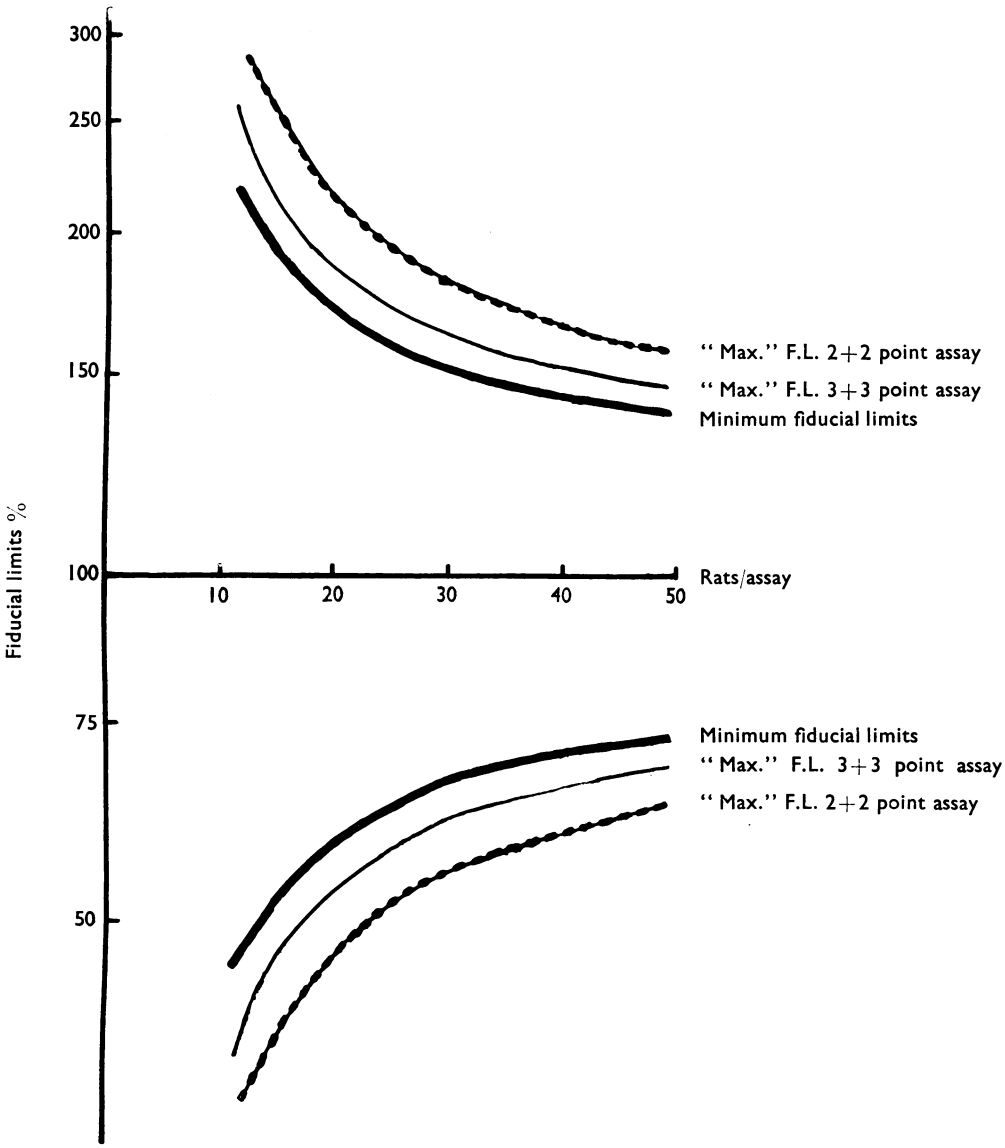

FIG. 2.—The effect of varying the number of rats per assay on the minimum and "maximum" fiducial limits (P=0.05). s^2=1,389.2; b=153.1.

TABLE III

THE EFFECT OF VARYING THE NUMBERS OF RATS PER ASSAY ON THE MINIMUM AND "MAXIMUM" FIDUCIAL LIMITS

$P=0.05$, $s^2=1,389.2$, $b=153.1$.

Rats/ Assay	2+2 pt. or 3+3 pt. Assay			2+2 pt. Assay			3+3 pt. Assay		
	Minimum Variance V(M) Min.	Weight W	Minimum Fiducial Limits	"Maximum" Variance V(M) Max.	Weight W	"Maximum" Fiducial Limits	"Maximum" Variance V(M) Max.	Weight W	"Maximum" Fiducial Limits
12	0·019756	51	47–212%	0·039511	25	35–288%	0·027162	37	39–254%
18	0·013170	76	57–177%				0·018109	55	51–197%
24	0·009878	101	62–161%	0·019756	51	51–197%	0·013582	74	57–176%
30	0·007902	127	66–152%				0·010866	92	61–164%
36	0·006585	152	68–146%	0·013170	66	58–172%	0·009055	110	64–156%
42	0·005644	177	71–142%				0·007761	129	66–151%
48	0·004939	202	72–139%	0·009878	101	63–159%	0·006791	147	68–147%

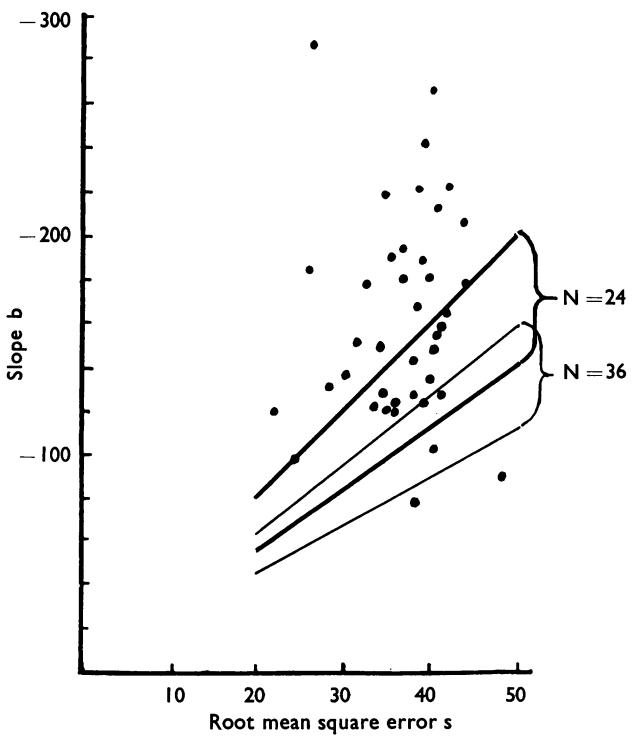

FIG. 3.—Values of s and b required to give fiducial limits of 50 to 200% when twenty-four or thirty-six rats per assay were used. ● represents individual values of s and b in forty assays of subcutaneous corticotrophin.

Fig. 2, which is constructed from the results in Table II, shows the number of rats required to give any desired degree of accuracy. Fig. 3 gives the values of s and b necessary to ensure the required fiducial limits of at the most 50 to 200% if twenty-four or thirty-six rats per assay are used. The individual values of s and b found in forty assays were plotted on the graph to indicate the number of assays where the desired precision would have been obtained using twenty-four or thirty-six rats per assay.

DISCUSSION

The results of forty assays of subcutaneous corticotrophin have been analysed by the method involving depletion of adrenal ascorbic acid in intact rats pretreated with hydrocortisone acetate. The total weight for the assays (1,340 rats) was found to be 6,283.2, so that the mean weight per rat was 4.69. This figure compares favourably with the value of 2.57 per rat in similar assays using hypophysectomized rats (Mussett and Perry, 1956), indicating that this modification appears to be about 1.5 to 2 times as efficient as the original Sayers (Munson modification) technique.

If limits of the order of 50 to 200% are required it can be seen from Fig. 2 that these limits can be obtained with twenty-four rats provided that the values of s and b are representative. However, because of the variations from assay to assay, it has been found necessary to use a rather larger number of rats to ensure a 200% upper fiducial limit. When the upper fiducial limit is 200%, $\log \frac{200}{100} = 0.3010 = t\sqrt{V(M)}$. When the error is minimal $V(M) = \frac{s^2}{b^2}\left[\frac{1}{N_S} + \frac{1}{N_T}\right]$ and therefore $\frac{s^2}{b^2} = \left(\frac{0.3010}{t}\right)^2 \div \left(\frac{1}{N_S} + \frac{1}{N_T}\right)$. By substituting N_S, N_T, and the appropriate value of t ($P = 0.05$) it was calculated that using twenty-four rats per assay $\frac{s^2}{b^2} = 0.12446$, so that if $s = 20$ then $b = 56.7$, and if $s = 50$, $b = 141.7$. All values of s and b above the line joining these points will give fiducial limits of 50 to 200% or less when twenty-four rats per assay are used and the doses have been correctly chosen. Similarly if a "maximal" variance occurs then the corresponding points are $s = 20$, $b = 80.2$ and $s = 50$ and $b = 200.4$. When the forty values of s and b are plotted on the graph, twenty-two of the forty assays would have given the required precision if twenty-four rats per assay had been used, but the majority of the remainder lie between the two lines ($N = 24$) and may give fiducial limits greater than 50 to 200%. When the number of rats per assay is increased to thirty-six all but five assays lie above the upper line. In practice it had been found necessary to use thirty-two rats per assay.

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THE STRUCTURE-ACTIVITY RELATIONSHIPS OF THE ANTIVIRAL CHEMOTHERAPEUTIC ACTIVITY OF ISATIN β-THIOSEMICARBAZONE

BY

D. J. BAUER AND P. W. SADLER

From the Wellcome Laboratories of Tropical Medicine, London, N.W.1, and the Courtauld Institute of Biochemistry, London, W.1

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As part of an investigation devoted to the development of new antiviral agents a compound of established antiviral activity has been subjected to systematic structural modification. The structure-activity data so obtained have been used in the design of new compounds, some of which are described. The compound chosen was isatin β-thiosemicarbazone, which has high activity against neurovaccinia infection in mice, and a 4-point parallel-line assay of *in vivo* chemotherapeutic activity has been developed, which has enabled the activity of the derivatives to be determined against isatin β-thiosemicarbazone as a standard. The overall dimensions of the isatin β-thiosemicarbazone molecule appear to be nearly maximal for the retention of high activity, as all substituents in the aromatic ring decrease the activity irrespective of their nature or position. The projection of the $-CS.NH_2$ group in relation to the ring nitrogen was found to be critical, as the α-thiosemicarbazone was inactive. A number of modifications of the side-chain were investigated: all led to reduction or loss of antiviral activity. The antiviral activity showed a positive correlation with chloroform solubility over a considerable range. The most active compound encountered was 1-ethylisatin β-thiosemicarbazone, with an activity of 286 (isatin β-thiosemicarbazone≡100). Isatin β-thiosemicarbazone showed no activity against 15 other viruses, and 20 related compounds showed no activity against ectromelia.

The antiviral effect of isatin β-thiosemicarbazone (I) against vaccinia virus was first observed by Thompson, Minton, Officer, and Hitchings (1953), who found that mice receiving a diet containing 0.6% of the compound were protected against intracerebral infection with about 100 LD50 of the virus. Their experiments were not designed to demonstrate the maximum effect of the compound, and it was subsequently shown (Bauer, 1955) that mice treated with isatin β-thiosemicarbazone by subcutaneous injection of 2 doses of 125 mg./kg. each day throughout the incubation period would survive intracerebral infection with about 10,000 LD50 of vaccinia virus without showing signs of illness. The compound also gave complete protection against 100 to 1,000 LD50 of virus when given in a single dose of 25 mg./kg. 18 hours after infection.

The activity of isatin β-thiosemicarbazone against vaccinia infection is thus fully equal to the effect of the sulphonamides and tetracyclines against the agents of the psittacosis-lymphogranuloma group. These organisms have long been described as viruses, but present opinion assigns them to the Rickettsiales, and isatin β-thiosemicarbazone thus remains the only true example of an antiviral agent active in experimental animals which confers a protection against infection which is sufficiently powerful to be of clinical interest.

It is therefore important to investigate the relationship between structure and antiviral activity, since the information gained, apart from its intrinsic importance in the chemotherapy of pox virus infections, may make it possible to establish principles governing activity which can be applied to other classes of antiviral agents yet to be discovered. The first steps in this direction were taken by Thompson *et al.* (1953), who considered that the antiviral activity required both the thiosemicarbazone side-chain and a cyclic component. In the present work the relationship of structure to activity has been studied in much greater detail. A number of related compounds

and derivatives of isatin β-thiosemicarbazone have been prepared and tested, the relationship between structure and activity has been fully elucidated, and some of the derivatives have been found to possess a therapeutic activity considerably greater than that of the parent compound.

METHODS

Synthesis of Compounds

The melting points and analytical data of new substituted isatin β-thiosemicarbazones prepared in the present investigation are given in Table I, together with literature references to the preparation of those isatins which are already known.

Ring-substituted Compounds.—These were prepared by refluxing equimolar quantities of thiosemicarbazide with the appropriate substituted isatin in 50% aqueous ethanol for several hours. The products which separated on cooling were collected and washed well with hot water. Crystallization from butanol afforded samples for analysis. *5-Carboxymethylisatin:* prepared from *p*-aminophenylacetic acid by the Sandmeyer synthesis, orange needles, m.p. 224°; found: C, 59.4; H, 3.5; N, 7.3; Calc. for $C_{10}H_7O_4N$: C, 59.9; H, 3.5; N, 7.0. *5-Ethoxycarbonylmethylisatin:* esterification of 5-carboxymethylisatin with ethanol and concentrated sulphuric acid gave 5-ethoxycarbonylmethylisatin as orange plates, m.p. 132°.

N-*substituted Compounds.*—*1-Pentylisatin:* equimolar quantities of 1-sodioisatin and pentyl bromide were heated under reflux for 48 hr. The mixture was filtered and the filtrate was extracted with a small volume of 2N sodium hydroxide, which on acidification with concentrated hydrochloric acid gave 1-pentylisatin as red plates, m.p. 47°. 1-Isopropyl- and 1-isobutylisatin were prepared similarly. *1-(2-Hydroxyethyl)-isatin:* equimolar quantities of 1-sodioisatin and 2-chloroethanol were heated under reflux for 48 hr. The mixture was filtered, the filtrate was reduced to small volume and the product separated on the addition of 60/80° petroleum ether. Crystallization from aqueous methanol gave orange needles, m.p. 118°. *5-Carboxymethyl-1-methylisatin:* methylation of 5-carboxymethylisatin with dimethyl sulphate in 2N sodium hydroxide gave 5-carboxymethyl-1-methylisatin as red needles, m.p. 180°. *5-Ethoxycarbonylmethyl-1-methylisatin:* esterification of 5-carboxymethyl-1-methylisatin with ethanol in concentrated sulphuric acid gave 5-ethoxycarbonylmethyl-1-methylisatin as bright orange needles, m.p. 143°. *1-Methyl-4-trifluoromethylisatin:* methylation of 4-trifluoromethylisatin (Sadler, 1956) with dimethyl sulphate in 2N sodium hydroxide gave 1-methyl-4-trifluoromethylisatin as large orange plates, m.p. 156°.

Compounds Substituted in the Side-chain.—*Isatin β-2-phenylthiosemicarbazone:* equimolar quantities of 2-phenylthiosemicarbazide (Mautner and Kumler, 1956) and isatin were heated under reflux in ethanol for 2 hr. The product was removed by filtration and washed with ethanol. Crystallization from butanol gave yellow plates, m.p. 210° (yield 80%). Found: C, 60.9; H, 4.2; S, 10.7; Calc. for $C_{15}H_{12}ON_4S$: C, 60.8; H, 4.1; S, 10.8. *1-Methylisatin β-2-phenylthiosemicarbazone:* equimolar quantities of 2-phenylthiosemicarbazide and 1-methylisatin were heated under reflux for 4 hr. in 50% aqueous ethanol. The ethanol was removed by distillation and the product which crystallized on cooling was recrystallized from 50% aqueous methanol: yellow needles, m.p. 175° dec. (yield 70%). Found: C, 61.9; H, 4.6; S, 10.3; Calc. for $C_{16}H_{14}ON_4S$: C, 61.7; H, 4.5; S, 10.3. The following compounds were prepared similarly. *Isatin β-4-methylthiosemicarbazone:* yellow needles from ethanol, m.p. 260° (decomp.). Found: C, 51.3; H, 4.4; N, 23.8; S, 13.4; Calc. for $C_{10}H_{10}ON_4S$: C, 51.3; H, 4.3; N, 23.9; S, 13.7. *Isatin β-4-allylthiosemicarbazone:* yellow needles from ethanol, m.p. 211°. Found: C, 55.5; H, 4.7; N, 20.7; S, 11.9; Calc. for $C_{12}H_{12}ON_4S$: C, 55.3; H, 4.7; N, 21.5; S, 12.3. *Isatin β-4-phenylthiosemicarbazone:* yellow needles from ethanol, m.p. 255° (decomp.). Found: C, 60.9; H, 4.2; N, 18.6; S, 10.5; Calc. for $C_{15}H_{12}ON_4S$: C, 60.8; H, 4.1; N, 18.9; S, 10.8. *Isatin β-S-methylthiosemicarbazone:* yellow needles from ethanol, m.p. 257° (decomp.). Found: C, 51.3; H, 4.4; N, 23.5; S, 13.9. Calc. for $C_{10}H_{10}ON_4S$: C, 51.3; H, 4.3; N, 23.9; S, 13.7.

Miscellaneous Compounds.—*Isatin α-thiosemicarbazone* (prepared by Dr. C. G. Raison). Hot solutions of isatin α-anil (2.22 g. in 10 ml. alcohol) and thiosemicarbazide (0.91 g. in 10 ml. water) were mixed and boiled for 1 hr. When cold, the separated crystals were recrystallized from aqueous dimethylformamide. The compound formed brick-red crystals, m.p. 216–217° (decomp.). Found: C, 48.8; H, 3.8; S, 14.4. Calc. for $C_9H_8ON_4S$: C, 49.1; H, 3.7; S, 14.5. *3-Formyl-1-methyloxindole thiosemicarbazone:* equimolar quantities of 3-formyl-1-methyloxindole (Julian, Pikl, and Boggess, 1934) and thiosemicarbazide were refluxed in aqueous ethanol. The product, which separated on cooling, was recrystallized from aqueous ethanol, yellow plates, m.p. 219°. Found: C, 53.6; H, 4.5; S, 12.3; Calc. for $C_{11}H_{12}ON_4S$: C, 53.2; H, 4.8; S, 12.9. *1-Acetylindoxyl thiosemicarbazone:* equimolar quantities of 1-acetylindoxyl (Vorländer and Drescher, 1901) and thiosemicarbazide were refluxed in aqueous ethanol. The product separated from the hot reaction mixture and was recrystallized from a large volume of ethanol, white plates, m.p. 242°. Found: C, 53.2; H, 4.9; S, 12.9; Calc. for $C_{11}H_{12}ON_4S$: C, 53.2; H, 4.8; S, 12.9. *β-1-Benzimidazolylpropionthioamide:* a solution of 0.1 mole of β-1-benzimidazolylpropionitrile (Efros, 1953) in 20 ml. of 30% (w/v) ammoniacal ethanol was saturated with hydrogen sulphide and allowed to stand for 2 days. The ethanol was removed under reduced pressure and the residue was crystallized several times from hot water giving white needles, m.p. 151° (yield 25%). Found: C, 59.1; H, 5.3; S, 15.5. Calc. for $C_{10}H_{11}N_2S$: C, 58.5; H, 5.4; S, 15.6.

TABLE I

SUBSTITUTED ISATIN β-THIOSEMICARBAZONES

Literature references are to the preparation of the corresponding isatins.

Substituent	Literature Reference	m.p.°	Formula	Required %			Found %		
				C	H	S	C	H	S
5-Fluoro ..	Holt and Sadler (1958)	275	$C_9H_7ON_4SF$	45·4	2·9	13·4	46·0	3·2	13·2
6-Fluoro ..	Sadler (1956)	257	$C_9H_7ON_4SF$	45·4	2·9	13·4	45·4	2·9	13·8
7-Fluoro ..	Holt and Sadler (1958)	263	$C_9H_7ON_4SF$	45·4	2·9	13·4	45·6	3·0	13·4
4-Chloro ..	Sadler (1956)	288	$C_9H_7ON_4SCl$	42·4	2·7	12·6	42·4	2·2	12·7
6-Chloro ..	,,	270	$C_9H_7ON_4SCl$	42·4	2·7	12·6	42·5	2·4	12·7
7-Chloro ..	Holt and Sadler (1958)	272	$C_9H_7ON_4SCl$	42·4	2·7	12·6	42·2	2·7	12·2
4-Bromo ..	Sadler (1956)	294	$C_9H_7ON_4SBr$	36·1	2·3	10·7	36·2	2·2	10·5
6-Bromo ..	,,	290	$C_9H_7ON_4SBr$	36·1	2·3	10·7	36·2	2·3	10·9
7-Bromo ..	Holt and Sadler (1958)	275	$C_9H_7ON_4SBr$	36·1	2·3	10·7	36·5	2·3	10·3
4-Iodo ..	Sadler (1956)	281	$C_9H_7ON_4SI$	31·2	2·0	9·2	32·0	2·3	9·7
5-Iodo ..	Borsche *et al.* (1924)	270	$C_9H_7ON_4SI$	31·2	2·0	9·2	31·5	2·2	9·0
6-Iodo ..	Sadler (1956)	275	$C_9H_7ON_4SI$	31·2	2·0	9·2	31·7	2·2	9·1
7-Iodo ..	Holt and Sadler (1958)	263	$C_9H_7ON_4SI$	31·2	2·0	9·2	30·7	1·6	9·0
6-Methyl ..	Sadler (1956)	270	$C_{10}H_{10}ON_4S$	51·3	4·3	13·7	51·1	4·4	13·5
7-Methyl ..	Holt and Sadler (1958)	275	$C_{10}H_{10}ON_4S$	51·3	4·3	13·7	51·1	4·4	13·6
1-Methyl ..	Hantzsch (1921)	245	$C_{10}H_{10}ON_4S$	51·3	4·3	13·7	50·5	4·5	13·7
1-Ethyl ..	Michaelis (1897)	201	$C_{11}H_{12}ON_4S$	53·2	4·8	12·9	53·1	5·1	12·8
1-Propyl ..	,,	193	$C_{12}H_{14}ON_4S$	54·9	5·4	12·2	55·1	5·1	12·1
1-Isopropyl ..	—	225	$C_{12}H_4ON_4S$	54·9	5·4	12·2	54·9	5·3	12·1
1-Pentyl ..	—	184	$C_{14}H_{18}ON_4S$	57·9	6·2	11·0	57·7	6·0	11·0
1-Hydroxymethyl	Reissert and Handeler (1924)	230	$C_{10}H_{10}ON_4S$	48·0	4·0	12·8	48·1	4·3	13·2
5-Amino ..	Giovannini and Portmann (1948)	>320	$C_9H_9ON_5S$	45·9	3·8	13·6	46·0	4·4	12·9
5-Hydroxy ..	,, ,,	284	$C_9H_8O_2N_4S$	45·8	3·4	13·5	45·5	3·4	13·4
1-Benzyl ..	Antrick (1885)	268	$C_{16}H_{14}ON_4S$	61·9	4·5	10·3	62·5	4·5	9·4
1-(2-Hydroxyethyl)	—	247	$C_{11}H_{12}O_2N_4S$	50·0	4·5	12·1	49·9	4·6	12·2
1-Carboxymethyl	Langenbeck (1928)		$C_{11}H_{10}O_3N_4S$	47·5	3·6	11·5	47·7	3·9	11·0
5-Carboxymethyl	—	273	$C_{11}H_{10}O_3N_4S$	47·5	3·6	11·5	47·1	3·6	11·1
1-Acetyl ..	Suida (1878)	244	$C_{11}H_{10}O_2N_4S$	50·4	3·8	12·2	50·8	4·1	12·3
1-Ethoxycarbonylmethyl	Ainley and Robinson (1934)	183	$C_{13}H_{14}O_3N_4S$	51·0	4·5	10·5	51·0	4·6	10·4
1-Diethoxycarbonylmethyl	,, ,,	200	$C_{16}H_{18}O_5N_4S$	50·8	4·8	8·5	50·6	4·4	8·4
1-(2-Cyanoethyl)	Carlo and Lindwall (1945)	234	$C_{12}H_{11}ON_5S$	52·7	4·0	11·7	52·6	4·0	11·8
4,5-Benz ..	Martinet (1919)	275	$C_{13}H_{10}ON_4S$	57·8	3·7	11·9	57·8	3·7	11·9
6,7-Benz ..	,,	280	$C_{13}H_{10}ON_4S$	57·8	3·7	11·9	57·3	3·7	11·7
5-Ethoxycarbonylmethyl	—	215	$C_{13}H_{14}O_3N_4S$	51·0	4·5	10·5	51·3	4·6	10·3
5-Carboxymethyl-1-methyl	—	264	$C_{12}H_{12}O_3N_4S$	49·3	4·1	10·9	49·6	3·8	10·5
5-Ethoxycarbonylmethyl-1-methyl	—	183	$C_{14}H_{16}O_3N_4S$	52·6	5·0	10·0	53·3	5·3	9·8
1-Methyl-4-trifluoromethyl	—	263	$C_{11}H_9ON_4SF_3$	43·7	3·0	10·6	44·6	3·1	10·7
5-Carboxy ..	Giovannini and Portmann (1948)	273	$C_{10}H_8O_3N_4S$	45·5	3·0	12·1			
7-Carboxy ..	,, ,,	296	$C_{10}H_8O_3N_4S$	45·5	3·0	12·1	45·3	2·9	12·2

Physical Investigations

Infra-red Absorption Spectra. — Spectra were determined by using a Perkin-Elmer 21 double-beam recording spectrometer fitted with a rock salt prism.

Water/lipid Partition Coefficients.—Solubilities in chloroform and partition coefficients of the compounds between chloroform and water were determined spectroscopically at 25° using matched quartz cells in a Hilger Uvispek.

Biological Methods

Viruses.—The IHD strain of neurotropic vaccinia virus adapted to intracerebral passage in mice was used. A stock suspension was prepared from an infected mouse brain homogenized in normal horse serum or tissue culture medium (10% bovine serum, 0.25% lactalbumin hydrolysate, 5% papain digest broth, 80% Earle solution) and mixed with 3 volumes of glycerol. The stock preparation was stored at −20°. Mice were inoculated intracerebrally with a suitable dilution of this preparation in tissue culture medium, and the time elapsing between infection and death was recorded to the nearest half-day. Animals which were alive and well at the end of 14 days were considered to have survived indefinitely.

The origin of the other neurotropic viruses used has been described previously (Bauer and Bradley, 1956); some were insufficiently stable as glycerol suspensions, and inoculations were made from intact brain tissue stored at −20° and homogenized at the time of use.

Treatment.—Most of the test compounds were only slightly soluble in water, and were made up as suspensions. The dose chosen was inoculated subcutaneously in a volume of 0.1 ml. twice daily for 5 days, the first dose being given 2 to 5 hr. after infection.

Preliminary Test of Antiviral Activity.—In previous work (Bauer, 1958) it has been shown that the mean reciprocal survival time of mice infected intracerebrally with neurovaccinia virus has a linear regression upon the logarithm of the dilution of virus used for infection, and that this relationship can be used as the basis of a test of chemotherapeutic activity, since treatment with an active compound will cause a significant reduction of the mean reciprocal survival time over a very wide range of virus doses. The compounds examined in the present work received a preliminary evaluation in a test of this nature, in which groups of 6 mice infected intracerebrally with about 1,000 LD50 of neurovaccinia virus were treated with doses of 125 mg./kg. and the survival times were compared with those of a control group of 6 mice which were similarly infected but left untreated. Compounds which gave no significant ($P>0.05$) reduction of the mean reciprocal survival time at this dose were considered to be inactive.

Determination of Dose-response Curves. — Compounds which showed activity in the preliminary test were examined further at lower doses. It was found that the mean reciprocal survival time gave a linear regression on the logarithm of dose of compound between the minimum dose producing an observable effect and the dose giving indefinite survival of all animals. The curve obtained with 1-propylisatin β-thiosemicarbazone is shown in Fig. 1, in which the response obtained at doses of 0.25, 0.5, and 2.5 mg./kg. is shown in comparison with the responses observed in the corresponding control groups.

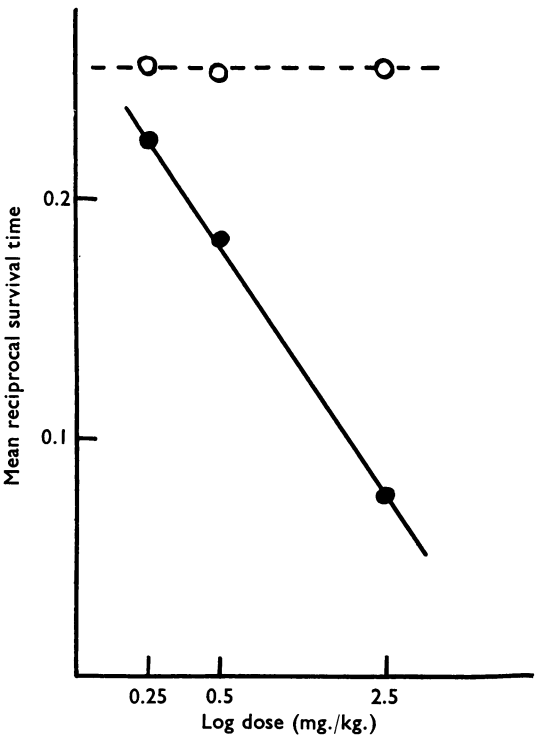


FIG. 1.—Dose-response curve of antiviral activity of 1-propylisatin β-thiosemicarbazone in mice infected intracerebrally with about 1,000 LD50 of neurotropic vaccinia virus. Ordinate, mean reciprocal survival time ; abscissa, log dose of compound. Treated ●——●; controls, O— — —O.

Assay of Antiviral Activity.—The regression lines obtained for the dose-response curves clearly formed a suitable basis for an assay of antiviral activity based on the conventional 4-point design, in which the active compounds were assayed against isatin β-thiosemicarbazone, which was chosen as a standard. From the dose-response curve of a given compound 2 doses were selected, one being twice the other, which would give responses within the region of linearity when 1,000 LD50 of virus was used as the infecting dose. Two doses were similarly selected for isatin β-thiosemicarbazone ; 1.25 and 2.5 mg./kg. were found to be suitable, although in some assays 1 and 2 mg./kg. were used. Four groups each of 6 mice were inoculated intracerebrally with about 1,000 LD50

of virus; 2 groups were treated with the test compound and 2 with isatin β-thiosemicarbazone, both in the selected doses. The survival times were converted to reciprocals (indefinite survival giving a reciprocal of zero) and the joint regression coefficient (b) was calculated for the 24 responses. The relative potency (R) was then obtained in the usual way as a function of the horizontal distance between the individual regression lines for test compound and standard from the relation

$$\log R = \log x_S - \log x_T - (\bar{y}_S - \bar{y}_T)\, b^{-1}$$

where x_S is the mean dose of isatin β-thiosemicarbazone, x_T is the mean dose of test compound, y_S is the mean response with isatin β-thiosemicarbazone and y_T the mean response with the test compound. The value obtained for R was then multiplied by 100 to give the activity based on isatin β-thiosemicarbazone taken as 100, and the result was finally multiplied by the ratio of the molecular weights of test compound and standard to give the activity expressed on an equimolar basis. The results of a typical assay are shown graphically in Fig. 2. The upper line represents the response obtained with doses of 1.25 and 2.5 mg./kg. of isatin β-thiosemicarbazone, and the lower line the responses with 5 and 10 mg./kg. of 7-methylisatin β-thiosemicarbazone; b was found to be −0.039, $\bar{y}_S$ 0.2176 and $\bar{y}_T$ 0.0747, giving a value of 0.89 for R, equivalent to a relative activity on an equimolecular basis of 94 for the 7-methyl derivative in comparison with the parent compound.

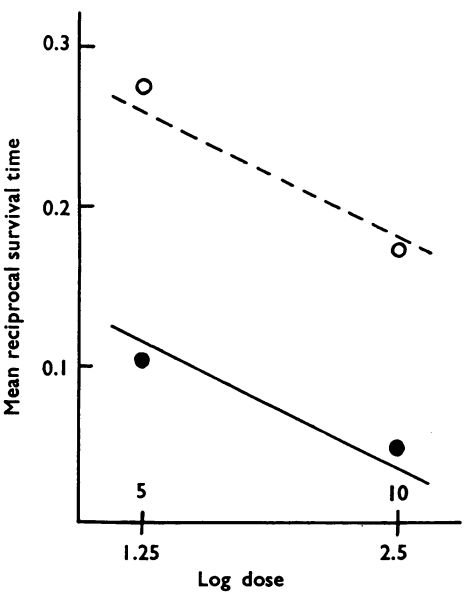


FIG. 2.—Four-point assay of antiviral activity of 7-methylisatin β-thiosemicarbazone in mice infected intracerebrally with about 1,000 LD50 of neurotropic vaccinia virus. Ordinate, mean reciprocal survival time; upper abscissa, log dose of test compound; lower abscissa, log dose of isatin β-thiosemicarbazone. ●——●, 7-methylisatin β-thiosemicarbazone; O— — —O, isatin β-thiosemicarbazone.

RESULTS

Effect on Antiviral Activity of Substitution in the Aromatic Ring

The introduction of substituents into the aromatic ring, as shown in Table II, usually results in reduction or total loss of activity. Substitution in the 5- position has a particularly marked effect, activity being lost except with substituents, such as fluorine, with small atomic or group radii. Activity is reduced to a lesser extent by substitution in the 4- and 6- positions, and some of the 7- substituted compounds still retain quite high activity.

An attempt was made to correlate the effect of substitution on antiviral activity with σ values, which are also given in Table II. The σ value, derived originally by Hammett (1949) from studies of the rates of hydrolysis of a series of substituted benzoic esters, affords a numerical measure of the

TABLE II

ANTIVIRAL ACTIVITY AND σ-VALUES OF RING SUBSTITUTED ISATIN β-THIOSEMICARBAZONES

(No substituent ≡ 100)

Substituent	4-Position	6-Position		5-Position	7-Position	mσ Value for 5- and 7-Positions
	Activity	Activity	pσ Value	Activity	Activity	
Amino ..				0		−0·161
Methyl ..	3·6	0·3	0·170	0	94	−0·069
Ethyl ..				0	~50	−0·043
Carboxymethyl ..				0		−0·027
Ethoxycarbonylmethyl ..				0		
Hydroxy ..				0		−0·002
Methoxy ..		0	−0·268	0·03		0·115
Fluoro ..		43·1	0·062	35·5	⩾20	0·337
Iodo ..	0	3·9	0·276	0	75·3	0·352
Carboxy ..				0	0	0·355
Chloro ..	9·9	11·7	0·227	4·2	98·3	0·373
Bromo ..	67·3	10·5	0·232	3·1	16	0·391
Trifluoromethyl ..					34·5	0·415
Nitro ..				0	0	0·710

effect of a substituent upon the reactivity of a side-chain in an aromatic system. The 5- and 7-substituents are listed together with the *meta* σ values, as both positions are *meta* to the β-carbon atom. The 6- substituents, which are *para* to the β-carbon atom, are given *para* σ values. No σ factors are quoted for the *ortho* substituents in the 4- position, as both steric and field effects are superimposed, to varying extents, on polar effects, making precise evaluation of these factors difficult (O'Sullivan and Sadler, 1957a). The 5- and 7-substituents are arranged in order of increasing *meta* σ values.

Table II shows that there is no parallel between antiviral activity and the σ values of the substituents. Considerable discrepancies occur between the data for the 5- and 7- substituents although their σ factors are identical, and, although a fairly wide range of σ values is covered in the 5- substituted series, ranging from the low value with the amino group through the intermediate values with the halogen substituents to the high value with the nitro group, most of these show zero activity. However, two generalizations emerge. Firstly, the smallest substituent (fluorine) in both the 5- and 6-positions decreases the activity the least (all other substituents quoted have much greater atomic or group radii), and secondly, substituents in the 7-position exert a noticeably smaller effect. This suggests that steric effects are far more important than the inductive and mesomeric effects of the substituents. Supporting evidence for this is that neither the 4,5- nor the 6,7-naphthisatin β-thiosemicarbazone possesses antiviral activity (see below). The observation that most activity is retained in the 7- substituted derivatives might be related to the relatively high solubility of the parent isatins. These compounds probably owe their enhanced solubilities to steric hindrance of the normal hydrogen-bonded dimer (O'Sullivan and Sadler, 1956), which suggests that the *N*-alkyl derivatives might have greater activity as these cannot dimerize in this fashion.

Effect of N-substitution upon Antiviral Activity

The activity of a number of *N*-substituted isatin β-thiosemicarbazones is shown in Table III. Alkylation in this position produces a marked rise in activity, reaching a maximum with 1-ethylisatin β-thiosemicarbazone, which with a relative activity of 286 is the most active compound so far encountered. Activity falls off as the chain is lengthened further, and the 1-pentyl derivative is practically inactive. High activity is retained in the 1-(2-hydroxyethyl) compound, but most of the other substituents which are either large or strongly polar have only residual or zero activity. This supports the hypothesis that isatin β-thiosemicarbazone should be monomeric and not form *inter*molecular bonds if high activity is to be retained.

TABLE III

ANTIVIRAL ACTIVITY OF *N*-SUBSTITUTED ISATIN β-THIOSEMICARBAZONES

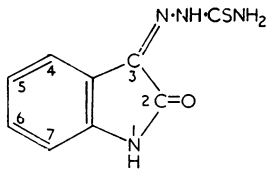
Substituent	Antiviral Activity
None	100
Methyl	202
Ethyl	286
Isopropyl	44
Propyl	28·5
Pentyl	3·4
Hydroxymethyl	42
1-Methyl-4-trifluoromethyl	48·4
2-Hydroxyethyl	204
Acetyl	87
Ethoxycarbonylmethyl	0
Diethoxycarbonylmethyl	0
2-Cyanoethyl	0
Carboxymethyl	0·02
5-Ethoxycarbonylmethyl-1-methyl	0
5-Carboxymethyl-1-methyl	0

Effect of Modifications in the Pyrrolidine Ring

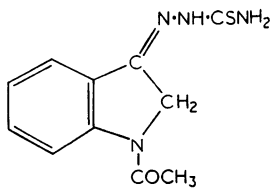
Modifications were next made at the 2- and 3-positions. The inactivity of 1-acetylindoxyl thiosemicarbazone (II) readily demonstrated that the α-carbonyl group was essential. Extension of the side-chain at the 3- position resulted in loss of activity, as 3-formyl-1-methyloxindole thiosemicarbazone (III) and 3-formylindole thiosemicarbazone were completely inactive. These and other similar results suggest that isatin β-thiosemicarbazone exerts its antiviral activity in the form of a six-membered, resonance-stabilized, hydrogen-bonded ring (IV), a type of bonding which has previously been demonstrated in the isatin β-oximes (O'Sullivan and Sadler, 1957b). Support for this hypothesis is provided by an analysis of the infra-red absorption data of isatin β-thiosemicarbazone and relevant reference compounds both in the solid state and in weakly polar solvents; however, the stability of such *intra*molecular bonds in an aqueous medium is uncertain. Isatin α-thiosemicarbazone may possess a similar *intra*molecular bonded structure (V), but it had no antiviral activity. This suggests not only that an all-planar configuration is

necessary but also that the orientation of the $-CS.NH_2$ group relative to the ring nitrogen is critical.

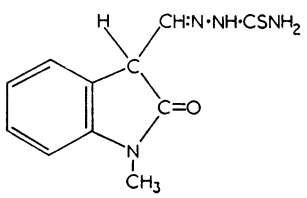
(I)



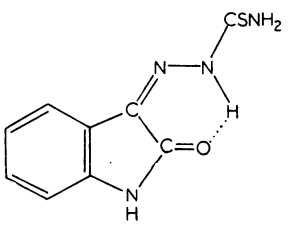
(II)



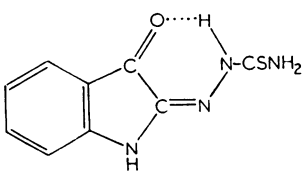
(III)



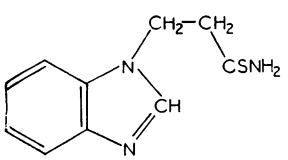
(IV)



(V)



(VI)



Effect of Substitution in the Side-chain

Finally, modifications were made to the thiosemicarbazone moiety, and the effects on antiviral activity are shown in Table IV. If *intra*molecular hydrogen bonding is of importance then replacement of the hydrogen atom on the nitrogen atom at the 2- position of the side-chain by another atom or group should result in loss of activity, as the molecule may no longer be held in configuration IV. Reference to Table IV shows this to be the case. The rest of the results presented indicate that an unsubstituted $-CS.NH_2$ group is essential for retention of activity.

TABLE IV

EFFECT OF SUBSTITUTION IN THE SIDE-CHAIN ON THE ANTIVIRAL ACTIVITY OF ISATIN β-THIOSEMICARBAZONE

R′–	R″=	R‴–	Antiviral Activity
H–	S=	H–	100
H–	O=	H–	0
H–	S=	C_6H_5	0
H–	HN=	H–	0
H–	S=	CH_3–	0
C_6H_5	S=	CH_3–	?
C_6H_5	S=	H–	0
H–	S=	CH_2=CH–CH_2–	4

R′–	R″–	R‴–	Antiviral Activity
—	CH_3–S–	H–	0

Miscellaneous Compounds

The following compounds were inactive: isatin, thiosemicarbazide, isatin β-amidinohydrazone, isatin β-hydrazone, di-β-isatinazine, 3-formyl-1-methyloxindole thiosemicarbazone, 1-acetylindoxyl thiosemicarbazone, α-naphthisatin β-thiosemicarbazone, β-naphthisatin β-thiosemicarbazone, isatin β-semicarbazone, isatin α-thiosemicarbazone, β-1-benzimidazolylpropionthioamide.

Activity of Isatin β-Thiosemicarbazone Against Other Viruses

In tests carried out in mice by the same method as the preliminary test of activity against neurovaccinia virus, isatin β-thiosemicarbazone in repeated doses of 100–125 mg./kg. had no therapeutic effect upon intracerebral infection with

poliomyelitis, influenza (NWS strain), rabies (Flury strain), Ilhéus, Wyeomyia, Zika, California, pseudolymphocytic choriomeningitis (Sandom strain, identical with ectromelia), Ntaya, Semliki, herpes, dengue 1, Anopheles A, Anopheles B or MM viruses.

Activity of Substituted Isatin β-Thiosemicarbazones and Related Compounds Against Ectromelia

In view of the close antigenic relationship between vaccinia and ectromelia viruses many of the compounds were tested against intracerebral infection with ectromelia (pseudolymphocytic choriomeningitis) virus. The following compounds showed no detectable activity when tested at doses ranging in some cases up to 100 mg./kg.: isatin β-semicarbazone, isatin β-4-phenyl thiosemicarbazone, isatin β-amidinohydrazone, isatin β-hydrazone, di-β-isatinazine, 3-formyl-indole thiosemicarbazone, and the β-thiosemicarbazones of the following substituted isatins: 5-fluoro-, 5-chloro-, 5-bromo-, 5-methoxy-, 5-ethyl-, 5-nitro-, 4-methyl-, 7-chloro-, 7-nitro-, 1-methyl-, 1-hydroxymethyl-, 1-(2-hydroxyethyl)- and 1-(2-cyanoethyl)-isatin.

Discussion

Isatin is a diketonic compound and it has many of the properties of a simple diketone. A number of dicarbonyl compounds have been reported to possess antiviral activity against Newcastle disease virus and the PR8 strain of influenza virus in fertile eggs (McLimans, Underwood, Slater, Davis, and Siem, 1957), but they were also virucidal *in vitro* and the effect was presumably due to the degradation of the amino-acids of the virus by the Strecker reaction. A mechanism of this kind cannot explain the activity of isatin β-thiosemicarbazone, since isatin has no activity against vaccinia virus *in vivo*, and the antiviral activities of the substituted isatin β-thiosemicarbazones show no correlation with the dehydrogenase activity of the corresponding isatins, as shown in Table V. The logarithms of the chloroform solubilities of the compounds shown in Table V show a positive correlation with antiviral activity (correlation coefficient 0.775, $P<0.01>0.001$). The increased activity of the simple *N*-alkyl derivatives may therefore result entirely from their improved lipid solubility, which might more readily enable them

TABLE V

σ-VALUES AND DEHYDROGENASE ACTIVITIES OF SUBSTITUTED ISATINS, WITH ANTIVIRAL ACTIVITIES AND PARTITION COEFFICIENTS OF THE CORRESPONDING β-THIOSEMICARBAZONES

Figures in column three are obtained from the reciprocals of the times required for the systems to decolorize methylene blue in 10^{-4} M solution of the substituted isatin and 0·05 M DL-alanine (O'Sullivan and Sadler, 1957c).

Substituent	σ	Dehydrogenase Activity Relative to DL-Alanine	Antiviral Activity	Solubility in Chloroform (mg./100 ml.)	Chloroform Water Partition
4-Methyl	—	11	3·4	8	0·063
6-Methyl	−0·170	30	0·3	18	0·15
7-Methyl	−0·069	55	88·8	16	0·185
None	0·0	100	100	32	0·33
6-Fluoro	0·062	50	39·8	16	0·48
5-Methoxy	0·115	18	0·03	3	0·077
4-Chloro	—	105	8·6	10	0·34
6-Chloro	0·227	180	4	21	0·69
4-Bromo	—	200	49·5	10	0·205
5-Fluoro	0·337	—	35·5	4	—
7-Carboxy	0·355	—	0	0	∞
7-Chloro	0·372	210	85	29	0·097
1-Methyl	—	—	190	160	0·083
1-Acetyl	—	—	87	255	—
1-Pentyl	—	—	0	>200	0·0
1-Diethoxycarbonylmethyl	—	—	0	>200	0·33
1-Ethyl	—	—	286	2,170	—
1-(2-Hydroxyethyl)	—	—	204	25	—
1-Butyl	—	—	28·5	1,600	—
1-Isobutyl	—	—	44	2,100	—

to pass the blood-brain barrier and so reach the neurotropic strain of vaccinia used as the test virus. This would also account for the inactivity of the compounds bearing strongly polar substituents. The reduction in activity produced by substitution in the aromatic ring suggests that steric factors are of importance, in that this region of the molecule must not exceed certain dimensions if activity is to be retained.

The general configuration of the isatin molecule resembles that of the purines and also tryptophan and its derivatives, but there is no evidence available as to whether isatin β-thiosemicarbazone acts as an antimetabolite to these compounds; in particular, an action against 5-hydroxytryptamine seems to be excluded by the lack of antiviral activity of 5-hydroxyisatin β-thiosemicarbazone.

The requirement for sulphur in the side-chain suggests that metal chelation may be of importance for antiviral activity, particularly of copper, and it is of interest to note in this connexion that vaccinia virus contains copper (Hoagland, Ward, Smadel, and Rivers, 1941), a circumstance which might enable it to take up isatin β-thiosemicarbazone selectively, and that salts of copper have a therapeutic effect against neurovaccinia infection in mice (Bauer, 1958). However, even if chelation is concerned, the configuration of the ligand is also critical, since, whereas all thiosemicarbazones chelate copper, only a very limited number have antivaccinial activity.

Several possibilities exist for the *in vivo* conversion of the compound into biologically more active substances. For instance, simpler substituted thioureas may be obtained, which bear resemblance to some of the phenylthiourea derivatives recently described by Doub *et al.* (1958). These compounds have marked tuberculostatic activity (Youmans *et al.*, 1958), but this is of little import as antiviral and tuberculostatic activities rarely run parallel (Hurst and Hull, 1956); benzthiouracils could also be obtained, and substituted phenoxythiouracils have considerable antiviral activity (Bauer, 1955), but this route is rendered unlikely as the suggested mechanism for the formation of the benzthiouracil seems chemically improbable, although it might occur biologically. Formation of a tricyclic compound by loss of the elements of water from the α-carbonyl group and the terminal amino group of isatin β-thiosemicarbazone is possible, and it has been clearly demonstrated that both these groups are essential for the retention of activity. However, the resulting structure is not sufficiently close to growth inhibitors such as 2-thiodenine or 6-thiopurine (Elion *et al.*, 1953) to be considered as a purine antagonist. The relationship to thioalloxazine is also somewhat remote, but the enhanced activity which results from 2-hydroxyethyl substitution in the 1-position (Table III) makes the synthesis of related glycosides of considerable interest as potential riboflavine antagonists.

The structures of these compounds seem too remote from that of nicotinamide to be considered as competitive inhibitors; some support is given by the fact that similar arguments have been used in the case of the tuberculostatic compound isoniazid (isonicotinic acid hydrazide) (Zatman *et al.*, 1953). Obviously other heteroaromatic thioamides containing more than one nitrogen atom per heterocycle are worthy of investigation, but it would seem to be essential for the thiocarbamyl group to be attached directly to one of the rings, as β-1-benzimidazolylpropionthioamide (VI) is inactive. The lack of activity of isatin β-thiosemicarbazone against other viruses, particularly ectromelia, is difficult to explain in accordance with the commonly held view that viruses control the metabolism of the cell in which they are multiplying, for this implies that the final common path in virus multiplication must be the same for all viruses. A possible explanation is that isatin β-thiosemicarbazone chelates with the copper present in vaccinia virus and thereby exerts a detrimental effect upon virus multiplication; if this is the case, then the lack of activity against other viruses implies that they do not contain copper, or, if they do, then the copper is either inaccessible or held in a chelate of higher stability constant.

We wish to thank Miss M. E. Pitman for her assistance in the preparation of the compounds.

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PHARMACOLOGICAL PROPERTIES OF THALIDOMIDE (α-PHTHALIMIDO GLUTARIMIDE), A NEW SEDATIVE HYPNOTIC DRUG

BY

G. F. SOMERS

From the Pharmacological Research Laboratories, The Distillers Company (Biochemicals) Ltd., Speke, Liverpool 24

(RECEIVED SEPTEMBER 21, 1959)

Thalidomide (α-phthalimidoglutarimide, " Distaval," " Contergan ") is a new sedative hypnotic drug which produces no toxic effects when administered orally to animals in massive doses. This lack of toxicity may be due to a limited absorption. The drug has a quietening effect on the central nervous system, reducing the voluntary activity of laboratory animals and promoting sleep. Unlike the barbiturate drugs it does not cause an initial excitation in mice, incoordination or narcosis. It potentiates the actions of other central nervous system depressants, in particular the barbiturates. Its sedative effects are counteracted by central nervous system stimulants. It has no deleterious side effects and does not affect the heart, respiration or autonomic nervous system.

Thalidomide is a derivative of glutamic acid, its chemical structure being shown in Fig. 1.

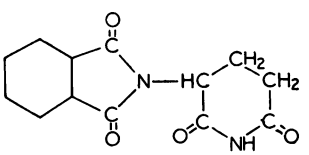

FIG. 1

It was first synthesized by Kunz, Keller, and Muckter (1956), who also described its main pharmacological properties. It occurs as a pure white, tasteless crystalline powder with a melting point of 271°. The substance is sparingly soluble in water, methanol, ethanol, acetone, and glacial acetic acid but readily soluble in dioxane, dimethyl formamide, pyridine and chloroform. It is insoluble in ether and benzene. Chemically it is related to bemegride (α-ethyl-α-methyl-glutarimide) and to glutethimide (β-ethyl-β-phenyl-glutarimide), but its pharmacological properties are different.

METHODS

Except where specifically stated thalidomide was used as a suspension in a 1% solution of carboxy-methylcellulose.

Acute Toxicity

The acute toxicity of thalidomide was determined by the oral, subcutaneous and intraperitoneal routes in male albino mice weighing between 19 and 21 g. Comparisons were made with phenobarbitone sodium and glutethimide. Where possible the regression of percentage mortality as probits on the logarithm of the dose was found, and the LD50s and limits of error ($P=0.95$) were calculated by the method of Litchfield and Wilcoxon (1949).

Subacute Toxicity

The subacute toxicity of thalidomide was determined in young rats which were dosed orally each day over four weeks. Their body weights were recorded twice weekly and at the end of the period the rats were killed and a post-mortem carried out. Microscopic preparations of the major organs were also examined.

The pharmacological actions of thalidomide on the central and autonomic nervous systems and on the cardiovascular system were studied in mice, rats and anaesthetized cats, according to the following methods.

Central Nervous System

Motor Activity.—The sedative effects of thalidomide were determined on the voluntary motor activity of mice by the photobeam method of Dews (1953). The apparatus was a perspex tank measuring 15 in. × 9 in. A beam of light shone across the short axis on to a phototransistor (Mullard OCP71). Interruption of the light beam activated a Thorp impulse counter (C. F. Palmer) and a magnetic digital counter. Groups of ten mice were placed in the tank 30 min. after dosing

and the counts recorded at 15 min. intervals over 45 min. For the calculations the counts between 15 and 45 min. were used, since the initial count was affected by the exploratory hyperactivity of the mice. A control group was always run concurrently with a treated group and the response of the treated group calculated as a percentage of the control value.

Narcotic Activity.—This was tested in mice by placing them on their backs and recording the number which had lost their righting reflex.

Movement Co-ordination and Holding Reflex.—The effects of thalidomide on motor co-ordination were compared with phenobarbitone and glutethimide by the rotating rod method of Gross, Tripod and Meier (1955). Groups of ten mice were treated with the respective drugs and placed at 30 min. intervals on to a wooden rod rotating at 15 revs./min. The times the individual mice were able to remain on the rod were measured and the average determined for each group. Mice remaining on the rod for more than 2 min. were regarded as unaffected.

Depression of the holding reflex was assessed by suspending mice by the front legs from a horizontal wire and observing their ability to bring up their back legs.

Anticonvulsant Activity.—This was determined by measuring the ability of thalidomide to protect mice against the convulsant actions of leptazol and strychnine.

Analgesic Activity.—Thalidomide was tested for analgesic activity in mice and rats by the hot plate method of Woolfe and Macdonald (1944), the tail pinching method as described by Bianchi and Francheschini (1954) and electrical stimulation of the feet (Dodds, Lawson, Simpson and Williams, 1945) and tail (Kraushaar, 1953). It was also tested for its ability to potentiate the analgesic actions of morphine and pethidine.

Hypothermic and Antipyretic Activities. — These were tested in normal rabbits and in rabbits made febrile by an injection of a suspension of inactivated *Escherichia coli* bacteria.

Autonomic Nervous System, Heart and Respiration

The pharmacological effects of thalidomide on the autonomic nervous system were studied in cats anaesthetized with chloralose. Blood pressure was recorded from the left carotid artery and respiration from a tracheal cannula by the method described by Paton (1949). The right preganglionic cervical sympathetic nerve was severed and the rostral stump stimulated with rectangular pulses of approximately 8 V and of 2.5 msec. duration for 5 sec. An electrocardiograph was taken from lead II using subcutaneously implanted needle electrodes. The record was made with a Cambridge direct-writing cardiograph. Thalidomide, because of its low water solubility, could not be given intravenously. It was, therefore, injected into the peritoneal cavity or directly into the duodenum.

Rabbit isolated hearts were perfused with Ringer-Locke solution containing 10 mg./l. of thalidomide and records were made of the heart beat and coronary flow.

Gastro-intestinal Tract

The effects of thalidomide on intestinal muscle were studied *in vitro* on the isolated duodenum of the rabbit and the isolated ileum of the guinea-pig. *In vivo* the charcoal meal test was used as described by Bryant, Felton and Krantz (1957).

Urinary System

Kidney effects of thalidomide were studied in rats hydrated with an oral dose of water (50 ml./kg.) and placed into individual metabolism cages.

Interactions with Other Drugs

Barbiturates.—The duration of hexobarbitone anaesthesia (125 mg./kg., intraperitoneally) was compared between groups of control and thalidomide treated mice.

Alcohol.—Possible potentiation of the toxic effects of alcohol was studied by determining the LD50s of alcohol in groups of mice given different doses of thalidomide.

Reserpine and Chlorpromazine. — These were studied in two ways. Firstly a comparison was made of the duration of catatonia in groups of mice given these drugs with and without thalidomide. Secondly the effects of thalidomide on the potentiating action of these drugs on hexobarbitone were determined as described by Kopmann and Hughes (1958).

Methylamphetamine and Methylphenidate.—These stimulant drugs were administered to mice previously treated with thalidomide and the effects on motor activity observed. The LD50s of these stimulant drugs were also determined in mice treated with thalidomide and compared with the values for normal mice.

Results

Toxicity

Acute Toxicity.—Thalidomide proved to be virtually non-toxic. As reported by Kunz *et al.* (1956), mice tolerated the maximum oral dose (5 g./kg.) that could be administered, without ill effects. They showed sedation without a preliminary excitation. Motility was considerably reduced, the mice bunching in a corner of the cage and going to sleep. There was no incoordination in their movements, and narcosis, catalepsy, and convulsions were absent. Sleeping mice could be easily aroused, and then they resumed normal but slow movements and showed little interest in their surroundings. In contrast both phenobarbitone and glutethimide caused initial excitation, marked ataxia, and incoordination of movement followed by narcosis and death. The LD50s of these two drugs are compared with thalidomide in Table I.

TABLE I

THE ACUTE ORAL TOXICITIES OF THALIDOMIDE, GLUTETHIMIDE AND PHENOBARBITONE IN MICE

Compound	LD50 (g./kg.)	Confidence Limits (P=0·95; g./kg.)
Thalidomide	>5	—
Glutethimide	0·52	0·46–0·58
Phenobarbitone sodium ..	0·27	0·25–0·29

Mice survived intraperitoneal doses as high as 4 g./kg. Guinea-pigs given 650 mg./kg. orally became quiet and sedated, while 400 mg./kg. injected intramuscularly produced no effects at all, insoluble material remaining unabsorbed at the site of the injection for over two days.

The low toxicity of thalidomide has been observed in man. Twenty cases of accidental or intentional overdosage have been reported (de Souza, 1959; Burley, personal communication), and all recovered uneventfully. It may well be that the absence of toxicity is due to a limited absorption, for the compound has a low solubility in body fluids, and when administered parenterally remains at the site of the injection. In the absence of a suitable assay method absorption studies have not yet been made.

Subacute Toxicity.—Two groups of ten rats given daily oral doses of 250 mg. and 1,000 mg./kg. respectively over a period of 21 days grew normally compared with untreated controls (Table II). Blood examinations showed no abnormal changes in the red and white cell counts.

TABLE II

THE EFFECT OF REPEATED ADMINISTRATION OF THALIDOMIDE ON THE GROWTH OF MALE RATS

Treatment	Daily Dose (mg./kg.)	Average Body Weights (in g.) at Stated Times from Beginning of Experiment			
		0 Days	7 Days	14 Days	21 Days
Controls ..	0	135	177	207	228
Thalidomide	250	142	179	214	226
„	1,000	138	171	197	226

There were no abnormal constituents in the urine. When the rats were killed no significant pathological changes were seen in the major organs or glands. Histological sections of the thyroid glands showed only a slight reduction in the colloid material, suggesting a slight depression of secretory activity. Murdoch and Campbell (1958) reported that thalidomide depressed the uptake of radioactive iodine by the thyroid in humans, but this has been shown to be common to tranquillizing drugs (Friedell, 1958). Greene and Farran (1958) stated that the effects on the human thyroid were negligible. The thyroid weights of our rats were normal, and histological changes in the glandular epithelium which one normally associates with an antithyroid drug were absent. A slight depression of glandular activity can be expected from a sedative drug due to a decreased demand for the thyroid hormone following the reduction in body activity.

Central Nervous System

Motor Activity.—Thalidomide caused a marked reduction in motor activity. The results obtained, using eight groups of mice at each dose level, are shown in Fig. 2. It proved impossible to compare the activity of thalidomide with phenobarbitone and glutethimide by this method, as mice varied considerably in their response to the latter drugs between hypermotility due to excitation and lack of movement due to narcosis. Kunz *et al.* (1956) using activity cages found the oral doses inducing sleep in mice to be 100 mg. for thalidomide, 40 mg. for phenobarbitone sodium, and 75 mg. for glutethimide, all per kg. body weight.

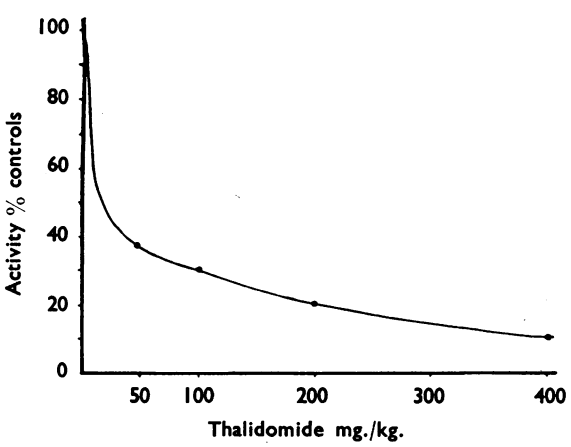

FIG. 2.—The effect of thalidomide on voluntary motor activity in mice. Each point is the mean of eight groups.

Narcotic Activity.—Thalidomide was devoid of a narcotic action. Even in maximum doses (5 g./kg.) it did not affect the righting reflex in mice. It behaved quite differently in this respect from glutethimide and phenobarbitone, which both had a strong narcotic effect.

Movement Co-ordination and Holding Reflex.—Even in very large doses thalidomide showed no effect on motor co-ordination. Mice given oral doses of 5 g./kg. were able to stay on the rotating rod for more than 2 min. With phenobarbitone and glutethimide the mice were unable to maintain their locomotion and balance and fell off the rod.

Anticonvulsant Activity.—Thalidomide did not antagonize leptazol-induced convulsions in mice, even in very large doses (1.6 g./kg.). Against spinal convulsions induced by strychnine there was a slight protection, probably due to the sedative action of thalidomide in reducing sensory stimuli. Similar results were obtained by Fincato (1957). Kunz *et al.* (1956) found it ineffective against electroshock seizures in the rat.

Analgesic Activity.—Thalidomide showed no analgesic activity in the tests described. In this respect it behaved similarly to glutethimide and to phenobarbitone, which do not reduce sensitivity to pain. It slightly potentiated the analgesic activities of morphine and pethidine in doses of 400 mg./kg. in mice.

Hypothermic and Antipyretic Activities.—Thalidomide did not reduce the basal temperature of rabbits in oral doses of 200 mg./kg. It showed a slight antipyretic activity, lowering and shortening the pyretic response to inactivated *E. coli* bacteria.

Cardiovascular, Respiratory and Autonomic Nervous System

In the anaesthetized cat administration of 125 mg./kg. of thalidomide intraperitoneally or direct into the duodenum did not affect the heart rate, blood pressure or respiration. The blood pressure responses to acetylcholine, adrenaline and histamine were unaltered, and the responses of the nictitating membrane to adrenaline and to stimulation of the preganglionic cervical sympathetic nerve remained the same. The drug therefore had no significant effects on the heart, respiration or autonomic nervous system, nor did it show any antihistamine activity.

The absence of cardiotoxicity was confirmed on the rabbit isolated heart where perfusion with Ringer-Locke solution containing 10 mg./l. of thalidomide produced no change in the amplitude and rate of the contractions. The coronary flow was also unaffected.

Gastro-intestinal Tract

In vitro thalidomide had a mild and short-lived spasmolytic action, a dose of 0.5 mg. in a 15 ml. bath reducing the spasmodic contractions of the guinea-pig isolated ileum to acetylcholine and histamine.

In vivo an oral dose of 500 mg./kg. of thalidomide given 30 min. before a charcoal meal did not significantly reduce the rate of transport through the stomach and intestines. The rate of travel was only reduced by 15%.

Urinary System

Thalidomide did not affect urinary output, the rate of excretion in water-loaded rats being the same after thalidomide as in the controls.

Interaction of Thalidomide with Other Drugs

Central Nervous System Depressants.—(1) Barbiturates. Thalidomide, in common with

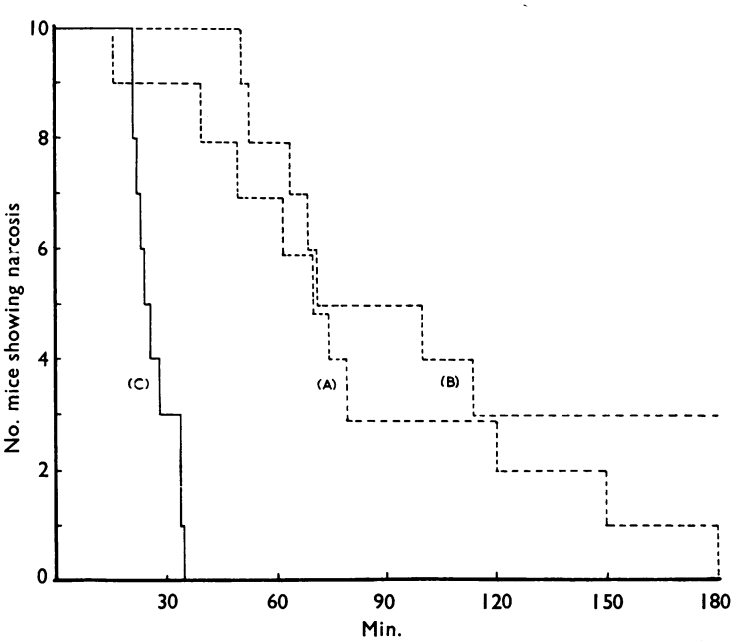

FIG. 3.—The effect of thalidomide (– – – (A) 400 mg./kg.; – – – (B) 1,600 mg./kg.) on hexobarbitone sodium (—— (C) 125 mg./kg.) narcosis in mice.

tranquillizing drugs, potentiated the narcotic action of barbiturates. Fig. 3 illustrates the results from a typical experiment with hexobarbitone showing that the anaesthetic action was considerably prolonged. The mean duration of narcosis in groups of the mice injected with 125 mg./kg. of hexobarbitone intraperitoneally 30 min. after oral doses of 400 and 1,600 mg./kg. of thalidomide were respectively 85 and 98 min. compared with 27 min. for the controls

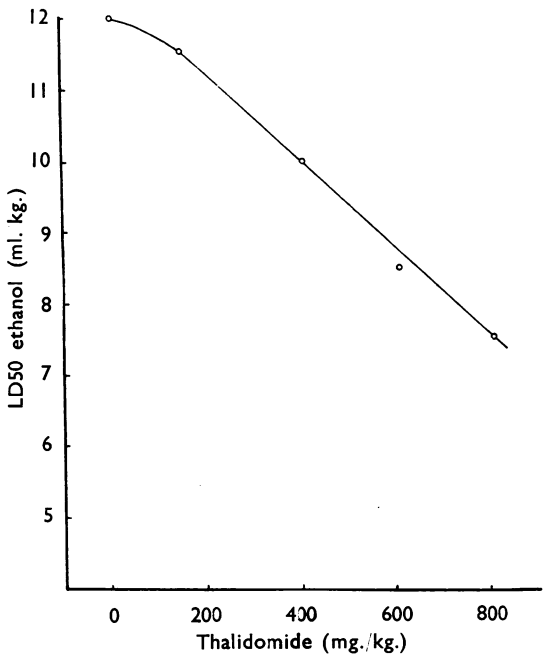

FIG. 4.—The effect of thalidomide on the acute toxicity of ethanol in mice.

TABLE III

THE EFFECT OF THALIDOMIDE ON THE DURATION OF CATATONIA IN MICE GIVEN CHLORPROMAZINE

There were ten mice in each group.

Group No.	Oral Dose of Thalidomide (mg./kg.)	Intraperitoneal Dose of Chlorpromazine (mg./kg.)	Percentage of Mice Showing Catatonia after :	
			24 hr.	48 hr.
1	0	40	50	0
2	100	40	40	0
3	200	40	60	20
4	400	40	90	30
5	800	40	100	90

(2) Alcohol. Thalidomide increased the oral toxicity of ethyl alcohol in mice. The increase in toxicity was linearly related to the dose of thalidomide (Fig. 4).

(3) Reserpine and chlorpromazine. A single oral dose of thalidomide increased the duration of catatonia produced in mice by chlorpromazine or reserpine (Tables III and IV). Similarly thalidomide increased the potentiating action of chlorpromazine and reserpine on hexobarbitone

TABLE IV

THE EFFECT OF THALIDOMIDE ON THE DURATION OF CATATONIA IN MICE GIVEN RESERPINE

There were ten mice in each group.

Group No.	Oral Dose of Thalidomide (mg./kg.)	Intraperitoneal Dose of Reserpine (mg./kg.)	Percentage of Mice Showing Catatonia after 24 hr.
1	Controls	2	0
2	100	2	20
3	200	2	50
4	400	2	60
5	800	2	70

TABLE V

THE EFFECT OF THALIDOMIDE ON THE POTENTIATING ACTION OF CHLORPROMAZINE ON HEXOBARBITAL NARCOSIS IN MICE

There were ten mice in each group. Thalidomide was given 2 hr. before, and chlorpromazine 1 hr. before, hexobarbital. The estimated standard error of a single mean duration of narcosis is $\pm 13\%$.

Group No.	Oral Dose of Thalidomide (mg./kg.)	Intraperitoneal Dose of Chlorpromazine (mg./kg.)	Intraperitoneal Dose of Hexobarbital (mg./kg.)	Geometric Mean Duration of Narcosis (min.)
1	0	0	125	21·0
2	0	1·5	125	37·9
3	100	1·5	125	36·7
4	200	1·5	125	52·6
5	400	1·5	125	49·4
6	800	1·5	125	70·8
7	100	0	125	30·0
8	200	0	125	30·5
9	400	0	125	33·0
10	800	0	125	44·7

TABLE VI

THE EFFECT OF THALIDOMIDE ON THE POTENTIATING ACTION OF RESERPINE ON HEXOBARBITAL NARCOSIS IN MICE

There were ten mice in each group. Reserpine and thalidomide were given 2 hr. before hexobarbital. The estimated standard error of a single mean duration of narcosis is ±15%.

Group No.	Oral Dose of Thalidomide (mg./kg.)	Intraperitoneal Dose of Reserpine (mg./kg.)	Intraperitoneal Dose of Hexobarbital (mg./kg.)	Geometric Mean Duration of Narcosis (min.)
1	0	0	125	19·4
2	0	0·4	125	31·1
3	100	0·4	125	41·5
4	200	0·4	125	42·2
5	400	0·4	125	47·3
6	800	0·4	125	51·6
7	100	0	125	26·7
8	200	0	125	32·2
9	400	0	125	24·9
10	800	0	125	31·2

narcosis in mice using the test described by Kopmann and Hughes (1958). (Tables V and VI.)

Central Nervous System Stimulants.—(1) Methylamphetamine. This stimulant drug rapidly counteracted the depressant action of thalidomide in mice. They quickly resumed normal motility and with increasing doses of methylamphetamine they became hyperactive and convulsed. The motility of mice made hyperactive with methylamphetamine (5 mg./kg., subcutaneously) was not depressed by an oral dose of thalidomide (500 mg.). The acute toxicity of methylamphetamine was also not reduced by the previous administration of thalidomide.

(2) Methylphenidate ("Ritalin"). The interaction of methylphenidate with thalidomide was very similar to that of methylamphetamine. Very high doses of thalidomide (1 g./kg.) slightly reduced hypermotility of mice given methylphenidate.

DISCUSSION

Thalidomide has been shown experimentally to be a sedative hypnotic drug acting differently from the barbiturates. It does not cause incoordination, respiratory depression or narcosis, and it is virtually non-toxic, possibly due to a limited absorption. The clinical value of this drug has been reported by Jung (1956), Stark (1956) and by Burley, Dennison, and Harrison (1959). Its safety has been confirmed in a number of cases of accidental and suicidal overdoses (de Souza, 1959; Burley, personal communication).

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THE ACTION OF SYMPATHETIC BLOCKING AGENTS ON ISOLATED AND INNERVATED ATRIA AND VESSELS

BY

S. HUKOVIĆ*

From the Department of Pharmacology, University of Oxford

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A preparation is described of isolated rabbit atria with both vagus and sympathetic nerves. The action on it of bretylium and of choline 2,6-xylyl ether bromide (TM10) was studied. A concentration of breylium sufficient to abolish the response to sympathetic stimulation also depressed the response to vagal stimulation. The effect was reversible, though more easily with choline xylyl ether. Both drugs abolished the accelerating action of acetylcholine in the presence of atropine, but they augmented the action of tyramine, and did not reduce that of amphetamine. In the vessels of the perfused rabbit ear they abolished the constrictor effect of nervous stimulation and of acetylcholine, but increased that of tyramine.

The substance choline 2,6-xylyl ether (TM10; hereafter called choline xylyl ether) was synthesized by Hey, and Willey discovered that it blocked the response to stimulation of postganglionic adrenergic fibres without affecting the actions of added adrenaline or noradrenaline (Hey and Willey, 1954). This action was studied in greater detail by Exley (1957). Recently bretylium, *N*-*o*-bromobenzyl-*N*-ethyl-*NN*-dimethylammonium *p*-toluene sulphonate, has been introduced by Boura, Green, McCoubrey, Laurence, Moulton, and Rosenheim (1959) as a substance having a similar action which can be used clinically. The action of these two substances on the isolated atria of the rabbit heart has been examined using in some experiments the sympathetically innervated preparation which has been recently described (Huković, 1959), and, in some, a doubly innervated preparation so that the vagal fibres or the sympathetic fibres could be stimulated at will. The substances have also been examined for their action on the perfused vessels of the rabbit ear.

METHODS

In all experiments on the atria the solution used was: NaCl 9.0 g., KCl 0.42 g., $CaCl_2$ 0.24 g., dextrose 2.0 g. and $NaHCO_3$ 0.5 g. in 1 litre. The temperature of the bath was 30°. The bath was bubbled with O_2. Rabbit's and guinea-pig's atria were set up in the isolated organ bath, as described by Burn (1952).

Rabbit atria with the vagus nerve (McEwen, 1956) and with the sympathetic nerve were prepared as follows. Rabbits were stunned and killed, and quickly tied out on a table. The skin was removed from the thorax and neck. The vagus nerve and the sympathetic chain of the right side were identified and tied. The trachea and the oesophagus were divided as they entered the thorax. The ventral wall of the chest was removed without disturbing the thymus and neighbouring tissues. The vertebral column was divided with strong scissors between the cervical and thoracic vertebrae. The main vessels were divided in front of the 3rd thoracic vertebra, and the vertebral column was again divided between the 2nd and 3rd thoracic vertebrae. All skeletal muscles were removed and the preparation was immersed in oxygenated solution at 30°. The preparation was left in this solution at least 5 min. The preparation was then cleaned again and fixed on the short part of an L-shaped rod, and then was again immersed in the solution where it remained. The proximal part of the vagus nerve was found and tied. The cervical sympathetic nerve was tied and freed of connective tissue as far as the stellate ganglion by blunt dissection, freeing the ganglion as well as possible. The vagus nerve was placed in one pair of electrodes approximately in the middle of its length. The second pair of electrodes were placed around the stellate ganglion itself. The electrodes were of a pattern described by Burn and Rand (1959) with a channel in the holder through which a small amount of solution was constantly siphoned from the bath so that the portion of nerve stimulated was continually irrigated by fully oxygenated solution. The other end of the atria was tied to a spring lever. The stimulus applied was 0.5 mA strength, at a frequency of 20 pulses/sec. Each stimulus was 5 msec. duration, and stimulation was maintained for 15 to 30 sec.

*Present address: Department of Pharmacology, University of Sarajevo, Yugoslavia.

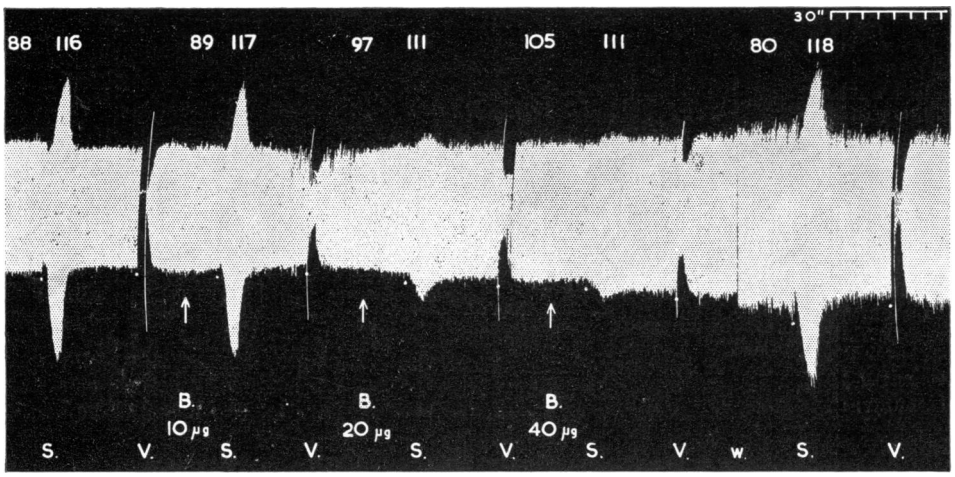

FIG. 1.—Isolated rabbit atria with sympathetic and vagus nerves. **S**, sympathetic stimulation, showing increase in rate (beats/min.; figures above the tracing) and amplitude. **V**, vagus stimulation, showing inhibition. **B**, addition of bretylium, the concentration per ml. being given. Note that in the concentration of 40 μg./ml., bretylium nearly abolished the effect of sympathetic stimulation and in addition reduced the effect of vagal stimulation. **W**, washing out of bath many times with recovery of effect of sympathetic and vagal stimulation.

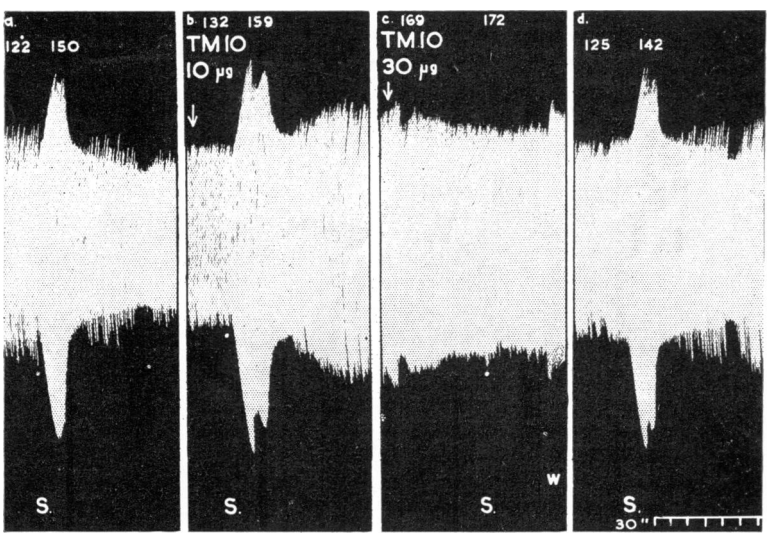

FIG. 2.—Isolated rabbit atria, showing block of effect of sympathetic stimulation (**S**) by choline xylyl ether (**TM10**). After 3 min. in bath 10 μg./ml. had no effect (second panel). A further 10 μg./ml. was added 5 min. later, making 20 μg./ml. in all; 3 min. after this second addition, the effect of sympathetic stimulation was diminished but not abolished (not shown). After 5 min. a third addition of 10 μg./ml. was made, making 30 μg./ml. in all. The effect of nerve stimulation was now abolished (third panel). After four washings (**W**) over 20 min., the effect of nerve stimulation was restored (last panel).

Rabbit ears were perfused with Locke solution of the composition described above with half the amount of dextrose. The arrangement was that described by Burn (1952) using Stephenson's recorder (1948). Stimulation was applied by a pair of electrodes to the great auricular nerve at the base of the pinna.

RESULTS

Bretylium in Doubly Innervated Preparation.—The effect of bretylium in isolated rabbit atria innervated by the vagi and by the sympathetic nerves is shown in Fig. 1. Stimulation of the sympathetic nerves increased the amplitude and also increased the rate from 88 to 116 beats/min. Stimulation of the vagus caused atrial arrest. Bretylium added in a concentration of 10 μg./ml. had no effect on sympathetic stimulation, but diminished the effect of vagal stimulation; in a concentration of 20 μg./ml., however, it diminished the effect of sympathetic stimulation without much further effect on vagal stimulation. Finally

bretylium in a concentration of 40 μg./ml. nearly abolished the effect of sympathetic stimulation; whereas the initial rise in rate was 28 beats/min., it was reduced to 6 beats/min. However, the effect of vagal stimulation was also smaller. After removing the bretylium from the bath, the initial effects both of sympathetic and of vagal stimulation were restored after many washings during 1 hr.

Choline Xylyl Ether on Sympathetic Stimulation. — The effect of choline xylyl ether on sympathetic stimulation is shown in Fig. 2. At first stimulation increased the rate by 28 beats/min. In the presence of 10 μg./ml. stimulation was unaffected, but a higher concentration, 30 μg./ml., blocked the effect of stimulation. After washing out the drug, the initial effect of stimulation was restored much more readily than when bretylium was used.

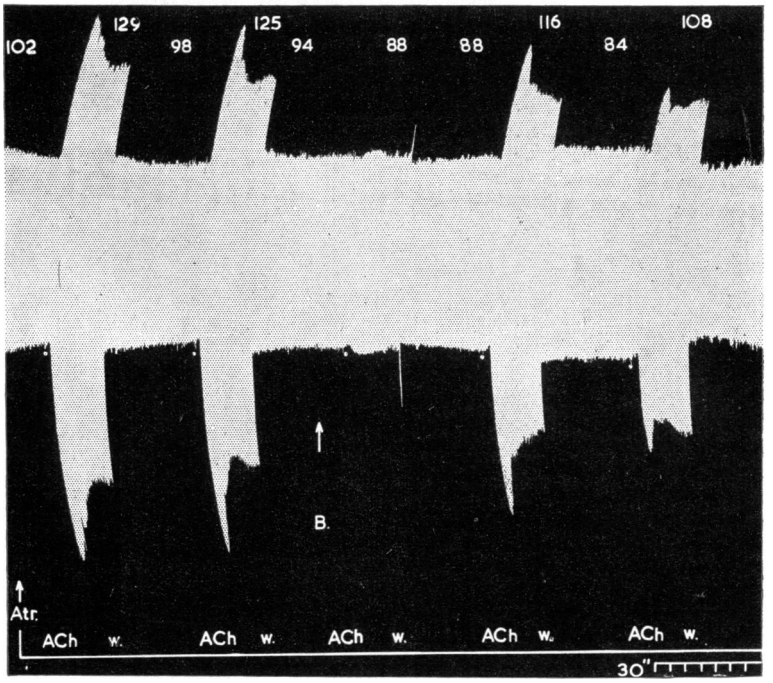

FIG. 3.—The effect of bretylium (**B**) in preventing the increase in rate and amplitude due to acetylcholine (**ACh**) in the presence of atropine. Atropine (**Atr**) 5 μg./ml., acetylcholine 200 μg./ml., bretylium 20 μg./ml. **W**, washing out.

The Response to Acetylcholine.—In the presence of atropine, acetylcholine causes stimulation of the isolated rabbit atria as shown in Fig. 3. The concentration of atropine was 5 μg./ml., and the concentration of acetylcholine was 200 μg./ml. This caused the rate to increase by 27 beats/min. in two successive trials. Bretylium (20 μg./ml.) was then added and in its presence acetylcholine was without effect. On washing out the bretylium the effect of acetylcholine was quickly restored. The action of choline xylyl ether was similar to that of bretylium; it also blocked the action of acetylcholine.

The Response to Tyramine and Amphetamine.—Concentrations of choline xylyl ether and of bretylium which blocked the accelerator action of acetylcholine had no blocking action on the accelerating action of tyramine. This is illustrated in Fig. 4. A concentration of tyramine, 2 μg./ml., applied twice caused a rise in rate of 16 and of 14 beats/min.; in the presence of bretylium, 20 μg./ml., tyramine, 2 μg./ml., caused a rise in rate of 23 beats/min.

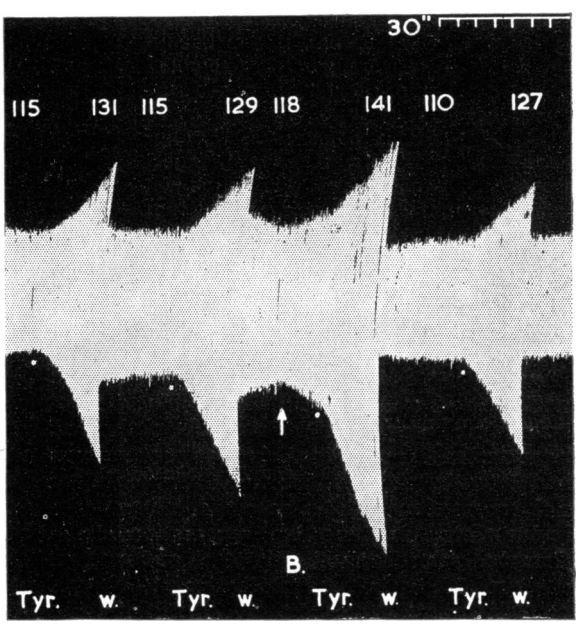

FIG. 4.—The effect of bretylium (**B**), 20 μg./ml., in increasing the effect of tyramine (**Tyr**), 2 μg./ml., on the rate and amplitude of the atria. **W**, washing out.

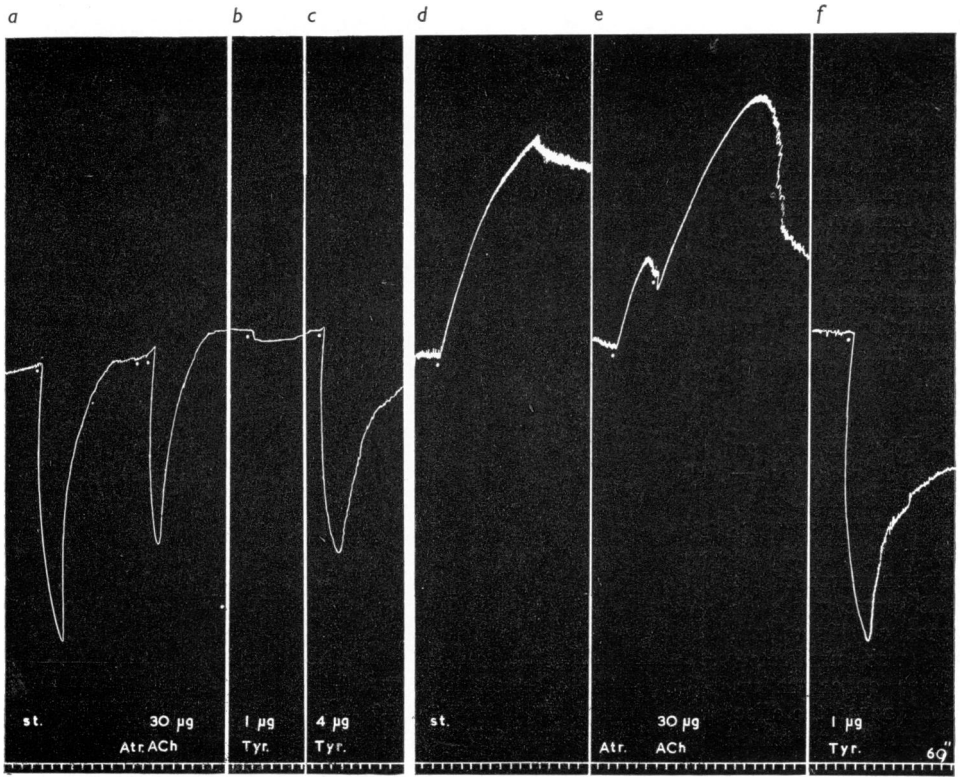

FIG. 5.—Record of outflow from veins of rabbit ear perfused with Locke solution at room temperature. **a,** Fall in outflow due to stimulation of great auricular nerve at the base of the ear (**st**); also fall in outflow due to injection of 30 µg. acetylcholine (**ACh**) after 8 µg. of atropine (**Atr**) into the solution entering the artery. **b,** No change in outflow after the injection of 1 µg. tyramine. **c,** Fall in outflow after injection of 4 µg. tyramine. The ear was then perfused overnight with Locke solution containing 1 µg./ml. bretylium. **d,** Rise in outflow following stimulation of the nerve. **e,** Rise in outflow following injection of 30 µg. acetylcholine after 8 µg. atropine. **f,** Fall in outflow after injection of 1 µg. tyramine.

After washing out the bretylium a further application of tyramine caused a rise in rate of 17 beats/min. Thus bretylium increased the action of tyramine.

The action of amphetamine in increasing the amplitude and rate of the atria was likewise not blocked by bretylium. Since the action of amphetamine is prolonged, a comparison was made between the action of amphetamine in one atrial preparation with its action in the presence of bretylium in another preparation. No difference was observed. In the presence of bretylium, 20 µg./ml., amphetamine, 5 µg./ml., increased the rate from 86 to 132 beats/min.

Vessels of Rabbit Ear.—Similar observations were made on the vessels of the rabbit ear. Bretylium and choline xylyl ether when added to the perfusion fluid blocked the constrictor effect of stimulating the great auricular nerve and also that of acetylcholine in the presence of atropine. However, they increased the effect of tyramine. Fig. 5 illustrates these actions of bretylium when it was present in the perfusion fluid in a concentration of 1 µg./ml. and had been perfused throughout the night. The dilator effect of stimulation in the presence of bretylium may have been due to stimulation of sensory fibres in the mixed nerve. The dilator action of acetylcholine in the presence of bretylium may have been due to the administration of insufficient atropine.

DISCUSSION

The use of an isolated atrial preparation with both vagal and sympathetic nerves intact made

it possible to compare the action of bretylium on the two innervations. In a concentration which almost completely blocked the effect of stimulating sympathetic fibres there was a considerable diminution in the effect of vagal stimulation also. Hence the selective action on the sympathetic terminations was only partial.

In a previous paper (Huković, 1959) it has been shown that when atrial preparations were made from rabbits treated with reserpine, stimulation of the sympathetic fibres often caused inhibition, and that this inhibitory effect was increased by eserine and abolished by atropine. This showed that cholinergic fibres were present. As Burn and Rand (1959) have suggested, such cholinergic fibres would normally have an adrenergic effect, since the acetylcholine they liberate would in turn liberate noradrenaline from the store in the neighbourhood of the nerve endings. These cholinergic fibres are indeed only recognized as cholinergic when treatment with reserpine has dispersed the store, and the liberated acetylcholine then exerts its own effect. Choline xylyl ether and bretylium inhibit the accelerating action of acetylcholine seen in the presence of atropine. This action has been shown to be exerted by release of noradrenaline from the store, since it is absent in preparations from rabbits treated with reserpine. Choline xylyl ether and bretylium do not appear to affect the store of noradrenaline itself, since they do not reduce the action of tyramine, and indeed they increase it. It is suggested, therefore, that they inhibit the accelerating action of acetylcholine mentioned above by preventing the liberation of noradrenaline from the store. They would also be expected to inhibit the noradrenaline-liberating action of cholinergic nerve fibres.

This work has been done during the tenure of a Fellowship from the British Council. I am indebted to Miss Roneen Hobbs for the experiments on the perfused rabbit's ear. This work was suggested and supervised by Professor J. H. Burn, to whom I am very grateful.

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ACTION OF TWO ETHYL THIOL ESTERS AGAINST EXPERIMENTAL TUBERCULOSIS IN THE GUINEA-PIG

BY

G. E. DAVIES AND G. W. DRIVER

From Imperial Chemical Industries Limited, Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire

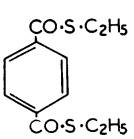
(RECEIVED OCTOBER 2, 1959)

Two thiol esters, ethyl dithiolterephthalate (Compound 13,130) and ditophal (ethyl dithiolisophthalate; Etisul), have been tested for their antituberculosis activity in guinea-pigs. Compound 13,130 was active at a dose of 100 mg./kg. twice daily by mouth in animals infected subcutaneously, but was inactive against an intracerebral infection at 50 mg./kg. Ditophal was active in the subcutaneously infected animals at 200 mg./kg. orally or 50 mg./kg. subcutaneously. Higher doses subcutaneously were irritant.

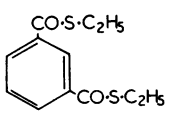
We have already described the antituberculosis effect of some thiol esters against experimental infections in mice (Davies, Driver, Hoggarth, Martin, Paige, Rose, and Wilson, 1956). Ditophal (ethyl dithiolisophthalate; Etisul) exerted an antituberculosis effect in mice comparable to that of isoniazid and streptomycin. It was effective when given in single doses, and against isoniazid-resistant strains. Ethyl dithiolterephthalate (Compound 13,130) is less effective than ditophal but nevertheless is a potent drug. In the present experiments we describe the action of the compounds on experimental tuberculosis in the guinea-pig, an animal in which the course of the disease is modified by the development of hypersensitivity.

METHODS

Two thiol esters have been used:



Compound 13,130



Ditophal

Compound 13,130 is an odourless, tasteless, crystalline solid; ditophal is a bland, odourless oil.

Subcutaneous Infection

Expt. 1 (Compound 13,130 Given Orally).—20 guinea-pigs, 260 to 404 g. in weight, were divided into five groups of four. The animals were infected subcutaneously over the sternum with 0.1 mg. wet weight of *Mycobacterium tuberculosis*, human strain, type C, suspended in 0.1 ml. of distilled water. The suspension was made by ball-milling bacilli harvested from a 14-day culture on Lowenstein's medium. Treatment was arranged as follows:

1. Two doses of 100 mg./kg. Compound 13,130 orally by stomach tube daily from the day of infection.
2. Two doses of 100 mg./kg. Compound 13,130 orally by stomach tube daily, starting from the 14th day after infection.
3. One dose of 20 mg./kg. streptomycin per animal subcutaneously daily, starting from the 14th day after infection.
4. Untreated controls.
5. Untreated controls.

Treatment ceased on the 90th day after infection. In this and all subsequent experiments the animals were not treated on Saturdays and Sundays.

Records were kept of weights, tuberculin reactions, appearance and ulceration of abscesses at the site of injection, enlargement of axillary lymph nodes and survival time.

All animals were examined *post mortem* and the degree of tuberculosis in the lungs, tracheo-bronchial lymph nodes, liver and spleen was estimated, each organ being given a maximum score of 5.

Two control animals were killed and examined on the 14th day in order to assess the degree of tuberculosis at the start of dosing in groups 2 and 3.

The experiment was terminated 120 days after infection.

Expt. 2 (Ditophal Given Orally).—40 guinea-pigs, 434 to 648 g. in weight, were divided into ten groups of four. The experiment was conducted as in Expt. 1, with the following dose schedules:

1. Two doses of ditophal, 200 mg./kg. orally daily from the day of infection.

2. Two doses of ditophal, 200 mg./kg. orally daily from 14 days after infection.
3. Two doses of ditophal, 100 mg./kg. orally daily from the day of infection.
4. Two doses of ditophal, 100 mg/kg. orally daily from 14 days after infection.
5. One dose of streptomycin, 40 mg./kg. subcutaneously daily from the day of infection.
6. One dose of streptomycin, 40 mg./kg. subcutaneously daily from 14 days after infection.
7. Two doses of isoniazid, 5 mg./kg. orally daily from the day of infection.
8. Two doses of isoniazid, 5 mg./kg. orally daily from 14 days after infection.
9. Untreated controls.
10. Untreated controls.

Expt. 3 (Ditophal Given Subcutaneously).—48 guinea-pigs, 440 to 630 g. in weight, were divided into six groups of eight and the experiment was carried out as in Expt. 1 with the treatment as follows:

1. Ditophal, 50 mg./kg. subcutaneously, once daily.
2. Ditophal, 50 mg./kg. subcutaneously, once weekly.
3. Ditophal, 10 mg./kg. subcutaneously, once daily.
4. Ditophal, 10 mg./kg. subcutaneously, once weekly.
5. Streptomycin, 40 mg./kg. subcutaneously, once daily.
6. Untreated controls.

Treatment ceased on the 120th day after infection.

Intracerebral Infection

Expt. 4 (Compound 13,130 Given Orally).—32 guinea-pigs, 300 to 400 g. in weight, were divided into four groups of eight and infected, under ether anaesthesia, by the intracerebral injection of 0.01 mg. wet weight of *M. tuberculosis* " Human C," suspended in 0.1 ml. of distilled water.

Treatment was started on the day after infection and was continued until death or for 5 weeks. The brains were removed *post mortem* and stained smears examined for the presence of *M. tuberculosis.* All animals were weighed once weekly.

TABLE I

THE EFFECT OF COMPOUND 13,130 AND STREPTOMYCIN ON GUINEA-PIGS INFECTED SUBCUTANEOUSLY

S=animal alive 120 days after infection. K14=animal killed at 14 days for assessment of degree of tuberculosis.

Group	Animal No.	Treatment	Degree of Tuberculosis (Maximum 5)					Survival Time in Days
			Lung	Liver	Spleen	Bronchial Lymph Nodes	Total	
1	1	Compound 13,130, two doses of	3	2	4	5	14	S
	2	100 mg./kg. orally daily from day	4	3	5	5	17	S
	3	of infection	2	2	4	5	13	S
	4		2	2	5	5	14	S
	Mean		2·75	2·25	4·5	5·0	14·5	120
2	1	Compound 13,130, two doses of	4	3	5	5	17	S
	2	100 mg./kg. orally daily from 14	1	1	2	2	6	S
	3	days after infection	3	5	5	5	18	112
	4		1	1	5	5	12	S
	Mean		2·25	2·5	4·25	4·25	13·25	118
3	1	Streptomycin 20 mg./kg. subcutan-	0	0	0	0	0	S
	2	eously once daily from 14 days	0	3	5	5	13	103
	3	after infection	1	1	5	5	12	S
	4		4	5	5	5	19	S
	Mean		1·25	2·25	3·75	3·75	11·0	116
4 and 5	1	Controls	0	0	0	5	5	K14
	2		0	0	0	5	5	K14
	3		5	5	5	5	20	76
	4		3	3	5	5	16	81
	5		4	3	3	5	15	70
	6		5	5	5	5	20	88
	7		4	2	3	5	14	S
	8		3	5	5	3	16	51
	Mean		4·0	3·84	4·33	4·66	16·8	81·0

TABLE II

THE EFFECT OF DITOPHAL GIVEN ORALLY, STREPTOMYCIN AND ISONIAZID ON GUINEA-PIGS INFECTED SUBCUTANEOUSLY

NS = death from some non-specific cause. S = alive 120 days after infection

Group	Animal No.	Treatment	Degree of Tuberculosis (Maximum 5)					Survival Time in Days
			Lung	Liver	Spleen	Bronchial Lymph Nodes	Total	
1	1	Ditophal, two doses 100 mg./kg.	2	1	2	5	10	65
	2	orally daily from day of infection	1	0	2	0	3	18 NS
	3		3	1	3	5	12	S
	4		2	2	3	5	12	59
	Mean		2·3	1·3	2·7	5·0	11·3	81·3
2	1	Ditophal two doses of 100 mg./kg.	0	0	2	0	2	21 NS
	2	orally daily from 14 days after	1	0	2	0	3	49 NS
	3	infection	2	1	3	5	11	S
	4		0	0	2	5	7	74
	Mean		1·0	0·5	2·5	5·0	9·0	97·0
3	1	Ditophal two doses of 200 mg./kg.	1	0	2	3	6	S
	2	orally daily from day of infection	1	0	1	5	7	S
	3		0	0	2	0	2	41 NS
	4		2	0	3	5	10	59
	Mean		1·3	0	2·0	4·3	7·7	99·7
4	1	Ditophal two doses of 200 mg./kg.	4	2	3	5	14	S
	2	orally daily from 14 days after	2	2	1	5	10	S
	3	infection	1	0	0	0	1	44 NS
	4		1	0	0	2	3	S
	Mean		2·3	1·3	1·3	4·0	9·0	120
5	1	Streptomycin 40 mg./kg. subcutan-	0	0	0	2	2	S
	2	eously once daily from day of	1	0	2	5	8	S
	3	infection	1	0	0	1	2	S
	4		0	0	0	1	1	S
	Mean		0·5	0	0·5	2·3	3·3	120
6	1	Streptomycin 40 mg./kg. subcutan-	1	0	0	1	2	S
	2	eously once daily from 14 days	0	0	0	0	0	S
	3	after infection	0	0	2	5	7	58 NS
	4		0	0	2	0	2	54 NS
	Mean		0·5	0	0	0·5	1·0	120
7	1	Isoniazid, two doses of 5 mg./kg.	0	0	0	0	0	S
	2	orally daily from day of infection	0	0	0	0	0	S
	3		0	0	0	0	0	29 NS
	4		0	0	0	0	0	29 NS
	Mean		0	0	0	0	0	120
8	1	Isoniazid, two doses of 5 mg./kg.	0	0	0	0	0	S
	2	orally daily from 14 days after	0	0	0	0	0	S
	3	infection	0	0	0	0	0	S
	4		0	0	0	0	0	S
	Mean		0	0	0	0	0	120
9 and 10	1	Controls	3	5	5	5	18	87
	2		2	3	3	5	13	54
	3		5	5	5	5	20	92
	4		3	5	5	5	18	37
	5		5	5	5	5	20	97
	6		2	5	5	5	17	56
	7		4	5	5	5	19	54
	8		5	5	3	5	18	S
	Mean		3·6	4·8	4·5	5·0	17·9	74·6

TABLE III

THE EFFECT OF DITOPHAL GIVEN SUBCUTANEOUSLY AND STREPTOMYCIN ON GUINEA-PIGS INFECTED SUBCUTANEOUSLY

NS = death from some non-specific cause. S = alive 120 days after infection.

Group	Animal No.	Treatment	Degree of Tuberculosis (Maximum 5)					Survival Time in Days
			Lung	Liver	Spleen	Bronchial Lymph Nodes	Total	
1	1	Ditophal 50 mg./kg. subcutaneously once daily	1	2	5	4	12	S
	2		2	5	5	5	17	111
	3		3	4	5	5	17	S
	4		2	3	5	5	15	S
	5		1	3	4	5	13	S
	6		2	2	4	4	12	S
	7		1	5	2	4	12	S
	Mean		1·7	3·4	4·3	4·6	14·0	118
2	1	Ditophal 50 mg./kg. subcutaneously once weekly	4	4	4	5	17	53
	2		5	5	5	5	20	93
	3		5	2	2	3	12	109
	4		2	4	3	4	13	70
	5		5	5	5	5	20	S
	6		1	5	5	5	16	52
	7		5	5	3	4	17	102
	8		4	4	3	4	15	109
	Mean		3·9	4·25	3·8	4·4	16·2	88·6
3	1	Ditophal 10 mg./kg. subcutaneously once daily	5	4	5	4	18	S
	2		5	4	3	5	17	S
	3		4	5	5	5	19	77
	4		5	4	3	5	17	88
	5		3	4	3	4	14	65
	6		4	2	2	4	12	S
	7		1	2	2	2	7	S
	8		4	5	5	4	18	92
	Mean		3·9	3·75	3·5	4·1	15·25	100
4	1	Ditophal 10 mg./kg. subcutaneously once weekly	5	1	1	5	12	98
	2		5	3	3	3	14	105
	3		5	5	4	5	19	68
	4		1	0	0	5	6	94
	5		4	2	2	3	11	S
	6		5	5	5	5	20	80
	7		4	2	3	4	13	S
	8		4	2	4	4	14	S
	Mean		4·1	2·5	2·75	4·25	13·6	100
5	1	Streptomycin 40 mg./kg. subcutaneously once daily	0	0	0	0	0	S
	2		0	0	0	0	0	S
	3		0	0	0	0	0	S
	4		0	0	0	0	0	S
	5		0	0	0	0	0	S
	6		0	0	0	0	0	S
	7		0	0	0	0	0	S
	8		0	0	0	0	0	S
	Mean		0	0	0	0	0	120
6	1	Controls	5	5	5	5	20	97
	2		5	5	4	5	19	S
	3		5	5	5	5	20	99
	4		3	5	5	5	18	49
	5		5	5	5	5	20	94
	6		3	3	5	5	16	S
	7		1	1	4	3	9	S
	8		4	5	4	4	17	75
	Mean		3·95	4·25	4·6	4·6	17·4	97

TABLE IV

THE EFFECT OF COMPOUND 13,130 ON THE SURVIVAL TIMES OF GUINEA-PIGS INFECTED INTRACEREBRALLY

S=animal surviving 60 days after infection. NS=death from some non-specific cause.

Group	Treatment	Survival Time in Days of Individual Animals								Mean Survival Time in Days
1	Compound 13,130, two doses of 50 mg./kg. orally daily	13	17	19	19	19	20	21	23	19
2	Compound 13,130, two doses of 50 mg./kg. orally twice daily+streptomycin 40 mg./kg. subcutaneously once daily	20	20	32	S	S	S	S	S	>44
3	Streptomycin 40 mg./kg. subcutaneously once daily	30	46	52	56	S	S	S	S	>49
4	Controls	12	15	NS 17	17	19	21	22	22	18

TABLE V

THE EFFECT OF DITOPHAL DOSED ORALLY ON THE SURVIVAL TIMES OF GUINEA-PIGS INFECTED INTRACEREBRALLY

NS=death from some non-specific cause.

Group	Treatment	Survival Time in Days of Individual Animals								Mean Survival Time in Days
1	Ditophal, two doses of 200 mg./kg. orally daily ..	1 NS	12 NS	13	13	26	27	43	49	28·5
2	,, ,, 100 ,, ,, ..	13	16	19	36	37	42	46	71	35·0
3	Streptomycin, two doses of 40 mg./kg. subcutaneously twice daily	19	22	22	26	40	43	44	56	34·0
4	Isoniazid, two doses of 5 mg./kg. orally daily ..	22 NS	39 NS	48	53	54	59	61	71	57·7
5	Untreated controls	15	15	17	19	19	19	21	23	18·6

The four groups were treated as follows:

1. Compound 13,130, 50 mg./kg. orally twice daily.
2. Compound 13,130, 50 mg./kg. orally twice daily plus 40 mg./kg. streptomycin subcutaneously once daily.
3. Streptomycin, 40 mg./kg. subcutaneously once daily.
4. Untreated controls.

Expt. 5 (Ditophal Dosed Orally).—40 guinea-pigs, 238 to 472 g. in weight, were arranged in groups of eight so that the mean weights of each group at the start of the experiment were roughly equal. They were infected intracerebrally, under ether anaesthesia, with 0.01 ml. mg. wet weight *M. tuberculosis,* suspended in 0.1 ml. of distilled water.

Treatment was started on the day after infection and continued for four weeks. The brains were removed *post mortem* and stained smears examined for the presence of *M. tuberculosis.* All animals were weighed once weekly.

The five groups were treated as follows:

1. Ditophal, 200 mg./kg. orally twice daily
2. Ditophal, 100 mg./kg. orally twice daily.
3. Streptomycin, 40 mg./kg. subcutaneously once daily.
4. Isoniazid, 5 mg./kg. orally twice daily.
5. Untreated controls.

RESULTS

Subcutaneous Infection

Both compounds showed a therapeutic effect against a subcutaneous infection even when given against an established infection, but both were less effective than streptomycin. (See Tables I, II, and III.)

The two thiol esters and streptomycin had no effect on the development of the tuberculin reaction or on the time of appearance and subsequent development of the abscesses at the site of inoculation or on the enlargement of the axillary lymph nodes. This is in contrast to the results obtained with isoniazid, where a small abscess appeared in only one guinea-pig and there was no enlargement of the axillary lymph nodes.

Intracerebral Infection

Compound 13,130 had no effect on an intracerebral infection of *M. tuberculosis* in guinea-pigs when dosed twice daily at 50 mg./kg. orally, nor did it potentiate the effect of streptomycin against such an infection (see Table IV).

Ditophal had a definite effect against an intracerebral infection when administered twice daily at 100 mg./kg. and 200 mg./kg. orally, which was comparable with that produced by streptomycin administered subcutaneously at 40 mg./kg. daily, but was inferior to the effect obtained with isoniazid administered twice daily at 5 mg./kg. (see Table V). Ditophal was without therapeutic effect when two weekly doses of 100 mg./kg. were given intracerebrally, or when given once daily subcutaneously at 50 and 10 mg./kg. or once weekly subcutaneously at 100 and 200 mg./kg.

Discussion

The striking antituberculosis effect obtained with ditophal in mice was not achieved in guinea-pigs. This was probably because the maximum dose of ditophal tolerated in guinea-pigs was limited by ulceration of the site of injection with doses greater than 50 mg./kg. The results do, however, indicate that the two thiol esters possess definite activity against tuberculosis in the guinea-pig, and further work with them seems justified.

Etisul is a trade mark of Imperial Chemical Industries.

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Davies, G. E., Driver, G. W., Hoggarth, E., Martin, A. R., Paige, M. F. C., Rose, F. L., and Wilson, B. R. (1956). *Brit. J. Pharmacol.*, **11**, 351.

Brit. J. Pharmacol. (1960), **15**, 128.

THE EFFECTS OF AMBUCETAMIDE ON HUMAN MYOMETRIAL AND OTHER PREPARATIONS, AND ITS ANTAGONISM TO THE MENSTRUAL STIMULANT

BY

V. R. PICKLES* AND H. J. CLITHEROE*

From the Physiology Department, University of Sheffield

(RECEIVED OCTOBER 5, 1959)

A plain-muscle stimulant, provisionally named the " menstrual stimulant," may be involved in the causation of dysmenorrhoea. Since ambucetamide [α-dibutylamino-α-(*p*-methoxyphenyl)-acetamide] has been claimed to alleviate this condition, its effects on the responses of human myometrial preparations to the menstrual stimulant have been studied. Ambucetamide regularly inhibited these responses, as well as those to vasopressin, and sometimes diminished the spontaneous rhythmical activity of the human myometrium. The rat and guinea-pig uterus, the guinea-pig and cat intestine preparations, showed more complex responses and may therefore be less suitable for testing antispasmodic drugs intended for treatment of dysmenorrhoea.

Ambucetamide [α-dibutylamino-α-(*p*-methoxyphenyl)acetamide; Dibutamide] is an antispasmodic drug which has been found to have some use in alleviating dysmenorrhoea (Carpenter and Janssen, 1955; Hoekstra, Fisher, Cull, Tisch, and Dickison, 1957). The " menstrual stimulant " is a plain-muscle stimulant of endometrial origin, overactivity of which is considered to be a possible cause of dysmenorrhoea (Chambers and Pickles, 1958; Pickles, 1959). It was therefore of interest to see whether ambucetamide antagonized the effect of menstrual stimulant on the human myometrium. If so, it would be of value to see whether the antagonism can be demonstrated on mammalian tissues which are more readily available in the laboratory.

A preliminary account of some of these findings has been reported (Clitheroe and Pickles, 1959).

METHODS

Human myometrial preparations, guinea-pig or rat uterus or guinea-pig duodenum preparations were suspended in a solution containing NaCl 0.82%, KCl 0.052%, $CaCl_2$ 0.008%, $NaHCO_3$ 0.047%, glucose 0.1%, and $MgSO_4$ 0.018%, as described by Pickles (1959).

The preparations of " menstrual stimulant " were prepared by making ether extracts of the lipids of human menstrual fluid. They were either unfractionated or treated as the preparation called "chromatographic fraction 5 " in the method of Chambers and Pickles (1958). The results with the two kinds of extract were qualitatively similar.

*Present address: Department of Experimental Medicine, University of Cambridge.

RESULTS

Human Myometrium.—In concentrations of 6 to 120 μg./ml., ambucetamide inhibited the responses of the human myometrium to the menstrual stimulant. In Fig. 1, a preparation, which had previously been giving regular responses to the same doses of menstrual stimulant, ceased to give any response after ambucetamide (25 μg./ml.) had been added to the bath 5 min. previously. This experiment was unusual in that the myometrium had been suspended for several hours and was by then showing little spontaneous rhythmical activity, which began again when the ambucetamide was washed out. A different kind of response,

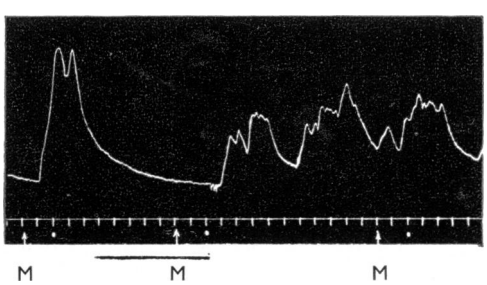

FIG. 1.—Human myometrial preparation *in vitro*. At M, equal doses of ether-soluble menstrual lipid (containing " menstrual stimulant ") were applied for 2 min. White dot indicates washing. Ambucetamide (25 μg./ml.) was applied for duration of horizontal bar. Time, 1 min.

probably bearing more similarity to that *in vivo* but a little more difficult to evaluate pharmacologically, is shown in Fig. 2. This experiment confirms earlier observations (Pickles, 1959) in that a single addition of menstrual stimulant caused the rhythmical contractions of the preparation to become better co-ordinated and more powerful. Such a response might continue almost indefinitely, but in this instance it was inhibited by the addition of ambucetamide.

The usual effect of ambucetamide on the spontaneous activity of a human myometrial preparation is shown in Fig. 3. The tone of the preparation always recovered more slowly than did the amplitude of the spontaneous rhythmical contractions after the ambucetamide was washed out. An inhibitory effect of ambucetamide on the spontaneous activity of the myometrium was clearly seen in about half of the 20 experiments in which it was tested. The antagonism to the menstrual stimulant was seen in each of 13 experiments.

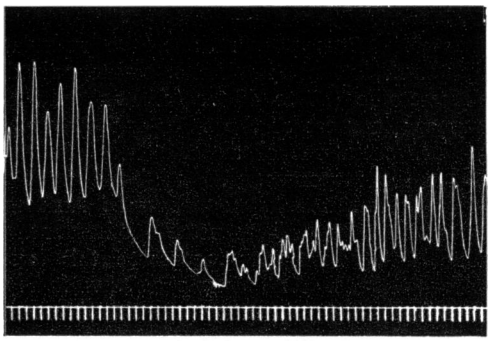

FIG. 3.—Human myometrial preparation *in vitro*. Ambucetamide (25 μg./ml.) was added for the duration of the horizontal bar. Time, 1 min.

In a similar series of 10 experiments, myometrial preparations were stimulated not with the menstrual stimulant but with vasopressin (Pitressin; Parke, Davis & Co.). Again, ambucetamide (12 to 48 μg./ml.) was found to have a long-lasting antagonistic effect.

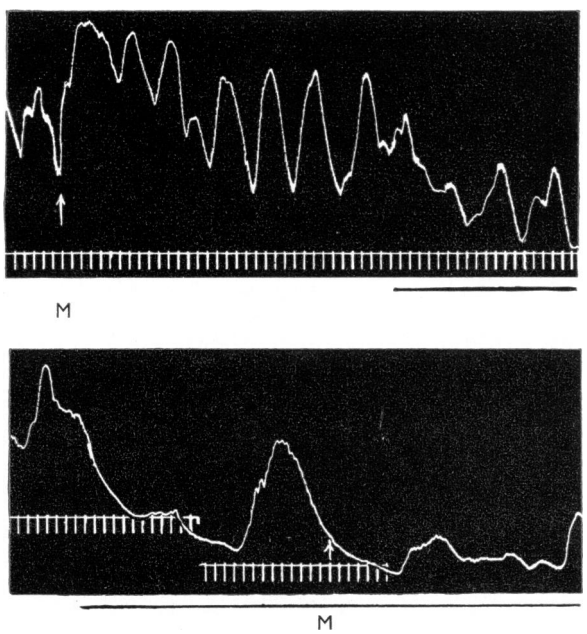

FIG. 2.—Human myometrial preparation *in vitro*. For 45 min. before the section of the record shown in the upper panel, the preparation had shown the poorly co-ordinated contractions as seen in the first 6 min. of the tracing. At M, 3 mg. of ether-soluble menstrual lipid was added to the 8 ml. organ bath; ambucetamide (20 μg./ml.) was added to the bath for the duration of the horizontal bar. The preparation was repeatedly washed during a period of 1.5 hr. between the end of the upper and the beginning of the lower record, but the preparation was not washed during either of the records above. Time, 1 min.

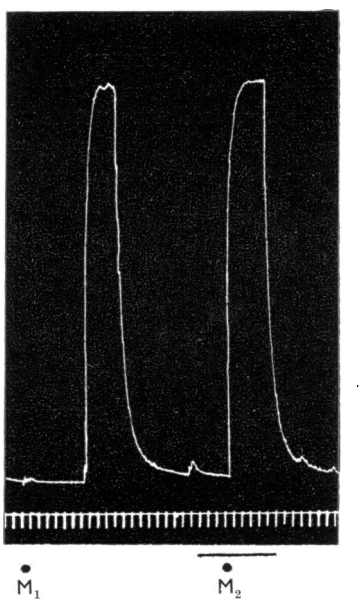

FIG. 4.—Isolated guinea-pig uterus. The ether-soluble menstrual lipid (0.1 mg.) was added to the 8 ml. organ bath at the black dots for 12 min. at M_1 and for 6 min. at M_2. Ambucetamide (12 μg./ml.) was added for the duration of the horizontal bar. Time, 1 min.

Guinea-pig Uterus. — In 18 experiments on guinea-pig uterus preparations, the stimulating action of menstrual stimulant was antagonized by ambucetamide (12 to 48 μg./ml.) in only three instances; in most of the other experiments, the same concentrations of ambucetamide tended to increase the responses. The guinea-pig uterus normally showed a latency of some minutes in its responses to the menstrual stimulant, but ambucetamide commonly decreased this latency to a few seconds. An example is shown in Fig. 4. In two further experiments, ambucetamide had no definite effect on the responses of the guinea-pig uterus to vasopressin.

Rat Uterus.—Like the guinea-pig uterus, the rat uterus gave variable results. In a series of 9 experiments, ambucetamide (12 to 48 μg./ml.) apparently increased the response to the menstrual stimulant in 4 and decreased it in 5 instances. The effect on the responses to vasopressin was likewise indefinite. Commonly the spontaneous activity increased after the ambucetamide had been washed out.

Guinea-pig Duodenum and Cat Small Intestine. —In both preparations, ambucetamide caused a slow increase in tone while the responses to the menstrual stimulant were diminished.

Discussion

The experiments on the human myometrial preparations are consistent with the finding that ambucetamide is of some value in alleviating dysmenorrhoea. They are also consistent with the view that dysmenorrhoea is due to excessive production of or sensitivity to the menstrual stimulant; but they do not provide positive evidence for this, since ambucetamide also antagonized the effect of vasopressin.

It is clear from the experiments on the isolated uterus of guinea-pigs and rats that these preparations may give responses quite different from those given by the human myometrial preparations. Misleading results might be obtained if guinea-pig or rat uterus preparations are used for testing antispasmodic drugs intended for use in dysmenorrhoea.

H. J. C. was the recipient of a grant from Messrs. Allen and Hanburys Ltd., to whom we wish to express our thanks. Ambucetamide was kindly supplied by Dr. P. F. D'Arcy.

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A STUDY OF ANTAGONISTS OF 5-HYDROXYTRYPTAMINE AND CATECHOL AMINES ON THE RAT'S BLOOD PRESSURE

BY

A. S. OUTSCHOORN* AND J. JACOB

From the Laboratory of Pharmacology, Service of Therapeutic Chemistry, Pasteur Institute, Paris

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The effects of 5-hydroxytryptamine on the blood pressure of anaesthetized rats depended on the dose and the initial level of blood pressure. At medium blood pressure levels, 5-hydroxytryptamine gave a depressor response and sometimes a pressor response which was more evident with large doses. The depressor effect was less apparent or even absent at low, and more pronounced at high, blood pressure levels, and the converse applied to the pressor components. Adenosine also gave a depressor and pressor response. Lysergic acid diethylamide, dihydroergotamine, 1-(3,4-dichlorophenyl)-2-isopropylaminoethanol (a dichloro analogue of isoprenaline), dibenamine and 1-benzyl-5-methoxy-2-methyltryptamine antagonized 5-hydroxytryptamine and catechol amines. Lysergic acid diethylamide and 1-benzyl-5-methoxy-2-methyltryptamine were more effective against 5-hydroxytryptamine, 1-(3,4-dichlorophenyl)-2-isopropylaminoethanol and dibenamine against catechol amines; dihydroergotamine was equally effective against both groups. These antagonists fell into two groups according to their action against the two types of effects (depressor and pressor) of 5-hydroxytryptamine: lysergic acid diethylamide and 1-(3,4-dichlorophenyl)-2-isopropylaminoethanol acted preferentially against depressor effects; 1-benzyl-5-methoxy-2-methyltryptamine and dibenamine preferentially against pressor; dihydroergotamine was not assignable to either group. Adenosine was affected similarly, but less than 5-hydroxytryptamine.

The effects of 5-hydroxytryptamine on the blood pressure of mammals are complex and vary according to the species (for references see Erspamer (1954) and Page (1958)). In dogs the main effects are pressor while cats and rabbits usually give depressor responses. According to Salmoiraghi, Page, and McCubbin (1956), the responses of rats are intermediate, being mixed pressor and depressor. It was thought useful to study various antagonists (lysergic acid diethylamide, dihydroergotamine, 1-(3,4-dichlorophenyl)-2-isopropylaminoethanol (a dichloro analogue of isoprenaline), dibenamine, 1-benzyl-5-methoxy-2-methyltryptamine and atropine) on the mixed responses in rats and to compare the antagonistic action to 5-hydroxytryptamine with that exerted against other drugs (catechol amines, acetylcholine); adenosine was also included as an agonist because it behaved like 5-hydroxytryptamine, and there may be some connexion between 5-hydroxytryptamine and adenosine triphosphate in platelets (Born, Ingram, and Stacey, 1958).

*Present address: Head, Pharmacological Laboratory, Medical Research Institute, Colombo, Ceylon.

METHODS

Rats of either sex, between 160 and 200 g. body weight, were used. The anaesthetic used was a mixture of barbitone sodium 10 mg. (as 4% solution) and urethane 75 mg. (as 25% solution), per 100 g. body weight of rat, injected subcutaneously, or half those amounts injected intraperitoneally. In the latter case, if after some time the rat showed signs of coming out of the anaesthetic, a further one fourth of the stated amounts was injected intravenously. The method used was that of Crawford and Outschoorn (1951) and the rats were heparinized. The blood pressure was recorded with a mercury manometer through a cannula in one carotid artery, the opposite carotid artery being tied off. Both vagi and the attendant sympathetic nerve fibres were left intact. Injections were made through a cannula in a femoral vein. In all the experiments the respiration of the animals was spontaneous.

The agonist drugs selected for study and the doses employed were 5 - hydroxytryptamine creatinine sulphate, calculated as base, 25 ng. to 20 μg.; adenosine, 50 ng. to 20 μg.; adrenaline hydrochloride as base, 100 to 200 ng.; noradrenaline hydrochloride as base, 50 to 100 ng.; isoprenaline hydrochloride as base, 50 to 100 ng.; acetylcholine chloride as salt,

100 to 500 ng. All doses of the agonist drugs are expressed per rat.

In studies on antagonists the agonist drugs were first given during a control period and the effects recorded. The chosen antagonist was given, at first in small dosage, and the agonists injected again. This process was repeated with increasing doses of the antagonist up to the limits of tolerance of the animal. Such limits were not necessarily the death of the rat, and were usually when the blood pressure had fallen low, compared with that during the control period. At very low blood pressures no conclusions could be drawn because even without antagonists the effects of drugs like acetylcholine were altered. For the experiments with antagonists doses of 0.5 to 2 μg. 5-hydroxytryptamine per rat and 5 to 20 μg. adenosine per rat were chosen. With these doses no tachyphylaxis occurred when the injections were made at intervals of 5 to 10 min.

All the antagonist drugs used, with the exception of atropine were broadly divided into three dose ranges each, as follows: dihydroergotamine methanesulphonate, 5 to 10 μg., 15 to 25 μg., and 35 to 80 μg.; lysergic acid diethylamide, 10 μg., 20 to 40 μg., and 60 to 80 μg.; dibenamine, 50 μg., 100 to 200 μg., and 300 to 400 μg.; 1-benzyl-5-methoxy-2-methyltryptamine hydrochloride, 100 to 200 μg., 300 μg. to 1.2 mg., and 2.0 to 2.8 mg.; 1-(3,4-dichlorophenyl)-2-isopropylaminoethanol hydrochloride, 125 to 500 μg., 750 to 900 μg., and 1.5 to 3.0 mg.; and atropine sulphate, 100 to 200 μg. These doses of antagonists are expressed per 100 g. body weight of rat. In the text the terms small doses, moderate doses and large doses are used to save repetition and refer to the three dose ranges mentioned above.

RESULTS

Effects of 5-Hydroxytryptamine and Adenosine in the Normal Anaesthetized Rat

The effects of 5-hydroxytryptamine and adenosine on the rat blood pressure were complex and depended on the dose and on the initial level of blood pressure.

Dependence on Dosage

With anaesthesia such as to keep the blood pressure at a medium level, doses ranging from the threshold to as high as 20 μg. per rat caused a great variety of responses. An analysis was made of the records at a particular drum speed (10 mm. per min. at the circumference). It would appear that the basic effect of 5-hydroxytryptamine was depressor. Depending on the dose employed, and varying in different rats (Fig. 1), two types of pressor components were superimposed on this depressor response. First there was a short "spike" which occurred early, either at the beginning or at any time during the depressor effect; that is to say the spike interrupted the fall of blood pressure; and second a "dome" which was prolonged and followed the hypotension.

On the basis of this analysis the effects of 5-hydroxytryptamine as related to dose were as follows (Fig. 2, upper record). Doses of 0.5 to 1 μg. per rat usually caused a fall of blood pressure only. Occasionally a spike was also present. The threshold dose was 250 to 500 ng. per rat. The effects of smaller doses (25 ng. per rat) were also observed, but were indistinguishable from injections of saline. Doses of 2 μg. per rat and above usually produced mixed effects. The

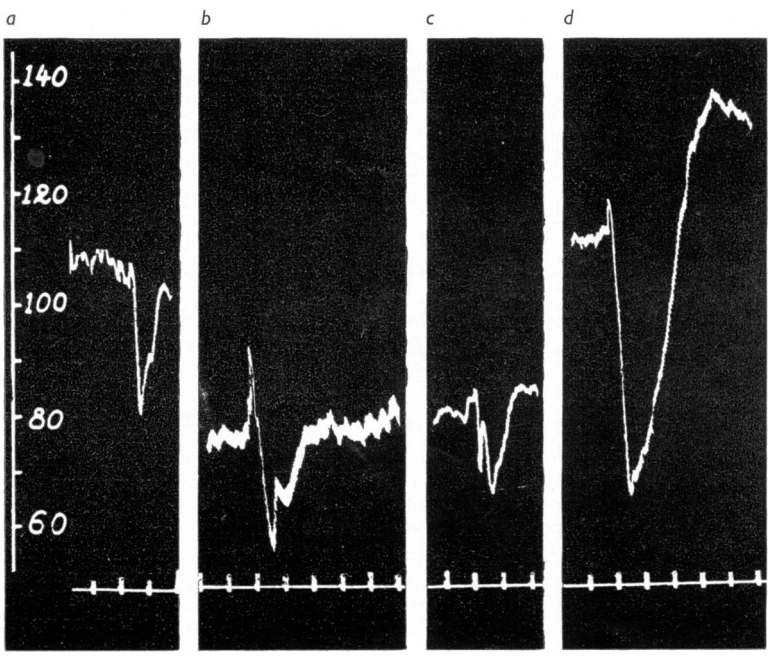

FIG. 1.—Blood pressure records from four different rats, 160, 200, 190 and 185 g., respectively, anaesthetized with urethane-barbitone sodium mixture. The figure illustrates the different responses to 2 μg. 5-hydroxytryptamine, from left to right: (a) depressor effect alone, (b) with superimposed early spike, (c) with late spike, and (d) with spike and dome. Pressure in mm. mercury. Time 0.5 min.

higher the dose the greater the proportion of pressor components. The results of injecting 5 to 10 μg. per rat were taller spikes and larger domes than with 2 μg. per rat. Sometimes the effect of these high doses was to obscure the depressor phase completely. In two rats out of fifty there occurred only pressor effects, whatever the dose of 5-hydroxytryptamine ; in these cases the record consisted of an early spike which was followed by a dome before the blood pressure could return to its original level.

The effects of adenosine were also mixed, and for certain doses they closely resembled those of 5-hydroxytryptamine. However, within a wide dose range they showed a different development of qualitative effects (Fig. 2, lower record). Increasing doses from 50 ng. per rat rarely caused any effect until doses of 2 to 5 μg. per rat were reached. These usually produced a sharp pressor spike. Larger doses (10 to 20 μg. per rat) resulted in a pressor spike being followed by a depressor phase. This was sometimes followed by a pressor dome, as in the case of 5-hydroxytryptamine. Still larger doses (up to 150 μg. per rat) showed a small spike followed by a profound fall. This fall was either prolonged or transient and was followed by a pressor dome. The latter type is illustrated in Fig. 2 (lower record). Thus, increasing the dose of adenosine increased its depressor effects, while increasing the dose of 5-hydroxytryptamine increased its pressor effects.

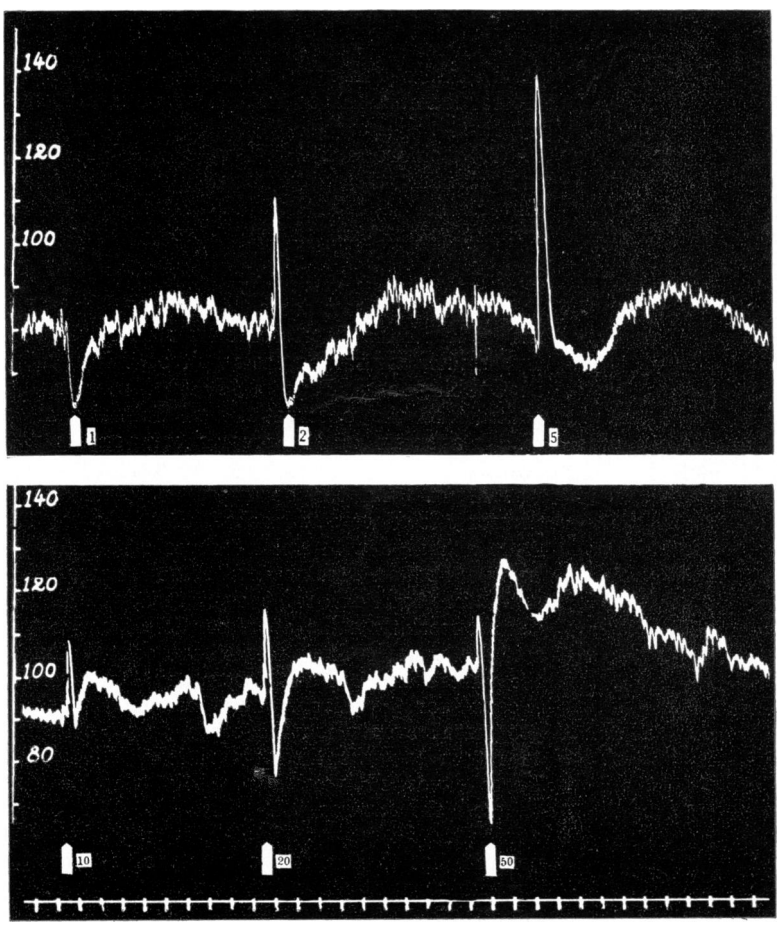

FIG. 2.—Blood pressure records from two rats; urethane-barbitone sodium anaesthesia. Upper record, 190 g. rat, effects of 1, 2 and 5 μg. 5-hydroxytryptamine. Between the 2nd and 3rd dose the drum was stopped for 4 min. Lower record, 200 g. rat, effects of 10, 20 and 50 μg. adenosine. Time 0.5 min.

Dependence on Blood Pressure Level

When the blood pressure of the rat was low, as when the animal had been for some hours under the experimental conditions, or if the injected anaesthetic mixture had a higher proportion of urethane than that mentioned, the effects of 5-hydroxytryptamine were different. Throughout the whole dose range used, even with the smallest effective doses, this substance produced predominantly pressor effects. It follows that a gradual fall of the blood pressure base line from medium levels to low ones was accompanied by an inversion of the effects even of small doses from depressor to pressor. On the contrary, if the blood pressure of the rat was high, as was obtained after anaesthesia with barbiturate only, the pressor components described were less apparent even with large doses of 5-hydroxytryptamine. In contrast to 5-hydroxytryptamine, the blood pressure response to 100 to 200 ng. adrenaline per rat or to 50 to 100 ng. of noradrenaline was of the same order over a wide range of blood pressure

levels (Fig. 3). Like 5-hydroxytryptamine, adenosine showed some change in the response with lowering of the blood pressure base line, but this was not so pronounced.

The dependence of the effects of 5-hydroxytryptamine on blood pressure level has already been outlined for other species by Page (1958), who related it to the influence of neurogenic vascular tone. Whatever may be its cause, this fact was kept in mind when interpreting the actions of antagonists. Thus reduction of depressor effects and potentiation or unmasking of pressor effects after antagonists were deduced only if on several occasions the blood pressure level at the time of injection of 5-hydroxytryptamine (or adenosine) was the same or higher than during the control period. Reduction of pressor effects, on the other hand, could be deduced even if the blood pressure was somewhat lower after the antagonist, but was more difficult to accept if the blood pressure was higher.

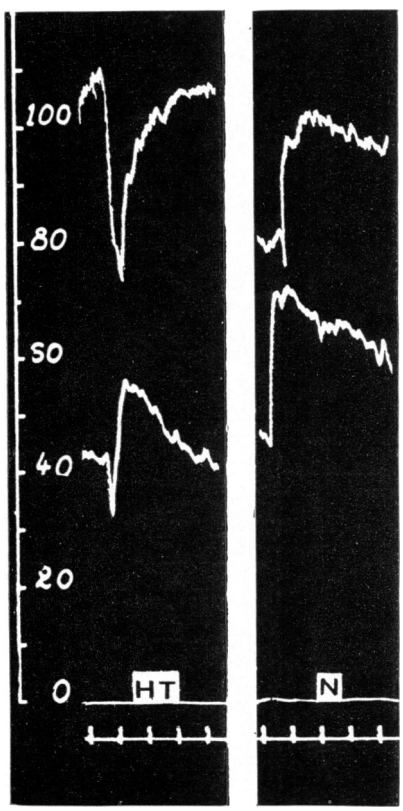

FIG. 3.—Blood pressure records from same rat, 170 g., anaesthetized with urethane. Effects of 1 μg. 5-hydroxytryptamine on left, and 100 ng. noradrenaline on right, at different blood pressures. Time interval between upper and lower records 40 min. Time 0.5 min. Note qualitative change in the response to 5-hydroxytryptamine in contrast to that to noradrenaline.

Actions of Antagonists

In view of the complexity of the effects of 5-hydroxytryptamine no attempt was made to make precise quantitative measurements, but only to determine and describe the main qualitative actions of the antagonists in the anaesthetized rat, breathing spontaneously. All the antagonists studied, with the exception of atropine, had some action against both 5-hydroxytryptamine and catechol amines. In addition, a remarkable feature was that early in the study of each antagonist it became clear that each had a preferential action against either the depressor or pressor component of the effect of 5-hydroxytryptamine; lysergic acid diethylamide and 1 - (3,4-dichlorophenyl) - 2 - isopropylaminoethanol antagonized the depressor response, while dibenamine and 1-benzyl-5-methoxy-2-methyltryptamine antagonized the pressor response.

Lysergic Acid Diethylamide

Small or moderate doses (10 to 40 μg. per 100 g. rat) reduced the depressor effects of 5-hydroxytryptamine, confirming the result of Salmoiraghi, McCubbin, and Page (1957). However, in contrast to the observations of Salmoiraghi *et al.*, antagonism of the pressor components was unusual (two cases out of twelve), and, if present, was slight. On the contrary, in several cases pressor effects were unmasked, that is to say the spike appeared with smaller doses of 5-hydroxytryptamine than were needed before the antagonist and occasionally a dome appeared as well. In some experiments the depressor effect of lysergic acid diethylamide itself might have contributed to the unmasking, but this factor could not wholly account for the phenomenon because unmasking progressed even when the blood pressure tended to return towards the control level (Fig. 4,A). These doses of lysergic acid diethylamide also reduced the pressor effects of 100 to 200 ng. adrenaline per rat and 50 to 100 ng. noradrenaline per rat, but the depressor effects of 5-hydroxytryptamine were more sensitive than the pressor effects of catechol amines, and of the catechol amines adrenaline was more sensitive than noradrenaline to this antagonist. There was also reduction of the depressor effects of adenosine and sometimes unmasking of pressor effects.

Large doses of lysergic acid diethylamide (up to 80 μg. per 100 g. rat) abolished the depressor effects of 5-hydroxytryptamine leaving purely pressor ones (" reversal "), but these pressor effects were reduced. At the same time the effects of these doses of adrenaline were abolished, and those of noradrenaline abolished or greatly reduced. The depressor effects of 50 to 100 ng. isoprenaline per rat and of 100 to 500 ng. acetylcholine per rat were unaffected throughout.

Dihydroergotamine

Small doses of dihydroergotamine (5 to 10 μg. per 100 g. rat) reduced the depressor effect of 5-hydroxytryptamine (Fig. 4,B) and to a less extent the depressor effects of adenosine, but, unlike lysergic acid diethylamide, there was no unmasking of pressor effects. Antagonism of pressor effects of 5-hydroxytryptamine was difficult to determine, owing to the pressor effect usually exerted by dihydroergotamine itself. These small doses of dihydroergotamine were also sufficient to reduce the pressor effects of adrenaline and noradrenaline, which were thus as sensitive to this antagonist as were the depressor effects of 5-hydroxytryptamine. In cases where there were depressor effects of adrenaline these were potentiated.

With moderate doses (15 to 25 μg. per 100 g. rat) antagonism of the depressor effects of 5-hydroxytryptamine and adenosine was increased only slightly, and with large doses (up to 80 μg. per 100 g. rat) no further antagonism occurred. Moderate and large doses, however, reduced further the pressor effects of adrenaline and noradrenaline as well as the depressor effects of isoprenaline, but no marked reduction of the (potentiated) depressor effects of adrenaline occurred. The depressor effects of acetylcholine were unaffected.

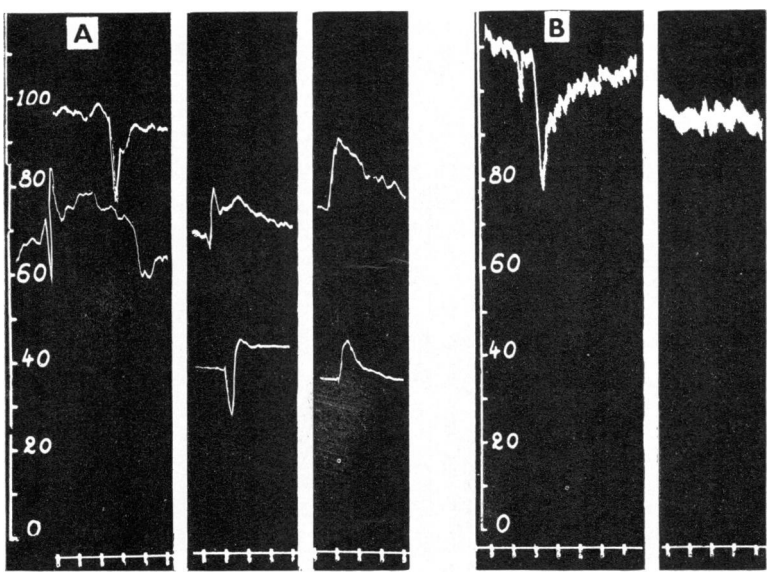

FIG. 4.—Blood pressure records of rats anaesthetized with urethane-barbitone sodium mixture. Time 0.5 min. **A**, Action of lysergic acid diethylamide on effects of 1 μg. 5-hydroxytryptamine. Upper tracings, 175 g. rat, from left to right, injection of 1 μg. 5-hydroxytryptamine 10 min. before, 27 min. after, and 1 hr. 12 min. after 35 μg. lysergic acid diethylamide. Lower tracings, 170 g. rat, injection of 1 μg. 5-hydroxytryptamine 27 min. before, 30 min. after, and 1 hr. 38 min. after 34 μg. lysergic acid diethylamide. Note middle panel shows possible combined actions of lysergic acid diethylamide and of fall of blood pressure; the panel on the right shows further changes possibly related to development of lysergic acid diethylamide antagonism. **B**, Action of dihydroergotamine on effects of 0.5 μg. 5-hydroxytryptamine; 185 g. rat; injection of 0.5 μg. 5-hydroxytryptamine before (left) and 1 hr. 27 min. after (right) dihydroergotamine (two injections, each 9.25 μg., 54 min. and 1 hr. 21 min. after control injection).

1-(3,4-Dichlorophenyl)-2-isopropylaminoethanol

This substance was described by Powell and Slater (1958) as an antagonist of the beta effects of catechol amines. In the present experiments it was found that small doses (125 to 500 μg. per 100 g. rat) markedly antagonized the depressor effects of isoprenaline, abolished a depressor effect of adrenaline if this was present, and reduced somewhat depressor effects of 5-hydroxytryptamine (Fig. 5,A). The pressor effects of adrenaline and noradrenaline were, on the contrary, potentiated and there was sometimes unmasking of pressor effects of 5-hydroxytryptamine. There was no action on the depressor effects of adenosine. Moderate doses produced the same results to a somewhat greater extent, but the depressor effects of adenosine were also slightly reduced with slight unmasking of its pressor effects.

The same actions, but to a still greater extent, occurred with large doses of 1-(3,4-

dichlorophenyl)-2-isopropylaminoethanol (up to 3 mg. per 100 g. rat), the depressor effects of 5-hydroxytryptamine being abolished almost completely, leaving only pressor effects (" reversal "); similar results were obtained with adenosine but to a less degree than with 5-hydroxytryptamine. There was also reduction of the pressor response to adrenaline and noradrenaline. The response to acetylcholine was unaffected. This antagonist therefore, in addition to reducing the depressor effects of catechol amines, also reduced the depressor action of 5-hydroxytryptamine (and to a less extent that to adenosine), and in large doses reduced the pressor reponses to catechol amines as well.

Dibenamine

The three dose levels of dibenamine (from 50 to 400 μg. per 100 g. rat) showed increasing reduction of the pressor effects of 5-hydroxytryptamine (Fig. 5,B) as well as of adrenaline and noradrenaline. The pressor effects of the catechol amines were sometimes abolished, and generally were more sensitive to this antagonist than the pressor effects of 5-hydroxytryptamine, adrenaline being more easily antagonized than noradrenaline. The depressor effects of 5-hydroxytryptamine were unaffected by small doses of dibenamine, but reduced slightly by moderate or large doses. There was no action against the depressor or pressor effects of adenosine.

1-Benzyl-5-methoxy-2-methyltryptamine

Small doses of 1-benzyl-5-methoxy-2-methyltryptamine (100 to 200 μg. per 100 g. rat) reduced the pressor effects of 5-hydroxytryptamine (Fig. 5,C) and the antagonism increased with the dose. With large doses (up to 2.8 mg. per 100 g. rat) the pressor effects of 5-hydroxytryptamine were abolished in all but one case out of nine. The pressor effects of adrenaline and noradrenaline, as well as of adenosine, were affected similarly, but to a less extent. The most sensitive to this antagonist was thus 5-hydroxytryptamine; adrenaline and adenosine were less sensitive and noradrenaline still less. The depressor effects of 5-hydroxytryptamine and adrenaline were increased with small doses of 1-benzyl-5-methoxy-2-methyltryptamine, the former slightly, the latter more markedly (tendency to " reversal "). Moderate and large doses (up to 2.8 mg. per 100 g. rat) of the antagonist had the opposite action on the depressor effects of 5-hydroxytryptamine which were now somewhat reduced, and so were those of isoprenaline, adenosine, and adrenaline; adrenaline being least affected and isoprenaline most affected. The depressor effects of acetylcholine were not antagonized.

Atropine

The administration of atropine (100 μg. per 100 g. rat) produced a marked reduction or abolition of the depressor effects of acetylcholine 250 to 500 ng. per rat, the pressor effects of adrenaline and noradrenaline being unaffected. A further injection of the same dose of atropine caused some reduction of the depressor effects of 5-hydroxytryptamine, and once a slight potentiation of its pressor effects. A particular action shown on adenosine was that the depressor effects

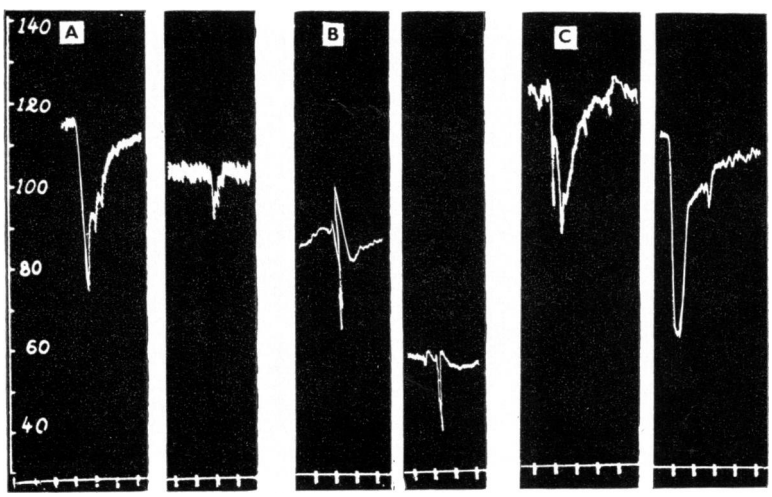

FIG. 5.—Blood pressure records from rats anaesthetized with urethane-barbitone sodium mixture. Time 0.5 min. **A**, Action of 1-3,4-dichlorophenyl)-2-isopropylaminoethanol on effects of 0.5 μg. 5-hydroxytryptamine; 180 g. rat. Injection 11 min. before (left), and 15 min. after (right) 1-(3,4-dichlorophenyl)-2-isopropylaminoethanol (360 μg.). **B**, Action of dibenamine on effects of 1 μg. 5-hydroxytryptamine; 195 g. rat. Injection 4 min. before (left) and 30 min. after (right) dibenamine (400 μg.). **C**, Action of 1-benzyl-5-methoxy-2-methyltryptamine on effects of 1 μg. 5-hydroxytryptamine; 180 g. rat. Injection before (left) and 55 min. after (right) 1-benzyl-5-methoxy-2-methyltryptamine (two injections, 360 μg. and 1.8 mg., 9 min. and 34 min. respectively after control injection).

were affected less than the pressor effects, which were potentiated (Fig. 6). A result of this was to make the slope of the dose response curve of adenosine steeper. This action could make the atropinized rat blood pressure preparation a useful one for the assay of adenosine.

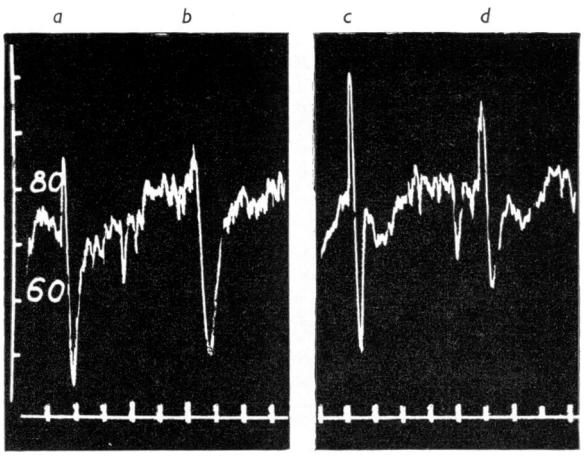

FIG. 6.—Blood pressure records from rat, 200 g., anaesthetized with urethane-barbitone sodium mixture. Action of atropine on effects of adenosine and 5-hydroxytryptamine. Responses to 20 μg. adenosine and 1 μg. 5-hydroxytryptamine alone at (a) and (b) respectively, and 1 hr. after atropine (two injections, each 200 μg., 5 min. and 35 min. after control injections) at (c) and (d).

The different patterns of antagonism shown are summarized in the Table, which embodies the salient features by means of a rough quantitative code. In addition to these results some further pecularities of lysergic acid diethylamide are noteworthy: (i) the antagonistic action of small or moderate doses required 0.5 to 1 hr. to develop fully, contrary to the other antagonists whose action appeared to be fully developed within much shorter times; this characteristic of lysergic acid diethylamide is illustrated in Fig. 4,A; (ii) a particular antagonistic action by lysergic acid diethylamide, once developed, appeared to persist for several hours; this was not the case for 1-(3,4-dichlorophenyl)-2-isopropylaminoethanol; in some experiments the action of small or moderate doses of the latter wore off completely in 45 to 60 min.; (iii) the dose range over which the whole sequence of antagonisms developed in each rat was smallest (2 to 4 fold) for lysergic acid diethylamide, much greater for 1-(3,4-dichlorophenyl)-2-isopropylaminoethanol (25 fold), the dose ranges of the other antagonists being intermediate (dibenamine 6 to 8 fold, dihydroergotamine 8 to 10 fold, 1-benzyl-5-methoxy-2-methyltryptamine 10 to 20 fold); (iv) the differences of sensitivity of the various agonist drugs to each antagonist were more clearly marked in the case of lysergic acid diethylamide than the others.

TABLE

RELATIVE SENSITIVITY OF DRUGS TO ANTAGONISTS

The pressor or depressor responses of the rat blood pressure to various agonists were not affected (**0**), or diminished by large (**1**), moderate (**2**) or small (**3**) doses of anta onist or the antagonism was doubtful (**?**). The antagonists revealed unma king (**U**) or potentiation (**P**) of effects with small or moderate doses, sometimes followed by antagonism (**U→1**), (**P→1**) with large doses of antagonist.

Antagonist	No. of Expts.	Catechol Amines				5-Hydroxy-tryptamine		Adenosine		Acetyl-choline
		Depressor (Beta)		Pressor (Alpha)		De-pressor	Pressor	De-pressor	Pressor	De-pressor
		Isopren-aline	Adren-aline	Adren-aline	Norad-renaline					
Lysergic acid diethylamide ..	(12)	0		2	2	3	U→1	2	U	0
Dihydroergotamine	(5)	1	P	3	3	3	?	2	0 or U	0
1-(3,4-Dichlorophenyl)-2-isopropylaminoethanol ..	(8)	3	3	P→1	P→1	2	U	1	U	0
Dibenamine	(5)			3	3	0 or 1	2	0	0	
1-Benzyl-5-methoxy-2-methyltryptamine	(9)	2	P→1	2	2	P→1	3	1	2	0

Discussion

The mixed effects of 5-hydroxytryptamine on the rat's blood pressure have been described by Salmoiraghi *et al.* (1956). Their results were substantially confirmed in our experiments for the particular dose range (2 to 5 μg. per rat) used by those authors. The study of a wider range of doses, however, disclosed a pattern of qualitative effects from mainly depressor responses resembling those in cats and rabbits to responses in which pressor components predominated as in dogs.

Part of the depressor effects of 5-hydroxytryptamine could be of parasympathetic origin because it was antagonized, though only to some extent, by atropine in maximally tolerated doses which completely inhibited the effects of injected acetylcholine. A vagal component of the effects of 5-hydroxytryptamine on the blood pressure of rats has already been shown by Salmoiraghi *et al.* (1956).

Part of both depressor and pressor effects of 5-hydroxytryptamine might also be of sympathetic origin in that all of the antagonists studied had some action against both 5-hydroxytryptamine and the catechol amines. In particular it would be tempting to draw conclusions as to the mediation of the effects of 5-hydroxytryptamine through catechol amines, depressor effects through hypotensive catechol amines, owing to the almost complete antagonism by 1-(3,4-dichlorophenyl)-2-isopropylaminoethanol, a selective beta inhibitor, and pressor effects through hypertensive catechol amines, owing to the antagonism by dibenamine. In this hypothesis the relative resistance of liberated substances compared with injected ones would have to be considered. The relationships between the effects of 5-hydroxytryptamine and the catechol amines appear, however, to be more complex, as not only corresponding effects but also opposite effects of these substances can be similarly antagonized by some agents, for example, the depressor effects of 5-hydroxytryptamine and the pressor effects of catechol amines by lysergic acid diethylamide and dihydroergotamine. This mixed antagonism shows that antagonism against corresponding effects does not necessarily mean the interruption at a particular point of a process of mediation. It can also be a clue to some relationship of another kind between 5-hydroxytryptamine and the catechol amines, for example, some common structural feature in their receptors. In this connexion one may recollect that on isolated organs, for example, the rat's uterus, typical inhibitors of catechol amines like dihydroergotamine and dibenamine also have a powerful antagonistic action against 5-hydroxytryptamine (Erspamer, 1953; Gaddum and Hameed, 1954), whose excitatory effects cannot be mediated by catechol amines which, under those conditions, are inhibitory (Gaddum, Peart, and Vogt, 1949). There are two further pieces of evidence that the depressor response to 5-hydroxytryptamine can be only partly, if at all, of sympathetic origin, namely, that this response was diminished by atropine and only partly affected even by large doses of dihydroergotamine.

It was reported by Salmoiraghi *et al.* (1957) that 10 to 50 μg. lysergic acid diethylamide per rat reduced the depressor effects of 5-hydroxytryptamine but failed to affect the response to 1 μg. of noradrenaline. In our experiments the pressor effects of 50 to 100 ng. noradrenaline per rat were also reduced and with larger doses of lysergic acid diethylamide almost abolished, so that the antagonist did not show an absolute specificity against 5-hydroxytryptamine. In fact none of the antagonists studied can be considered to possess a high degree of specificity. But it is possible to distinguish two substances (lysergic acid diethylamide and 1-benzyl-5-methoxy-2-methyltryptamine) which were relatively selective against 5-hydroxytryptamine, and two other substances (1-(3,4-dichlorophenyl)-2-isopropylaminoethanol and dibenamine) whose antagonism was directed rather more against the catechol amines. Dihydroergotamine was as powerful against 5-hydroxytryptamine as against the catechol amines and so occupied an intermediate position. 5-Hydroxytryptamine and adenosine, on the other hand, showed a common susceptibility or resistance to all the antagonists in this study, but adenosine in most cases was affected less than 5-hydroxytryptamine, and the development of effects which the two drugs showed on increasing the dose was in opposite directions. After most of the antagonists differences were diminished, but in the particular case of 1-(3,4-dichlorophenyl)-2-isopropylaminoethanol it was emphasized. In consequence, to distinguish between 5-hydroxytryptamine and adenosine in a solution containing one of them it might be useful to compare their effects before and after the administration of 1-(3,4-dichlorophenyl)-2-isopropylaminoethanol.

When classified according to their actions against the two effects of 5-hydroxytryptamine the antagonists studied fall into two main groups. One group containing lysergic acid diethylamide and 1-(3,4-dichlorophenyl)-2-isopropylaminoethanol antagonized preferentially the depressor effects of 5-hydroxytryptamine, while the other group containing 1-benzyl-5-methoxy-2-methyltryptamine and dibenamine antagonized prefer-

entially its pressor effects. It was difficult to classify dihydroergotamine. The compound antagonized the depressor effects of 5-hydroxytryptamine, but its action, if any, on the pressor effects of 5-hydroxytryptamine was obscured by its own pressor action. It is noteworthy that the two selective antagonists of 5-hydroxytryptamine already mentioned (lysergic acid diethylamide and 1-benzyl-5-methoxy-2-methyltryptamine) belong, according to this classification, to different groups. Lysergic acid diethylamide and 1-benzyl-5-methoxy-2-methyltryptamine differed from the selective antagonists of catechol amines in that after antagonism of the susceptible effect (depressor in the former case and pressor in the latter) they first potentiated the resistant effect, and finally, in doses 4 to 10 times as large, antagonized this as well. They thus showed a capacity for antagonizing all the effects of 5-hydroxytryptamine. The preferential actions of lysergic acid diethylamide and 1-benzyl-5-methoxy-2-methyltryptamine against different effects of 5-hydroxytryptamine agree well with the relative inefficacy of lysergic acid diethylamide in dogs (Salmoiraghi *et al.*, 1957), in which 5-hydroxytryptamine is mainly pressor, and with the efficacy of 1-benzyl-5-methoxy-2-methyltryptamine in the same species (Shaw and Woolley, 1956) as well as its weak action in rabbits (Jacob and Cugurra, 1960) where depressor effects predominate.

The actions of lysergic acid diethylamide and 1-benzyl-5-methoxy-2-methyltryptamine, antagonizing respectively the depressor and pressor actions of 5-hydroxytryptamine, bear some resemblance to those of the two kinds of antagonists of acetylcholine. Gaddum and Hameed (1954) put forward the theory that, as in the case of acetylcholine, there are at least two types of receptor with which 5-hydroxytryptamine combines. However, in view of the complexity of effects in the whole anaesthetized and spontaneously respiring rat and without more information on the modes of action of the antagonists, any attempt to correlate the types of antagonism here described with kinds of receptors would be only speculative.

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THE 5-HYDROXYTRYPTAMINE CONTENT OF MOUSE BRAIN AND WHOLE MICE AFTER TREATMENT WITH SOME DRUGS AFFECTING THE CENTRAL NERVOUS SYSTEM

BY

A. L. BARTLET*

From the Department of Pharmacology, School of Pharmacy, University of London, Brunswick Square, London, W.C.1, and the Department of Pharmacological Research, Parke, Davis and Company, Hounslow, Middlesex

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A number of drugs were examined for their ability to change the concentration of 5-hydroxytryptamine in mouse brain and in whole mice treated with 5-hydroxytryptophan. After β-phenyl*iso*propylhydrazine or iproniazid, two inhibitors of monoamine oxidase, the brain 5-hydroxytryptamine rose to a maximum value in 8 hr., after which it declined, although a slight rise remained for as long as 6 days. Dose-effect relationships, determined 6 hr. after administration, showed β-phenyl*iso*propylhydrazine to be approximately 60 times as effective as iproniazid in raising the brain 5-hydroxytryptamine. When mice were given 5-hydroxytryptophan and the amine content of the whole mice estimated, pretreatment with β-phenyl*iso*propylhydrazine increased their 5-hydroxytryptamine content whereas pretreatment with iproniazid did not change it. The concentration of the amine in mouse brain and in whole mice was lower after reserpine, but was raised when reserpine and β-phenyl*iso*propylhydrazine were given together. A small rise in brain 5-hydroxytryptamine was found after chlorpromazine ; when chlorpromazine was given with iproniazid, however, the resulting increase was less than that found after iproniazid alone. Brain 5-hydroxytryptamine was unaltered after prolonged treatment with morphine.

Monoamine oxidase is widely distributed in animal tissues and is an important route for the inactivation of 5-hydroxytryptamine (Davison, 1958 ; Keglević, Supek, Kveder, Iskrić, Kečkeš, and Kisić, 1959). Effective inhibitors of monoamine oxidase should therefore retard the inactivation of the amine *in vivo*, but this is only partially true for 1-*iso*nicotinyl-2-*iso*propylhydrazine (iproniazid), the most widely used inhibitor of monoamine oxidase. Although iproniazid is a strong and fairly specific inhibitor of this enzyme in homogenates (Zeller, Barsky, Fouts, Kircheimer, and Van Orden, 1952 ; Zeller, Barsky, and Berman, 1955), it has little effect on the inactivation of 5-hydroxytryptamine in tissue slices or in intact mice (Udenfriend, Weissbach, and Bogdanski, 1957). More recently, Horita (1958) has reported β-phenyl*iso*propylhydrazine to be a potent inhibitor of monoamine oxidase. In the present experiments, both β-phenyl*iso*propylhydrazine and iproniazid were examined as inhibitors of 5-hydroxytryptamine inactivation in intact mice.

*Present address: Department of Pharmacological Research, Parke, Davis and Company, Hounslow, Middlesex.

5-Hydroxytryptamine is present in the nervous system of a wide variety of species (Amin, Crawford, and Gaddum, 1954 ; Correale, 1956), and this has led to much interest in possible relationships between 5-hydroxytryptamine and drugs which affect brain function (Gaddum, 1954 ; Woolley and Shaw, 1954 ; Brodie, Pletscher, and Shore, 1955 ; Paasonen and Vogt, 1956 ; Pletscher, 1957). Chlorpromazine, reserpine and morphine, three drugs with actions on the central nervous system, have therefore been included as test substances in some of the present experiments.

METHODS

Materials.—Male albino mice, of body weight 10 to 20 g., were maintained on cube diet 41B with free access to water. The drugs used were chlorpromazine hydrochloride, β-phenyl*iso*propylhydrazine hydrochloride, iproniazid phosphate, reserpine, morphine sulphate, 5-hydroxytryptamine creatinine sulphate, (±)5-hydroxytryptophan, tryptamine hydrochloride, and (±)tryptophan. Quantities of tryptamine and 5-hydroxytryptamine have been expressed in terms of the base, but quantities of β-phenyl*iso*propylhydrazine, iproniazid, morphine and chlorpromazine refer to the

salts used. Drugs were freshly dissolved in distilled water, except reserpine, which was dissolved in a few drops of glacial acetic acid, diluted with 50 volumes of a mixture of equal parts of propylene glycol and ethyl alcohol, and made up to 100 volumes with distilled water. The stock solution of reserpine was diluted with distilled water immediately before use. Injections were given intraperitoneally in a dose volume of 0.1 ml./10 g. mouse, with the exception of 5-hydroxytryptophan, which was given in a dose volume of 0.2 ml./10 g. mouse.

Morphine Injected Mice.—Mice were injected daily with an increasing dose of morphine, under the supervision of Dr. S. J. Corne. The dose was increased as follows: 12.5 mg./kg. (10 days), 25 mg./kg. (21 days), 50 mg./kg. (7 days) and 100 mg./kg. (3 days). The final injection of morphine was given one hour before killing the mice and extracting 5-hydroxytryptamine.

Extraction of 5-Hydroxytryptamine.—Whole brains were dissected from mice which had been killed and bled out from the neck. The pooled brains from three mice were cut up finely with a scalpel on a glass slide and were extracted with 4 volumes of acetone (Analar). Next day, the acetone extract was separated from the residue and the residue was re-extracted overnight with 4 volumes of acetone. The second acetone extract was separated from the residue and the two extracts combined.

For the extraction of whole mice the acetone was acidified by the addition of 10 ml. N HCl/l. of acetone. Two mice were killed and homogenized with 4 g. sodium chloride and 4 volumes of acidified acetone. Next day the homogenate was filtered, and the residue extracted a second time overnight with 4 volumes of acidified acetone. On the following day the acetone extract was separated by filtration, and the residue on the filter paper washed with 30 ml. acidified acetone. The acetone extracts and washings were combined.

All extracts were kept at 0 to 4°. Just prior to the bioassay, an aliquot of acetone extract was evaporated to dryness at an external temperature of 35° and the residue re-extracted with a convenient volume of 0.9% sodium chloride.

Estimation of 5-Hydroxytryptamine. — The 5-hydroxytryptamine content of the saline extracts was determined on the isolated uterus of an oestrous rat. The uterus was suspended in a 15 ml. bath of oxygenated de Jalon solution at 29°. Atropine sulphate at a concentration of 10^{-6} was added to the de Jalon solution to increase the specificity of the preparation, which always responded to 0.02 to 0.03 μg. 5-hydroxytryptamine.

Rat Fundus.—The rat fundus preparation was made as described by Vane (1957). The fundus strip was suspended in a 10 ml. bath of oxygenated Tyrode solution at 37°, and contractions were recorded from a pendular lever (Paton, 1957). Atropine sulphate was added to the Tyrode solution to give a concentration of 10^{-6}.

Paper Chromatography.—Acetone extracts were evaporated to dryness at 35°, and the residue extracted with 0.25 ml. N HCl and 3 ml. ether. After shaking, the ether layer was discarded, and the aqueous phase transferred to an evaporating dish with 2 ml. alcohol. The extract was concentrated to 0.1 to 0.2 ml. and applied to a sheet of Whatman No. 1 paper together with marker spots of tryptamine, 5-hydroxytryptamine and tryptophan. Chromatograms were developed by the descending technique with *iso*propanol/ammonia (880)/water (20:1:2), or with *n*-butanol/acetic acid glacial/water (12:3:5). Occasionally two-way chromatograms were prepared with the same two solvent systems. All chromatograms were run for 5.5 to 7 hr. at a room temperature of 25.5 to 29.5°. Indoles were detected on the dried chromatograms by spraying with a 2% solution of *p*-dimethylaminobenzaldehyde in N HCl (Ehrlich's reagent), followed by heating at 80 to 85° for 3 to 5 min.

RESULTS

Identification of 5-Hydroxytryptamine in Extracts.—When bromolysergic acid diethylamide at a concentration of 10^{-7} was included in the de Jalon solution, the uterine responses to both 5-hydroxytryptamine and extracts were almost always abolished. Occasionally a brain extract still had a very slight stimulant action indicating the presence of a trace of interfering substance, but it was clear that the extracts reacted mainly with tryptamine receptors (Gaddum, 1953; Gaddum and Hameed, 1954).

Tryptamine has been identified in guinea-pig brain after administration of tryptophan and iproniazid (Hess, Redfield, and Udenfriend, 1959). Tryptamine and 5-hydroxytryptamine both react with tryptamine receptors and both are substrates of monoamine oxidase. The extracts were therefore examined for tryptamine, since this might have accounted for a significant part of their stimulant activity on the uterus. In extracts of mouse brains and whole mice 5-hydroxytryptamine was identified chromatographically as a blue spot with an R_F of 0.49 in butanol/acetic, and an R_F of 0.48 in *iso*propanol/ammonia. Tryptamine was not detected on these chromatograms. Even so, it seemed possible that tryptamine was present in small amounts. Tryptamine and 5-hydroxytryptamine can now be differentiated biologically, since Vane (1959) has shown that inhibitors of monoamine oxidase potentiate the response of the rat fundus preparation to tryptamine but not to 5-hydroxytryptamine. Tryptamine, 5-hydroxytryptamine, and brain extracts of normal mice and of mice treated with β-phenyl*iso*propylhydrazine were tested on rat fundus preparations both before and after the addition of iproniazid 10^{-5}. After

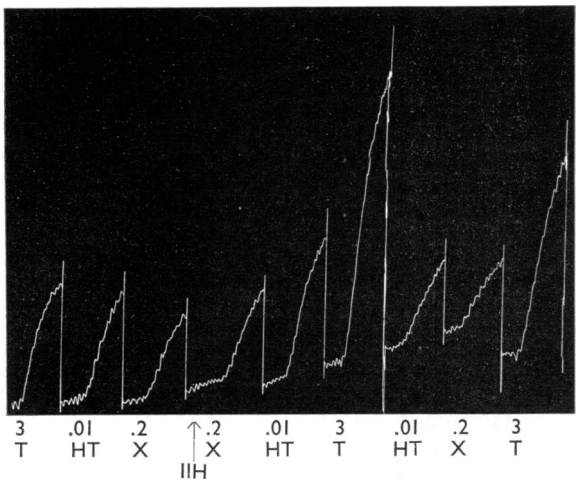

FIG. 1.—Rat fundus. 10 ml. bath. Interval 5 min. T, tryptamine (μg.). HT, 5-hydroxytryptamine (μg.). X, brain extract from control mice (ml.). From IIH, iproniazid 10^{-5} was present in the Tyrode solution. Iproniazid potentiated the response to tryptamine, but did not potentiate the responses to 5-hydroxytryptamine or extract.

iproniazid, the response of a fundus to tryptamine was potentiated whereas the contractions produced by 5-hydroxytryptamine and the extracts remained relatively unaffected (Fig. 1). This was consistent with the view that the active material in the extracts was 5-hydroxytryptamine rather than tryptamine.

The Effect of Drugs on Uterine Response to 5-Hydroxytryptamine.—The following drugs were added to the organ bath at the same time as 5-hydroxytryptamine: 0.5 μg. reserpine, 2 μg. chlorpromazine, 30 μg. iproniazid, 10 μg. β-phenyl-*iso*propylhydrazine, and 100 μg. morphine. The response to 5-hydroxytryptamine was reduced after chlorpromazine but was unaffected by the other drugs. When brain extracts of mice treated with chlorpromazine were tested there was no inhibition of the response to 5-hydroxytryptamine, however, showing that the extracts contained insufficient chlorpromazine to affect the uterus.

Recovery of 5-Hydroxytryptamine.—The extraction procedures adopted were similar to those of Correale (1956). The recovery of 5-hydroxytryptamine was tested by the addition of 0.5 μg. 5-hydroxytryptamine to three brain extracts (1 g. of brain approximately) and 200 μg. 5-hydroxytryptamine to two whole mice (30 g. approximately). In 5 determinations a mean of 73.2 ± 2.0% of the added 5-hydroxytryptamine was recovered from mouse brain, and, in 4 determinations, 64.5 ± 5.2% from whole mice. The recovery of added 5-hydroxytryptamine from whole mice remained unchanged at 63.0 ± 7.6% (4 determinations) when the mice were injected with β-phenyl*iso*propylhydrazine (100 mg./kg.). Differences in the 5-hydroxytryptamine content of the extracts may be taken to represent differences in the 5-hydroxytryptamine content of the mice, since β-phenyl*iso*propylhydrazine, an effective inhibitor of 5-hydroxytryptamine inactivation *in vivo*, did not alter the recovery of 5-hydroxytryptamine from the mice. Although Correale reported the recovery of 90–100% of added 5-hydroxytryptamine, other workers have obtained recoveries similar to those reported here, e.g. Twarog and Page (1953) recovered 60% of the added 5-hydroxytryptamine after extraction with acetone, and Welsh and Moorhead (1959) recovered 70% by a butanol extraction procedure.

The results given below have not been corrected for the loss of 5-hydroxytryptamine in the extraction procedure. A result has been taken as significant when P was less than 0.05.

TABLE I

INCREASES IN 5-HYDROXYTRYPTAMINE CONCENTRATION IN MOUSE BRAIN 1 TO 6 DAYS AFTER β-PHENYL*ISO*PROPYLHYDRAZINE AND IPRONIAZID

In 7 control mice the brain 5-hydroxytryptamine concentration was 0.624 ± 0·099 μg./g. brain.

Drug	Days after Administration of Drug	Mean Increase in 5-Hydroxytryptamine (μg./g. Brain ± S.E.). No. of Determinations in Brackets	Significance of Mean from Zero (P)
β-Phenyl*iso*propylhydrazine. (20 mg./kg.)	1	+0·486 ± 0·050 (2)	<0·001
	2	+0·063 ± 0·050 (2)	<0·25, >0·1
	3	+0·202 ± 0·050 (2)	<0·001
	4	+0·169 ± 0·045 (2)	<0·001
	5	+0·176 ± 0·045 (2)	<0·001
	6	+0·128 ± 0·045 (2)	<0·01, >0·005
Iproniazid (300 mg./kg.)	1	+0·379 ± 0·050 (2)	<0·001
	2	+0·146 ± 0·050 (2)	<0·005, >0·0025
	3	+0·311 ± 0·050 (2)	<0·001
	4	+0·175 ± 0·045 (2)	<0·001
	5	+0·187 ± 0·045 (2)	<0·001
	6	+0·165 ± 0·045 (2)	<0·0025, >0·001

5-Hydroxytryptamine in Mouse Brain.—Brain 5-hydroxytryptamine concentrations after administration of drugs have been expressed as differences between treated and control mice of the same experiment, the control values being given at the head of each table.

The effect of a single injection of β-phenyl*iso*propylhydrazine (20 mg./kg.) or of iproniazid (300 mg./kg.) on mouse brain 5-hydroxytryptamine was examined over a period of days (Table I). One day after β-phenyl*iso*propylhydrazine and iproniazid, increases in brain 5-hydroxytryptamine were 0.486 μg./g. and 0.379 μg./g. respectively, from a control value of 0.624 μg./g. Even 6 days after these monoamine oxidase inhibitors, small but significant increases in brain 5-hydroxytryptamine were found.

Increases in mouse brain 5-hydroxytryptamine concentration after β-phenyl*iso*propylhydrazine (100 mg./kg.) and iproniazid (300 mg./kg.) were also investigated at more closely spaced time intervals over a period of 24 hr. (Fig. 2). The maximum increases in amine were found 8 hr. after administration of either compound. However, for practical reasons the effect of different doses of β-phenyl*iso*propylhydrazine and iproniazid in raising the brain 5-hydroxytryptamine was investigated after 6 hr. (Fig. 3). The dose-effect curve for iproniazid had a flatter slope and a lower maximum than the corresponding curve for β-phenyl*iso*propylhydrazine. It was not possible therefore to give an accurate ratio of the potencies for these two compounds. However, the effects of 5 mg./kg. β-phenyl*iso*propylhydrazine and 300 mg./kg. iproniazid were of the same order, since they increased the brain 5-hydroxytryptamine by 0.347 μg./g. and by 0.389 μg./g., respectively.

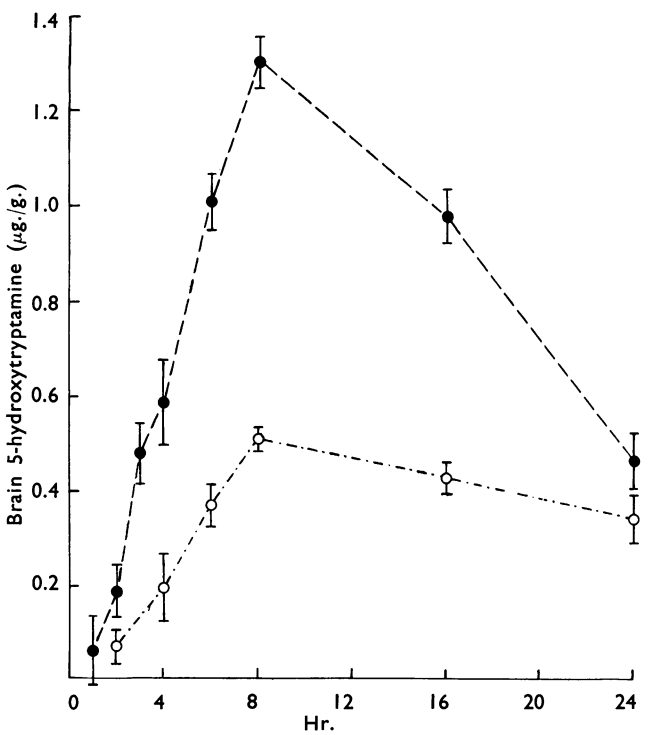

FIG. 2.—Increases in brain 5-hydroxytryptamine in mice after treatment with β-phenyl*iso*propylhydrazine (100 mg./kg.), marked ● – – – ●, or iproniazid (300 mg./kg.), marked O- · · · -O. The mean value for 5-hydroxytryptamine in control brains was 0.497±0.032 μg./g. (17 determinations). Ordinate, increase in brain 5-hydroxytryptamine (μg./g.). Abscissa, interval between injection of compound and extraction of 5-hydroxytryptamine (hr.). Each point is a mean value±S.E.

Table II shows differences in the 5-hydroxytryptamine concentration of mouse brain 6 hr. after the intraperitoneal injection of various drugs. The concentration of amine was lower after reserpine (2 mg./kg.), was higher after β-phenyl*iso*propylhydrazine (20 mg./kg.), and was increased by the combination of these two drugs at the same dose levels. When all the data were combined as in Table II, the rise in 5-hydroxytryptamine after β-phenyl*iso*propylhydrazine was higher than that after reserpine plus β-phenyl*iso*propylhydrazine. However, when the comparison was restricted to results determined in parallel, the difference between the two groups was not significant ($P>0.2$). A small but significant rise was found in each of 9 experiments after chlorpromazine (20 mg./kg.), but the increase in brain 5-hydroxytryptamine after chlorpromazine plus iproniazid was significantly less than the increase found after iproniazid alone ($P<0.02$, >0.01). No significant difference in 5-hydroxytryptamine concentration was found between the brains of control mice and mice treated with morphine.

5-Hydroxytryptamine in Whole Mice Injected with 5-Hydroxytryptophan.—Table III shows the effect of some drugs on the 5-hydroxytryptamine extracted from mice injected with 5-hydroxytryptophan. All mice received 5-hydroxytryptophan (150 mg./kg.), and 3 hr. later were killed and extracted for

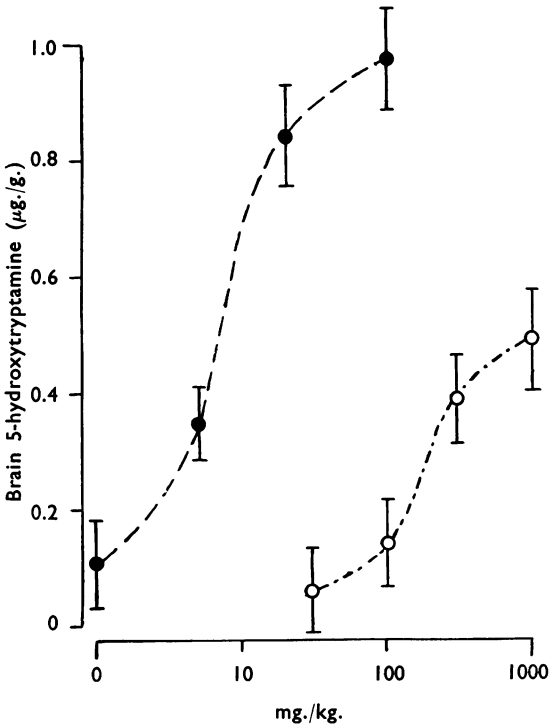

FIG. 3.—Increases in brain 5-hydroxytryptamine in mice 6 hours after varying doses of β-phenyl*iso*propylhydrazine, marked ●---●, and iproniazid, marked O-·-·-O. The mean value for 5-hydroxytryptamine in control brains was 0·449±0·029 μg./g. (10 determinations). Ordinate, increases in brain 5-hydroxytryptamine (μg./g.). Abscissa, doses of β-phenyl*iso*propylhydrazine and iproniazid (mg./kg.), plotted on a logarithmic scale. Each point is a mean value±S.E.

5-hydroxytryptamine. Drugs tested in the mice were administered intraperitoneally at specified periods before injecting the mice with 5-hydroxytryptophan. Treatment with reserpine (2 mg./kg.) lowered the 5-hydroxytryptamine concentration in mice; in animals treated with β-phenyl*iso*propylhydrazine (100 ml./kg.), it was increased above control levels, but values after treatment with iproniazid (300 mg./kg.) were not significantly different from the controls. 5-Hydroxytryptamine was also increased when β-phenyl*iso*propylhydrazine and reserpine were injected together; indeed, in mice injected with β-phenyl*iso*propylhydrazine plus reserpine it was not significantly different from β-phenyl*iso*propylhydrazine treated mice (P>0.2).

TABLE II

CHANGES IN 5-HYDROXYTRYPTAMINE CONCENTRATION IN MOUSE BRAIN 6 HOURS AFTER INTRAPERITONEAL INJECTION OF VARIOUS DRUGS

In 18 control mice the brain 5-hydroxytryptamine concentration was 0·478±0·027 μg./g. brain.

Drug	Dose (mg./kg., i.p.)	Changes in 5-Hydroxytryptamine (μg./g. brain±S.E.). No. of Determinations in Brackets	Significance of Mean from Zero (*P*)
Reserpine	2	−0·264±0·012 (6)	<0·001
Reserpine + β-phenyl*iso*propylhydrazine	2 20	+0·512±0·012 (6)	<0·001
β Phenyl*iso*propylhydrazine	20	+0·746±0·076 (12)	<0·001
Iproniazid	300	+0·445±0·057 (11)	<0·001
Iproniazid + Chlorpromazine	300 20	+0·286±0·065 (9)	<0·005, >0·002
Chlorpromazine	20	+0·108±0·021 (9)	<0·001
Morphine	(See methods)	−0·001±0·025 (3)	>0·9

DISCUSSION

Both β-phenyl*iso*propylhydrazine and iproniazid caused an accumulation of 5-hydroxytryptamine in mouse brain. Between 2 and 8 hr. after β-phenyl*iso*propylhydrazine (100 mg./kg.) or iproniazid (300 mg./kg.), the rise of 5-hydroxytryptamine in mouse brain was very rapid, 1.12 μg./g. brain accumulating after β-phenyl*iso*propylhydrazine. The accumulation of 5-hydroxytryptamine in brain after the monoamine oxidase inhibitors showed that it was synthesized at a high rate; it was calculated that the normal brain content accumulated in about 2.5 hr. after β-phenyl*iso*propylhydrazine. The amine reached its maximum concentration in brain 8 hr. after β-phenyl*iso*propylhydrazine or iproniazid and then declined. This decline may have been due to re-activation or re-formation of brain monoamine oxidase. Complete regeneration of brain

TABLE III

THE EFFECT OF SOME DRUGS ON 5-HYDROXYTRYPTAMINE LEVELS IN WHOLE MICE TREATED WITH 5-HYDROXYTRYPTOPHAN

All mice received 5-hydroxytryptophan (150 mg./kg.) 3 hr. before extraction of 5-hydroxytryptamine. In 8 control mice the concentration of 5-hydroxytryptamine was 3·91±0·26 μg./g. mouse.

Drug (hr. Before Injection of 5-Hydroxy-tryptophan)	Dose (mg./kg., i.p.)	5-Hydroxy-tryptamine (μg./g. Mouse ±S.E.). No. of Determinations in Brackets	Significance of Difference Between Means of Treated and Control Mice (*P*)
Reserpine (3 hr.)	2	2·76±0·25 (3)	<0·05, >0·02
Reserpine (3 hr.)+ β-phenyl*iso*propyl-hydrazine (1 hr.)	2 100	6·37±0·75 (3)	<0·0025, >0·001
β-Phenyl*iso*propyl-hydrazine (1 hr.)	100	7·73±0·64 (6)	<0·001
Iproniazid (1 hr.)	300	3·94±0·68 (4)	>0·90

monoamine oxidase was a lengthy process, however, since the brain 5-hydroxytryptamine was still slightly raised after 6 days.

When different doses of β-phenyl*iso*propyl-hydrazine and iproniazid were compared for their ability to raise the brain 5-hydroxytryptamine concentration, 6 hr. after administration of drug, β-phenyl*iso*propylhydrazine was found to be about 60 times as effective as iproniazid, since 5 mg./kg. β-phenyl*iso*propylhydrazine and 300 mg./kg. iproniazid both produced a rise of 0.3 to 0.4 μg. 5-hydroxytryptamine/g. brain. A similar order of effectiveness was found for these two compounds when they were examined as *in vitro* inhibitors of monoamine oxidase (Udenfriend, Witkop, Redfield, and Weissbach, 1958). The present results therefore support the view that the rise in 5-hydroxytryptamine in brain after β-phenyl*iso*propylhydrazine and iproniazid was due to inhibition of brain monoamine oxidase.

Log dose-effect curves were obtained by plotting increases in brain 5-hydroxytryptamine against doses of β-phenyl*iso*propylhydrazine or iproniazid. Sigmoid curves were obtained for both compounds, but the curve for iproniazid had a flatter slope and a lower maximum than the curve for β-phenyl*iso*propylhydrazine. The lower maximum of its curve suggested that iproniazid might inhibit both formation and inactivation of 5-hydroxytryptamine in the brain. According to Davison (1956), a metabolite of iproniazid inhibits enzymes requiring pyridoxal phosphate. This supports the present interpretation of the log dose-effect curve for iproniazid, since pyridoxal phosphate is a co-enzyme of the decarboxylase which forms brain 5-hydroxytryptamine.

Inhibition of 5-hydroxytryptamine inactivation by β-phenyl*iso*propylhydrazine must be widespread in the tissues of the mouse, since the 5-hydroxytryptamine content of animals injected with 5-hydroxytryptophan was increased after treatment with β-phenyl*iso*propylhydrazine (100 mg./kg.). This was a severe test of the effect of β-phenyl*iso*propylhydrazine, since the substrate was presented to the enzyme *in vivo*; iproniazid tested in the same way was without effect at 300 mg./kg.

After reserpine (2 mg./kg.), 5-hydroxytryptamine was reduced by 63% in mouse brain and by 30% in whole mice. When β-phenyl*iso*propyl-hydrazine was given with reserpine, the 5-hydroxytryptamine concentrations of mouse brain and whole mice were raised, suggesting that monoamine oxidase is involved in the disappearance of 5-hydroxytryptamine after reserpine. The 5-hydroxytryptamine content of mice injected with 5-hydroxytryptophan and β-phenyl*iso*propyl-hydrazine remained unaltered when the mice were pretreated with reserpine. This showed that reserpine (2 mg./kg.) had no appreciable effect on the decarboxylation of 5-hydroxytryptophan, which is in agreement with Brodie, Tomich, Kuntzman and Shore (1957). The difference between the increase in brain 5-hydroxytryptamine found after reserpine plus β-phenyl*iso*propyl-hydrazine and that found after β-phenyl*iso*propyl-hydrazine alone was not significant when the comparison was made between results determined in parallel.

The increase in brain 5-hydroxytryptamine concentration found after iproniazid plus chlorpromazine was significantly less than the increase found after iproniazid alone. This suggested a depression of 5-hydroxytryptamine formation after chlorpromazine. According to West (1958), chlorpromazine inhibits the 5-hydroxytryptophan decarboxylase of rat kidney *in vivo*. Such a decarboxylase inhibitor would be expected to cause a fall in brain 5-hydroxytryptamine within

a few hours, since the rate of formation of 5-hydroxytryptamine in brain has been shown to be high. After chlorpromazine, however, 5-hydroxytryptamine concentration in the brain was slightly raised above the corresponding control values. The reason for the small rise in brain 5-hydroxytryptamine after chlorpromazine is not understood. Possibly chlorpromazine inhibited some route of 5-hydroxytryptamine inactivation. According to Weissbach (1958), 5-hydroxytryptamine can be inactivated by cytochrome oxidase, an enzyme which is known to be inhibited by chlorpromazine *in vitro* (Bernsohn, Namajuska and Boshes, 1956; Dawkins, Judah, and Rees, 1959).

Costa and Himwich (1958) found a lowered 5-hydroxytryptophan decarboxylase activity in rabbit brain after convulsive doses of insulin, although the concentration of brain 5-hydroxytryptamine was raised or unaltered. Their results suggest that insulin, like chlorpromazine, depresses turnover of cerebral 5-hydroxytryptamine.

I would like to thank Professor G. A. H. Buttle for permission to work at the London School of Pharmacy, and Dr. G. B. West for his encouragement and welcome advice. I am also indebted to Dr. H. O. J. Collier for helpful suggestions, to Mrs. J. Skerry for technical assistance, to Dr. T. F. Grey for the β-phenyl*iso*propylhydrazine, and to Dr. B. T. Warner for statistical analysis.

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Brit. J. Pharmacol. (1960), **15**, 147.

GANGLION-BLOCKING PROPERTIES OF ATROPINE-LIKE DRUGS

BY

J. G. BAINBRIDGE AND D. M. BROWN

From the Beecham Research Laboratories, Ltd., Brockham Park, Betchworth, Surrey

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The ganglion-blocking properties of atropine, atropine methyl nitrate, oxyphenonium, poldine, methantheline and propantheline have been examined and compared with those of hexamethonium, mecamylamine, pentolinium and tetraethylammonium. The ganglion-blocking activity was assessed by stimulating the preganglionic nerve to the superior cervical ganglion and recording directly the percentage depression in the postganglionic action potentials. In this way any ambiguity due to the peripheral actions of the drugs was removed. As a group the atropine-like compounds are not markedly less potent than the ganglion-blocking agents, but their action is relatively transient. This work suggests that the ganglion-blocking action of atropine-like substances plays no part in their therapeutic effects.

Atropine-like drugs are frequently used in clinical practice for their peripheral anti-acetylcholine effects. The actions of atropine-like drugs at peripheral sites have been fully investigated, but their actions at autonomic ganglia have been examined in less detail [Marrazzi (1939), Dutta (1949), Konzett and Rothlin (1949), Ambache (1949), Cahen and Tvede (1952), and Paton (1954)]. We therefore thought it important to find out whether these drugs produce significant ganglion-block in doses which antagonize the peripheral actions of acetylcholine.

The measurement of ganglion-blocking action has usually been carried out on the cat nictitating membrane preparation, but there are several objections to this, particularly for atropine-like drugs which have a considerable peripheral action. For example, Cervoni, West, and Fink (1956) found that atropine directly depressed the smooth muscle of the nictitating membrane. This was confirmed by Thompson (1958) using the isolated nictitating membrane preparation. Even direct perfusion of the ganglion with the drug does not eliminate this error, since up to 50% may not reach the ganglion, being diverted possibly by arterio-venous anastomoses to peripheral sites (Paton, 1954).

In this investigation we have avoided complications due to peripheral actions of the drugs by measuring directly the postganglionic nerve potentials evoked by stimulating the preganglionic nerve to the superior cervical ganglion. We have studied the ganglion-blocking activity of several atropine-like drugs and some true ganglion-blocking agents.

METHODS

Stimulation and recording of postganglionic action potentials were similar to the method described by Eccles (1935).

Anaesthesia was induced in cats with ether, followed by intravenous chloralose/urethane solution (1% and 5% respectively) at approximately 5 ml./kg. The cervical sympathetic nerve and the postganglionic nerve from the superior cervical ganglion were dissected out. Care was taken to leave the blood supply to the ganglion intact. The cat was then transferred to a metal screening cage, stimulating and recording electrodes were placed in position and the exposed tissues were covered with liquid paraffin.

A rectangular pulse of approximately 0.1 msec. duration, 2 to 10 V. and 2 pulses/sec. frequency was applied to the preganglionic nerve. The recording electrodes on the postganglionic nerve led to a condenser-coupled amplifier and oscilloscope with recording camera.

Drugs were given intravenously into the femoral vein. The action potentials were photographed immediately before and at 1 min. after the injection, the effect of the compounds being maximal at this time. The action of the drug was expressed as the percentage reduction of the spike amplitude as measured from the photographic record.

Four to eight observations were made with each drug. The response was plotted against the dose on a log scale and a regression line was fitted by eye to the points obtained. The ED50 was read directly from the graph.

The duration of action of the atropine-like drugs was determined by injecting a dose equal to the ED50 and photographing the action potential before administration and at 1, 2, 4, and 8 min. after administration. The action potentials following hexamethonium were measured at 5 min. intervals until recovery. The percentage depression of spike amplitude was plotted against time, and from the graph the time taken from maximum depression to half recovery was estimated. Two experiments were carried out with each drug.

The following salts of the various drugs were used: pentolinium tartrate, hexamethonium bromide, mecamylamine hydrochloride, atropine sulphate, tetraethylammonium bromide, methantheline bromide, propantheline bromide, oxyphenonium bromide, and poldine methosulphate (Nacton).

The doses given below are expressed in terms of these salts.

Results

Fig. 1 illustrates the action potentials before and after the administration of 0.2 mg./kg. of hexamethonium and 4 mg./kg. of atropine. This dose of atropine reduced the spike potential by approximately 80%, and hexamethonium by about 35%.

Fig. 2 shows the dose-response lines obtained for most of the drugs investigated. The slopes of the lines would appear to be all of the same order.

The doses of atropine-like drugs and ganglion-blocking agents giving a 50% reduction in spike amplitude, as determined from the dose-response lines, are given in Table I.

It will be noted that the atropine-like drugs, as a group, are somewhat less potent than the true ganglion-blocking agents, but the difference is not marked. In the case of atropine methyl nitrate this drug is even more potent than mecamylamine and hexamethonium. However, the two groups of drugs differ considerably in their duration of action. The atropine-like drugs have a short effect of approximately 2 to 6 min., while the duration of action of the true ganglion-blocking drugs administered at the ED50 level is upwards of 30 min.

A comparison of the duration of action of atropine-like drugs with hexamethonium was made and the results are given in Table II. As the time of complete recovery was rather variable, and as the initial slope of the recovery curve was more constant, the " mean half recovery time " as defined above was worked out.

Table I

RELATIVE GANGLION-BLOCKING POTENCY OF ATROPINE-LIKE DRUGS AND TRUE GANGLION-BLOCKING AGENTS WITH RESPECT TO HEXAMETHONIUM

Drug	ED50 (mg./kg.)	Equipotent Molar Ratio (Hexamethonium = 1.0)
Pentolinium	0·07	6·55
Mecamylamine ..	0·23	0·733
Hexamethonium ..	0·31	1·0
Atropine methyl nitrate	0·14	2·22
Propantheline	0·70	0·545
Methantheline	0·70	0·510
Tetraethylammonium ..	0·80	0·226
Atropine	2·4	0·249
Oxyphenonium ..	2·4	0·154
Poldine (Nacton) ..	3·8	0·097

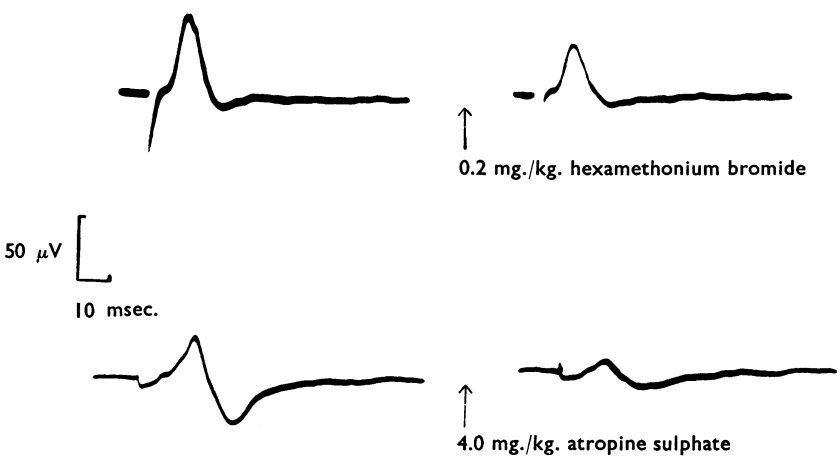

Fig. 1.—Postganglionic action potentials. Top records: before and after the intravenous injection of 0.2 mg./kg. of hexamethonium. Bottom records: before and after 4 mg./kg. of atropine.

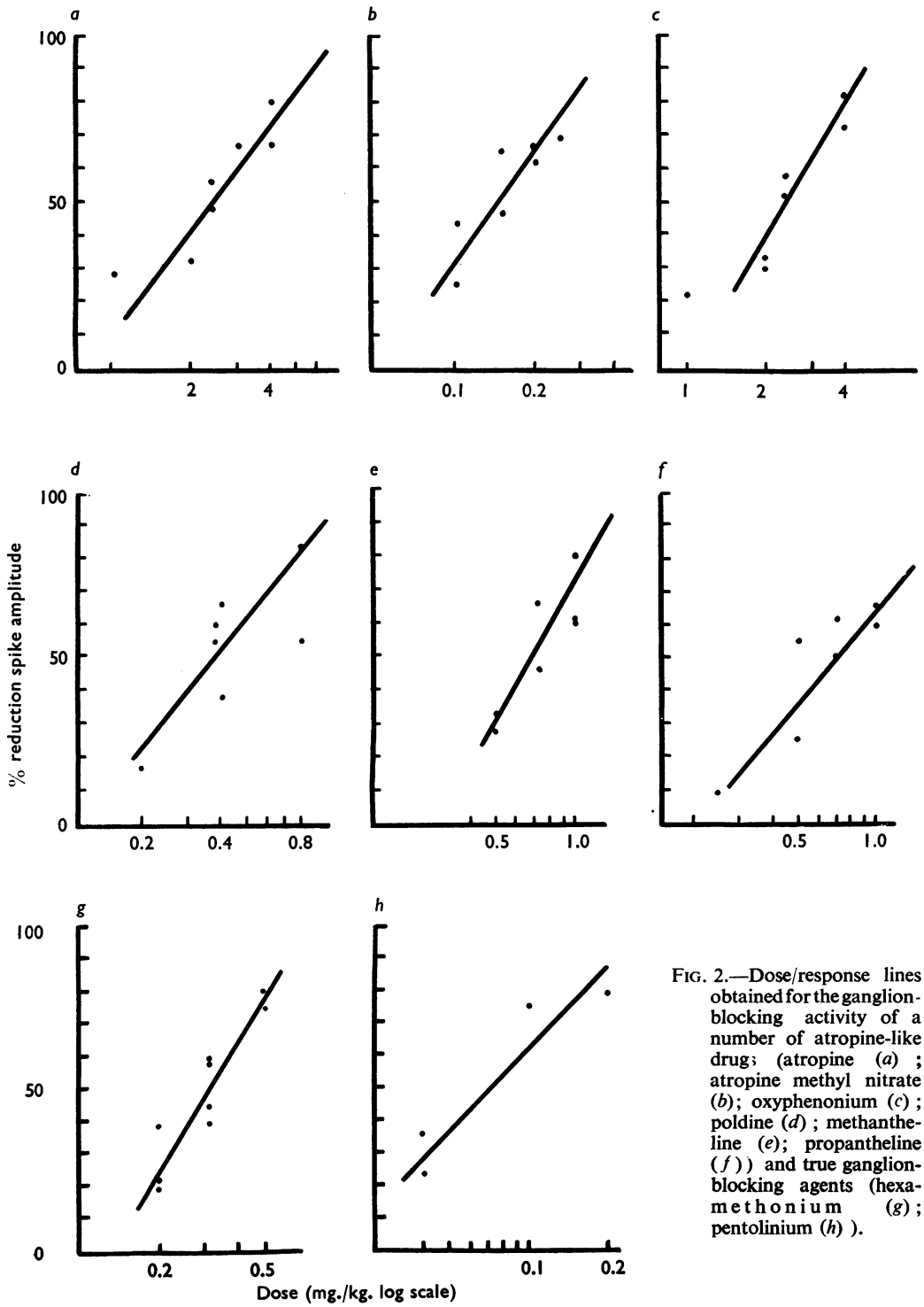

FIG. 2.—Dose/response lines obtained for the ganglion-blocking activity of a number of atropine-like drugs (atropine (*a*); atropine methyl nitrate (*b*); oxyphenonium (*c*); poldine (*d*); methantheline (*e*); propantheline (*f*)) and true ganglion-blocking agents (hexamethonium (*g*); pentolinium (*h*)).

TABLE II

TIME TAKEN FOR THE POSTGANGLIONIC ACTION POTENTIAL TO RECOVER BY 50% AFTER THE ADMINISTRATION OF THE ED50 OF SOME ATROPINE-LIKE DRUGS AND HEXAMETHONIUM

Drug	Mean Half Recovery Time (Min.)
Oxyphenonium	5·5
Atropine	4·2
Atropine methyl nitrate	4·7
Poldine	5·8
Propantheline	3·3
Methantheline	2·3
Hexamethonium	31·0

DISCUSSION

Atropine and various anti-acetylcholine drugs have been shown by a direct method to block transmission through the superior cervical ganglion.

Fig. 3*a* shows the relation between toxic, ganglion-blocking, and peripheral anti-acetylcholine activity of the atropine-like drugs, and Fig. 3*b* shows the relationship between the toxic and ganglion-blocking doses for the true ganglion-blocking drugs. The toxic dose is the intravenous LD50 for mice, and the figure for anti-acetylcholine activity is the dose required to obtain 50% inhibition of the vasodepressor response to intravenous acetylcholine in the cat (Acred, Atkins, Bainbridge, Brown, Quinton, and Turner, 1957). The ganglion-blocking dose is the ED50 value as given in Table I.

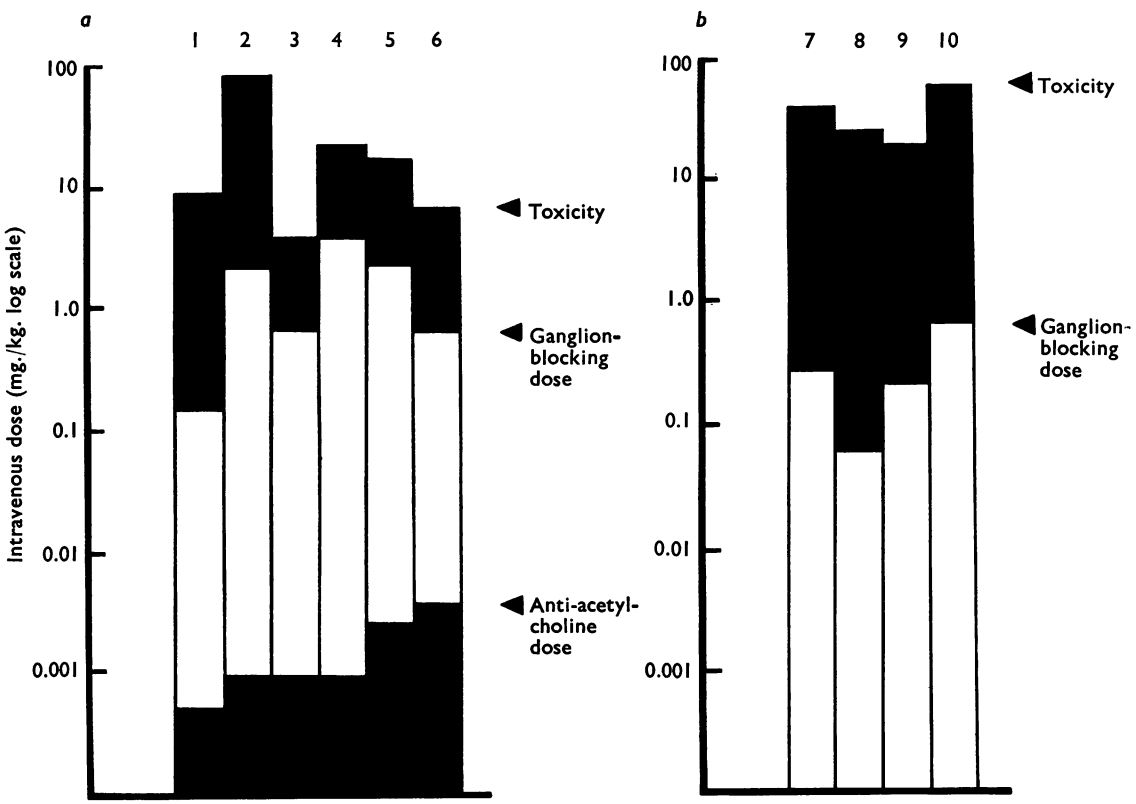

FIG. 3.—Diagram showing (*a*) relationship between toxicity, ganglionic and peripheral activities of atropine-like compounds (1, atropine methyl nitrate; 2, atropine; 3, propantheline; 4, poldine; 5, oxyphenonium; 6, methantheline); and (*b*) toxic and ganglionic activity of ganglion-blocking agents (7, hexamethonium; 8, pentolinium; 9, mecamylamine; 10, tetraethylammonium bromide).

In the case of the ganglion-blocking agents the ratio between toxic and ganglion-blocking doses is large, ranging from 400 for pentolinium to 90 for mecamylamine and tetraethylammonium, whereas in the atropine-like group the ratio varies from 6 for propantheline to 60 for atropine methyl nitrate. ED50 values for the ganglion-blocking activity for the two groups actually overlap, atropine methyl nitrate being remarkably potent whereas tetraethylammonium is less potent than methantheline and propantheline. The ratio between ganglionic and peripheral ED50 values in the atropine-like group is of the order of 1,000, and so it is most improbable that ganglion-block contributes to their therapeutic actions.

No simple relation exists between ganglionic and peripheral potencies in the atropine group. Quaternization of the nitrogen in atropine leads to an increase of toxicity and peripheral anti-acetylcholine activity and to a proportionately much greater ganglion-blocking potency. The presence of a quaternary nitrogen atom is, however, not essential to a high ganglion-blocking potency (for example, mecamylamine and pempidine).

The effect of anti-acetylcholine drugs at ganglia could be due to depolarization or competition with acetylcholine. Paton and Perry (1953) have shown that acetylcholine, nicotine, and tetramethylammonium block ganglia by depolarization, all causing an initial stimulation of the ganglion cells. Other known ganglion-blocking agents neither depolarize nor cause an initial stimulation and are assumed to act competitively. We have no evidence that the atropine-like drugs cause an initial stimulation of the superior cervical ganglion, although we did produce an initial stimulation of the intestine *in vivo* after intravenous administration (Acred *et al.*, 1957). It is possible that this is a nicotine-like effect. On the other hand, Konzett and Rothlin (1949), perfusing the superior cervical ganglion and recording from the nictitating membrane, overcame the block produced by atropine with higher concentrations of acetylcholine, suggesting a competitive mode of action.

The ganglion-blocking action of atropine can be attributed partly to its local anaesthetic activity (Paton, 1954). However, most of the compounds we have investigated are quaternary esters and have no local anaesthetic properties (Acred *et al.*, 1957; Barlow, 1955). Further, while atropine has about half the local anaesthetic potency of procaine the latter has a much smaller ganglion-blocking action than atropine both by intravenous and close intra-arterial injection to the ganglion (see also Dutta, 1949). Hence the available evidence as to mode of action suggests a competitive block such as probably occurs at peripheral sites, although other modes of action are possible (Paton, 1954).

Although the ganglionic action of atropine could hardly become manifest in clinical use it could explain the observation that in anaesthetized cats intravenous doses greater than 1 mg./kg. often cause a transient fall of blood pressure.

We can conclude that the ganglion-blocking action of atropine-like drugs does not add significantly to their peripheral actions. In the first instance, the dose required to produce a ganglion-block is so great that the degree of side-effects alone would prohibit their use. In the second instance, the duration of block is so short that in no way could it be said to influence the therapeutic action. Finally, the precise mechanism of the block has not as yet been elucidated.

The authors wish to thank Miss A. Trenchard for technical assistance.

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Brit. J. Pharmacol. (1960), **15**, 152.

PHARMACOLOGICALLY ACTIVE PEPTIDES IN THE BLOOD AND URINE OF ANIMALS INFECTED WITH *BABESIA RODHAINI* AND OTHER PATHOGENIC ORGANISMS

BY

L. G. GOODWIN AND W. H. G. RICHARDS

From the Wellcome Laboratories of Tropical Medicine, London

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The blood and urine of mice and rats infected with *Babesia rodhaini* contain substances which stimulate the isolated guinea-pig ileum and rat duodenum. The amount of active material excreted increases as the infection increases. The active substances are stable to boiling with hydrochloric acid but not with alkali; they pass through a cellophane membrane and are soluble in hot ethanol. They are destroyed rapidly by papain and less rapidly by chymotrypsin, but are unaffected by trypsin or pepsin. Their action on smooth muscle is not affected by atropine, eserine, antihistamines, iproniazid, bretylium or by lysergic acid diethylamide. The active substances are probably peptides and there is evidence that the urine contains a mixture of peptides, some of which relax and some of which contract the rat duodenum. Similar active peptides appear in the urine of mice infected with *Plasmodium berghei*, *Trypanosoma rhodesiense*, *Streptococcus pyogenes* and Rift Valley fever virus.

Maegraith, Gilles and Devakul (1957) made a study of the pathological processes in puppies and dogs infected with *Babesia canis*. They showed that in severe and fatal cases the clinical progress bore " little direct relation to the prevailing degree of parasitic infection of the erythrocytes," and that the plasma often became lytic for both parasitized and normal erythrocytes. After severe lysis, the erythrocyte count, haemoglobin and packed cell volume often remained within normal limits, although the animal passed into a state resembling oligaemic shock. An injection of noradrenaline caused immediate, though temporary, recovery from this condition; a similar dramatic effect was reported by Maegraith, Devakul and Leithead (1956) in monkeys infected with *Plasmodium knowlesi*.

Maegraith *et al.* (1957) concluded that the pathological processes are non-specific and are common to other acute medical states, and that " the initiating factors may be physiologically active soluble substances of relatively simple nature derived from the parasite or as a consequence of host-parasite reaction or arising from tissue damage."

Severe or fatal shock also occurs in cattle infected with piroplasms, and may be brought about by treatment with effective drugs (Stephan and Esquibel, 1929). A similar phenomenon is sometimes observed in mice infected with *B. rodhaini* (Beveridge, 1953). It has been suggested that the destruction of parasites by the drug liberates substances which are toxic to the host. The liberation of " toxins " from protozoal parasites has often been suggested to explain physiological disturbances in the host, but pharmacological studies of the nature of these " toxins " have been few.

The present paper describes a preliminary study of the active substances present in the blood and urine of animals infected with *B. rodhaini* and other pathogenic organisms. Pharmacologically active peptides are shown to be present.

METHODS

Infections

Babesia rodhaini.—The strain used was the rat-adapted strain described by Beveridge (1953). Mice (20 g.) or rats (50 g.) were inoculated intraperitoneally with 0.1 ml. of infected rat blood containing 100 to 600m. parasitized red cells. The strain has increased in virulence since 1953 and now kills all mice in about 5 days. Rats did not die; maximal parasitaemia was reached in 5 to 6 days and then declined. In most experiments the parasites were counted daily in stained blood smears.

Plasmodium berghei.—The strain was obtained from the London School of Hygiene in 1949 and maintained in mice by blood passage. Mice were

infected by the intraperitoneal injection of 0.1 ml. of infected blood containing about 500 million parasitized red cells. The infection reached its maximum in the blood in 8 days; most of the mice died 6 to 8 days after inoculation.

Trypanosoma rhodesiense.—The strain (C) was isolated in Entebbe in 1939 and maintained in mice by blood passage. Mice were infected by the intraperitoneal injection of 0.5 ml. of diluted infected blood containing about 500,000 trypanosomes; the mice died 4 days after inoculation.

Streptococcus pyogenes.—A culture of a freshly isolated strain was obtained from University College Hospital. Mice were inoculated intraperitoneally with 0.05 ml. of a 24-hr. subculture on Loeffler's serum agar. The mice died 4 days after inoculation.

Rift Valley Fever.—The strain was maintained by passage of infected liver. Mice were inoculated intraperitoneally with infected liver suspension; they died 4 days after inoculation.

Intravascular Haemolysis without Infection

Groups of 10 mice were injected intravenously with preparations which caused or simulated intravascular haemolysis and gave rise to haemoglobinuria. The preparations included haemoglobin solution, lysed mouse red cells, washed rabbit red cells, saponin and phenylhydrazine.

Collection of Urine

Urine was collected in a glass metabolism cage from groups of 10 to 50 mice. Faeces were separated with a terylene net screen, or by means of a polythene funnel with a curved spout similar to the glass funnel described by Brittain (1959). Urine was usually collected in a bottle containing 0.05 ml. of N HCl, and was neutralized to *p*H 7.2 before testing. In some experiments the collecting vessel was immersed in alcohol and solid carbon dioxide so that the urine was frozen as soon as it reached the bottle. Mice were fed daily for 3 hr. outside the cage; the rest of the time between inoculation and death was spent in the cage; the urine collected was not contaminated with food. Water was unrestricted at feeding time; in the metabolism cage it was provided in a bottle or 1 ml. was given daily into the stomach of each mouse to provide a good urine flow. Collected specimens of urine were stored at $-15°$. In some experiments, urine was collected with a pipette directly from the mouse after gentle pressure on the abdomen. In some experiments the urine was passed through alumina columns (Savory and Moore, chromatographic grade) before testing.

Preparation of Extracts

Blood and urine samples were collected in siliconed apparatus. Extracts of urine, blood and tissues were prepared by Code's modification (1937) of Barsoum and Gaddum's (1935) method for the assay of histamine.

Urine was dialysed for 24 hr. in a cellophane sac against an equal volume of water and the fluids on both sides of the membrane tested. Urine was also dialysed against running water for 24 hr.

Extracts of blood and urine were also prepared with hot ethanol. Two volumes of boiling absolute ethanol were added to the sample, and boiling continued for 10 min. The liquid was centrifuged and filtered and the filtrate evaporated to dryness under reduced pressure at less than 50°. The residue was dissolved in a small amount of glacial acetic acid and precipitated by the addition of 9 vols. of anaesthetic ether. The precipitate was washed with ether, dried and dissolved in de Jalon solution.

A crude separation of peptides was made by the method of Gaddum and Horton (1959). 25 ml. of urine of mice infected with *Babesia* was passed through a column (10×1 cm.) of amberlite IRC 50 resin adjusted to *p*H 6.0. The liquid which passed through the column was reserved. The column was washed with phosphate buffer at *p*H 6.0 and then adjusted to *p*H 9.0 by the addition of the calculated amount of N NaOH. Elution was then carried out with M phosphate buffer at *p*H 9.0 and serial fractions of 2.5 ml. of eluate were collected. Part of each fraction was used for test and part was evaporated to dryness, extracted with hot ethanol, and the extract evaporated and dissolved in de Jalon solution for test. All samples and extracts were adjusted to *p*H 7.2 before test.

Isolated Organs

Guinea-pig ileum was suspended in a bath of Tyrode solution at 37°. Rat duodenum was prepared by the method of Horton (1959) and suspended in a 3 ml. bath of de Jalon solution at 31°. Atropine (10^{-6}) was used for both preparations to depress spontaneous movements. Atropine, mepyramine, triprolidine, eserine, iproniazid, bretylium and lysergic acid diethylamide were tested as antagonists, usually at a concentration of 10^{-4} in the bath.

Enzymes

Urine specimens and hot ethanol extracts were incubated for 1 to 24 hr. at 37° with proteolytic enzymes and the effect on the response of the rat duodenum tested. Controls were set up containing all reagents except the enzyme and containing normal mouse urine instead of infected mouse urine.

Chymotrypsin.—0.5 mg. was added to 0.25 ml. of sample in 0.1 M $NaHCO_3$ buffer.

Trypsin.—10 mg. was added to 0.25 ml. of sample in 0.1 M $NaHCO_3$ buffer.

Pepsin.—5 mg. was added to 0.5 ml. of sample in N/10 HCl.

Papain.—10 mg. was added to 0.5 ml. of sample in buffer at *p*H 6.0 containing 2 mg. of cysteine.

Samples of urine were also heated with N/10 NaOH in the boiling water bath for 10 min. and neutralized. Their activities were compared with unheated samples containing equivalent amounts of salt.

Haemoglobin was determined in urine by the acid haematin method.

Potassium was determined in blood and urine in the flame photometer.

Results

It seemed possible that liberation of histamine might be responsible for shock in infected animals, and accordingly an attempt was made to determine the histamine content of urine, blood, skin, nose and feet of rats and mice during the course of infection with *B. rodhaini*. It was soon apparent that the urine and tissues of infected animals contained substances which interfered with the assay of histamine. An example is shown in Fig. 1; an extract of blood which contained no detectable histamine caused a slow contraction of the guinea-pig ileum which persisted on washing out. Sensitivity to subsequent standard doses of histamine was greatly increased.

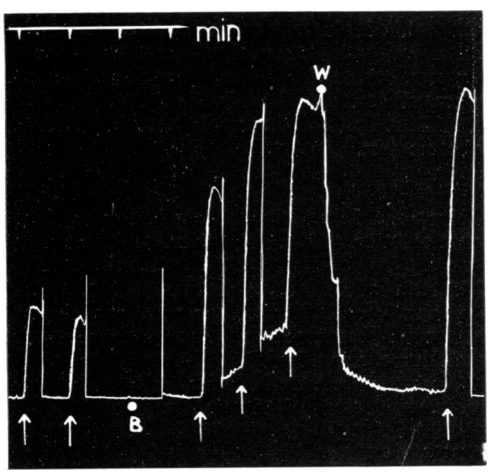

FIG. 1.—The effect of an extract of blood from mice infected with *Babesia* on the response of the guinea-pig ileum to histamine. Tyrode solution containing atropine 10^{-6}; 10 ml. bath. At arrows: 30 ng. histamine. At B: 1 ml. extract of *Babesia* blood (Code process). Wash periods: 30 sec., except at W, which was for 2.5 min.

Fig. 2 shows that hot ethanol extracts of infected blood caused larger contractions of guinea-pig ileum than an extract of normal blood.

Large amounts of active material were found in the urine of infected mice and rats. Fig. 3 shows the contractions of the guinea-pig ileum caused by a series of daily specimens of urine collected from mice during the 5 days of the infection. Fig. 4 shows that the appearance of

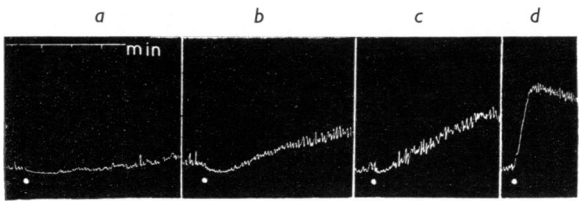

FIG. 2.—The effect of hot ethanol extracts of the blood of mice infected with *Babesia* on the isolated guinea-pig ileum. Tyrode solution containing atropine 10^{-6}; 10 ml. bath. 1 ml. ethanol extracts of mouse blood at (*a*) normal blood; (*b*) blood containing 1% of parasitized erythrocytes; (*c*) blood containing 17% of parasitized erythrocytes. At (*d*), 500 ng. histamine.

active substances in the urine preceded the rise of parasitaemia and haemoglobinuria. This occurred both in mice which died 5 days after inoculation and in rats which recovered from the infection spontaneously.

In many experiments with guinea-pig ileum, a slight relaxation or inhibition of spontaneous movements preceded the contraction caused by *Babesia* urine. The initial relaxation was much more marked when the urine was tested on the isolated rat duodenum (Horton, 1959). Relaxation was usually slight at the beginning of the infection and increased from day to day; on the day before death the relaxation was much greater than the subsequent contraction (Fig. 5).

The responses of different specimens of rat duodenum were variable. The typical reaction to *Babesia* urine was a relaxation followed by a contraction, but with some preparations relaxation predominated and, with others, contraction. The response depended partly on the load on the lever; lightly loaded preparations were more

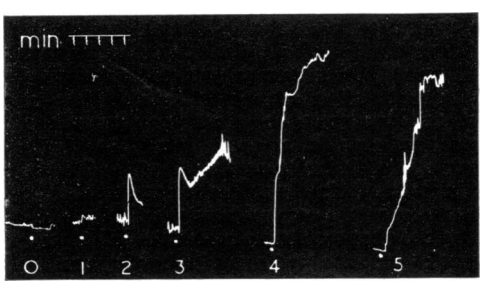

FIG. 3.—The effect of urine from mice infected with *Babesia* on the isolated guinea-pig ileum. Tyrode solution containing atropine 10^{-6}; 3 ml. bath. At 0, 0.1 ml. urine collected before infection; at 1, 2, 3, 4 and 5, 0.1 ml. urine collected on 1st, 2nd, 3rd, 4th and 5th days of the infection. The mice died after the 5th day.

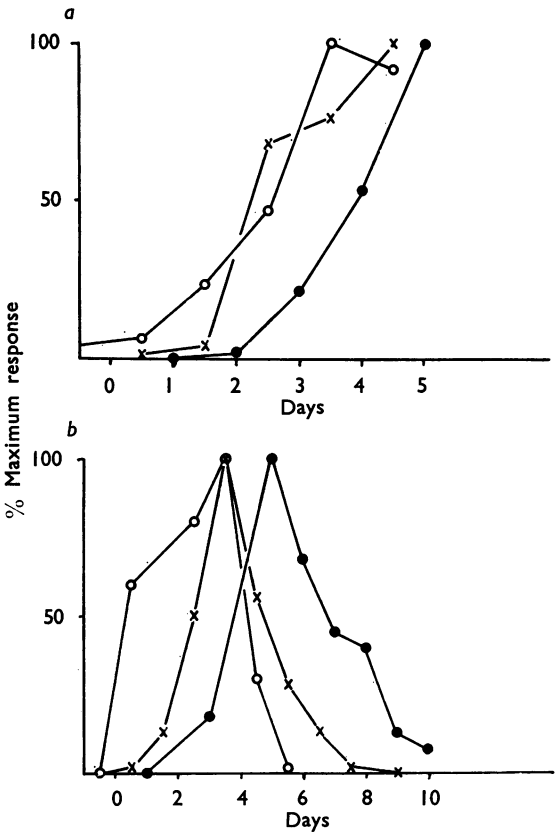

FIG. 4.—The progress of *Babesia* infections in mice (*a*) and rats (*b*) as measured by parasitaemia (●——●), haemoglobinuria (X——X) and the activity of the urine on the isolated guinea-pig ileum (O——O). Measurements are plotted as percentages of the maximum. The maximum percentage of parasitized erythrocytes in mice was 94% (5th day) and in rats 42% (6th day). The maximum degree of haemoglobinuria in mice was 21.2 mg./ml. and in rats 7.5 mg./ml. The effect on the ileum was measured as the height of the contraction produced in experiments similar to that illustrated in Fig. 3.

likely to relax well and more heavily loaded preparations were more likely to contract. A load of 0.5 g. as recommended by Horton (1959) was found to be the optimum for showing both responses.

Normal mouse or rat urine sometimes gave a small contraction and sometimes a small relaxation; the effect was never as large as that of *Babesia* urine. Urine collected from mice inoculated intraperitoneally with normal mouse blood showed no increase in active substances.

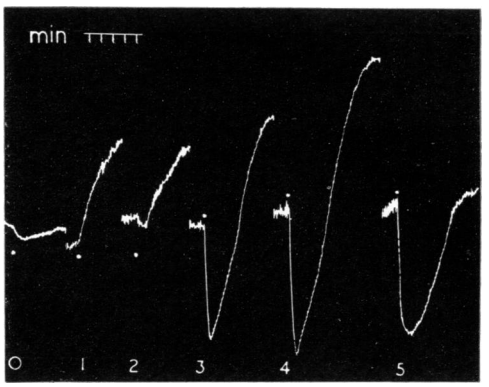

FIG. 5.—The effect of urine from mice infected with *Babesia* on the isolated rat duodenum. De Jalon solution with atropine 10^{-6}; 3 ml. bath. The samples of urine were the same as those in Fig. 3; the dose was 0.05 ml.

Bradykinin always gave a relaxation which seldom recovered as far as the original base-line until the bath was washed out.

The variable effect of *Babesia* mouse urine on the rat duodenum and the day-to-day qualitative changes which took place as the infection progressed suggested that there was probably more than one active substance in the urine. When a specimen of mouse urine which caused contraction of the rat duodenum was passed through an Amberlite IRC 50 column at *p*H 6.0 the filtrate gave a pure relaxation. When the column was eluted at *p*H 9.0 the first fraction collected caused relaxation; this fraction would be expected to contain kinins (Gaddum and Horton, 1959). The second fraction gave a contraction followed by a relaxation and subsequent fractions gave pure contractions (Fig. 6). The maximal response was given by the fifth sample collected. Hot ethanol extracts of the fractions gave responses of the rat duodenum almost identical with those of the fractions from which they were prepared. The activity of the fractions was unchanged by passage through alumina columns; the active substances passed readily through a cellophane membrane.

When urine from infected mice, or the material extracted from it by hot ethanol, was incubated with trypsin or pepsin, the activity was not destroyed in 24 hr. Papain destroyed all activity in 1 hr. Incubation with chymotrypsin for 1 hr. increased activity; incubation for 24 hr. destroyed almost all activity (Fig. 7). Boiling with alkali reduced, but did not abolish, activity. Active substances were present in urine collected

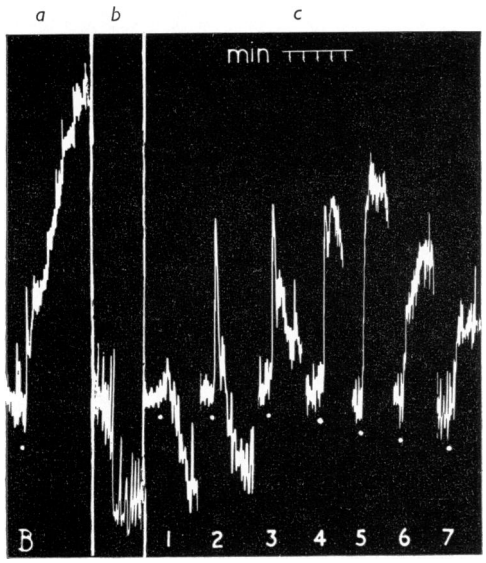

FIG. 6.—The separation of active fractions from the urine of mice infected with *Babesia*. Rat duodenum in de Jalon solution with atropine 10^{-6}; 3 ml. bath. (*a*): at B, untreated *Babesia* urine; (*b*): fluid which passed through Amberlite IRC 50 column at *p*H 6.0; (*c*): at 1–7, successive fractions collected from the column washed with buffer at *p*H 9.0. All doses 0.03 ml.

directly from the mouse by pressure on the abdomen and were stable in the refrigerator at −15°. Specimens stored in a refrigerator at 4° showed partial conversion of relaxing to contracting activity.

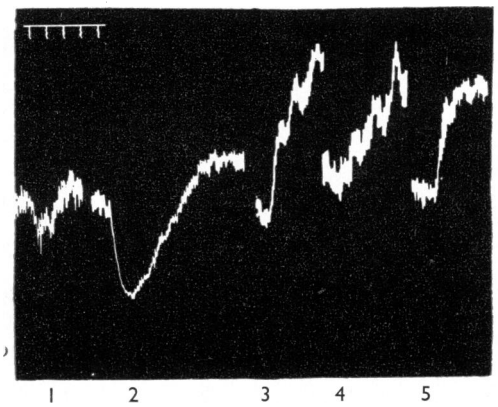

FIG. 8.—Active substances in the urine of mice in which haemoglobin has been released intravascularly. Isolated rat duodenum in de Jalon solution with atropine 10^{-6}; 3 ml. bath. Doses 0.02 ml. Time in min. 1, normal mouse urine; 2, *Babesia* mouse urine; 3, 4 and 5, urine from mice injected intravenously with lysed mouse blood, saponin, and rabbit erythrocytes respectively.

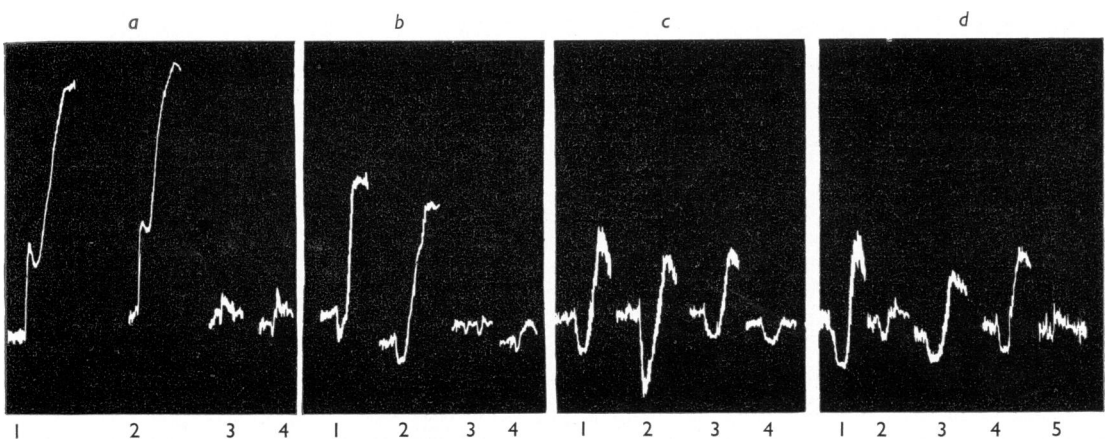

FIG. 7.—Effect of enzymes on urine of mice infected with *Babesia*, assayed on isolated rat duodenum. De Jalon solution with atropine 10^{-6}; 3 ml. bath. All doses 0.03 ml. (*a*) Pepsin: 1, *Babesia* urine incubated 24 hr. alone; 2, *Babesia* urine incubated 24 hr. with pepsin; 3, normal urine incubated 24 hr. alone; 4, normal urine incubated 24 hr. with pepsin. (*b*) Trypsin: 1, *Babesia* urine incubated 24 hr. with trypsin; 2, *Babesia* urine incubated 24 hr. alone; 3, normal urine incubated 24 hr. alone; 4, normal urine incubated 24 hr. with trypsin. (*c*) Chymotrypsin: 1, *Babesia* urine incubated 1 hr. alone; 2, *Babesia* urine incubated 1 hr. with chymotrypsin; 3, *Babesia* urine incubated 24 hr. alone; 4, *Bab sia* urine incubated 24 hr. with chymotrypsin. (*d*) Papain: 1, *Babesia* urine, fresh; 2, *Babesia* urine, incubated 1 hr. with papain; 3, *Babesia* urine, incubated 1 hr. alone; 4, *Babesia* urine, incubated 24 hr. alone; 5, *Babesia* urine, incubated 24 hr. with papain.

Experiments in which haemoglobin, lysed washed mouse erythrocytes, rabbit erythrocytes or haemolytic drugs were injected intravenously into mice showed that in all instances pharmacologically active substances were excreted in the urine. The quantities were rather less than would have been expected in *Babesia* urine showing the same degree of haemoglobinuria, and the relaxing component was apparently absent (Fig. 8). Haemoglobin, in larger amounts than those present in *Babesia* urine, when given alone or with normal mouse urine had no effect on the isolated guinea-pig ileum or rat duodenum. Urine mixed with fresh mouse blood had activity which in some instances was similar to that of *Babesia* urine.

Atropine, mepyramine, tripolidine, eserine, iproniazid, bretylium and lysergic acid diethylamide at a concentration of 10^{-4} in the bath had no effect on the relaxation or contraction of rat duodenum caused by *Babesia* urine or extracts of urine (Fig. 9). Higher concentrations of lysergic acid diethylamide had no effect on the relaxation but sometimes reduced the subsequent contraction of the rat duodenum.

Mice infected with *Plasmodium berghei, Trypanosoma rhodesiense, Streptococcus pyogenes* and Rift Valley fever virus all excreted substances in the urine which caused contraction or relaxation of the rat duodenum (Fig. 10). In acute fatal infections, the amount of relaxant material increased greatly towards the end.

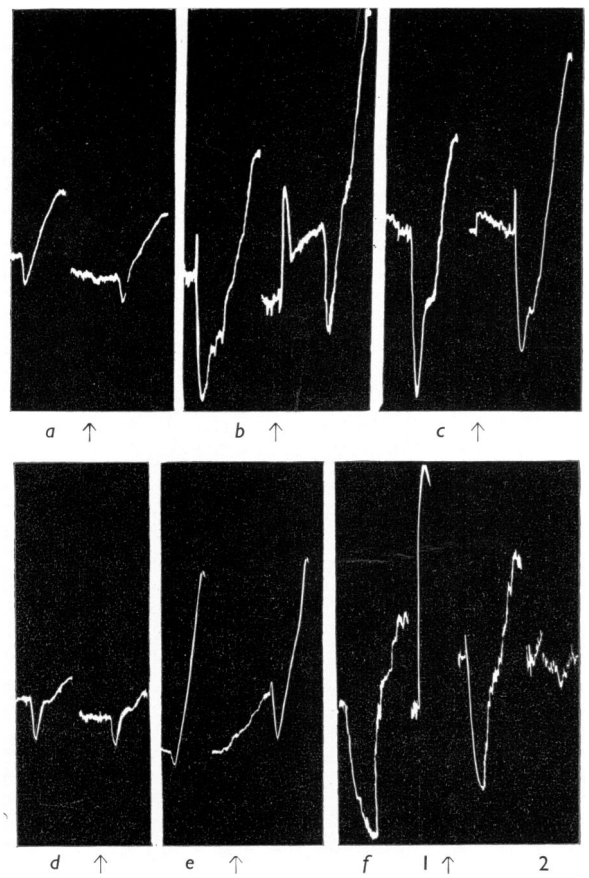

FIG. 9.—The lack of effect of various drugs on the response of the isolated rat duodenum to urine of mice infected with *Babesia*. De Jalon solution with atropine 10^{-6}; 3 ml. bath. The effect of a dose of 0.02 ml. of urine was recorded and the bath washed out. The drug was added (↑) to give a concentration of 10^{-4} in the bath and a second dose of 0.02 ml. urine then added without washing out. (*a*) Atropine, (*b*) mepyramine, (*c*) iproniazid, (*d*) eserine, (*e*) bretylium, (*f*) lysergic acid diethylamide; 5-hydroxytryptamine was given at 1 (0.005 ml. 10^{-4} solution) and at 2 (0.01 ml. 10^{-4} solution); the bath was washed out between the second dose of urine and the second dose of 5-hydroxytryptamine.

DISCUSSION

The cause of death of animals infected with protozoa is often obscure. Post-mortem examinations frequently show that the organs, although not normal, exhibit little evidence of severe impairment of function. *Babesia rodhaini* infections in mice are often heavy, and death may often be accounted for by the severity of the anaemia. However, the death of puppies infected with *B. canis,* and of mice treated with a non-toxic dose of an effective drug, cannot be explained in this way. The results of the present investigation show that the blood and urine of infected animals contain substances which are active on smooth muscle, some of which are probably peptides. They are stable to boiling with hydrochloric acid but not with alkali, they are extractable with hot ethanol, they pass readily through cellophane, they are destroyed by papain and chymotrypsin but not by trypsin or pepsin. Their action on smooth muscle is not affected by antihistamines, atropine, eserine, or by lysergic acid diethylamide in doses which abolish the action of 5-hydroxytryptamine. Identification of the active constituents will depend upon the separation of relatively pure fractions from the mixture of substances which has been shown to be present. Until this is done, quantitative parallel assays are of doubtful value.

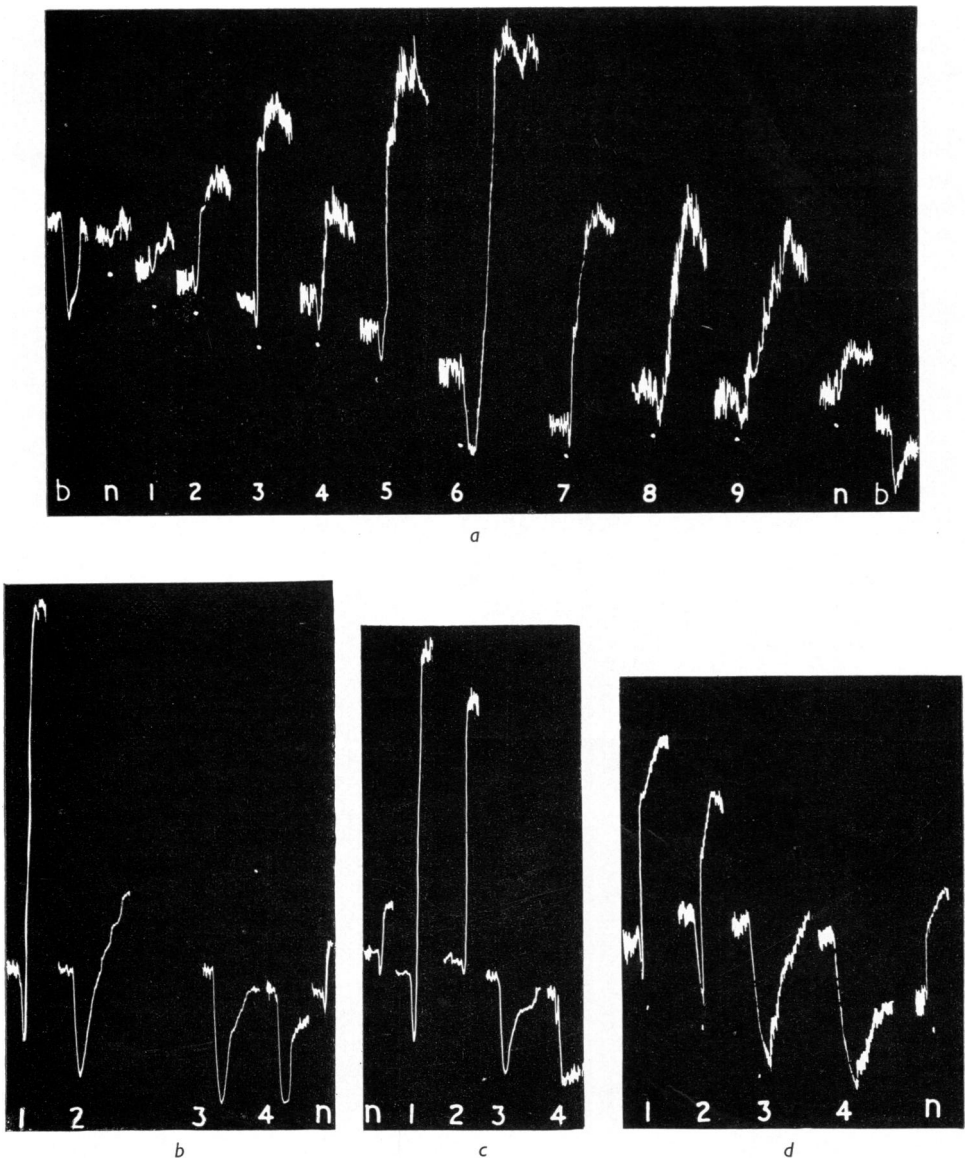

FIG. 10.—Active substances in the urine of mice infected with various organisms. Isolated rat duodenum in de Jalon solution with atropine 10^{-6}; 3 ml. bath; doses of urine 0.02 ml. b= bradykinin (10^{-6} in bath); n=normal mouse urine collected in metabolism cage; 1–9=urine samples collected daily until death. (*a*) *Plasmodium berghei*; (*b*) *Streptococcus pyogenes*; (*c*) Rift Valley fever; (*d*) *Trypanosoma rhodesiense*.

It is of interest to speculate upon the origin of the active substances which appear in the blood and urine of infected animals. *Babesia* and malaria parasites live within the red cells and destroy haemoglobin; they must possess powerful proteolytic enzymes. At schizogony, the erythrocyte bursts and the merozoites, together with their metabolic residues, are released into the plasma. It is possible that protein residues may be released from the broken erythro-

cytes and also that free enzymes derived from the parasite may attack plasma proteins and release peptides. In addition, the presence of broken cells and parasites in the plasma may start the chain of reactions which give rise to the release of kinins from plasma globulins. The experiments in which intravascular haemolysis was induced in mice by the injection of drugs or of lysed or incompatible red cells show that haemolysis is followed by the appearance of active substances in the urine.

Beraldo (1952) described substance U, a peptide in dog urine which was formed by the mixture of urine with traces of blood. He suggested that the release of substance U might explain some of the symptoms of pathological conditions in which blood and urine come into contact (Beraldo, 1955). The present experiments are clearly related to Beraldo's work and extend it into the field of the pathology of infectious diseases.

Pharmacologically active peptides occur not only in the urine of animals infected with haemolytic organisms, but also in trypanosome and virus infections. It is likely that the sensitization of guinea-pig ileum to histamine demonstrated in Fig. 1 is connected with the histamine sensitization which occurs in pertussis infections of mice (Parfentjev and Goodline, 1948). Matsui and Kuwajima (1959) have recently shown that the histamine sensitizing factor is "a toxin which has nothing to do with preventive antigens."

It seems possible that active peptides are liberated from the proteins of the host in all serious illnesses and that their pharmacological effects may be associated with the symptoms and signs of infectious disease.

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METABOLISM AND EXCRETION OF DI(*p*-AMINOPHENYL) SULPHOXIDE IN DIFFERENT ANIMAL SPECIES

BY

A. A. LEVI AND G. A. SNOW

From Imperial Chemical Industries Limited, Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire

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Rabbits, rats and guinea-pigs were treated with di(*p*-aminophenyl) sulphoxide and their urines examined by an analytical method which permits the simultaneous determination of this compound and of dapsone [di(*p*-aminophenyl) sulphone] which is a possible product of metabolic oxidation. The method gives for each drug the total of free compound plus acid-labile conjugates. All three species excreted unchanged drug together with dapsone. With rats and guinea-pigs about 33% of the excretion is dapsone, but with rabbits only 6 to 12%. The rate of combined excretion is much greater in rabbits than in the other two species. These results are discussed in relation to the significance of di(*p*-aminophenyl) sulphoxide as a drug in the treatment of leprosy.

Di(*p*-aminophenyl) sulphoxide has received favourable preliminary reports in clinical trials against leprosy (Buu-Hoï, Khuyen, and Xuong, 1955; Davey, Kissaun, and Moneta, 1957; Laviron, Lauret, Kerbastard, and Jardin, 1957), and there has been a suggestion that its action might be in some ways different from that of dapsone, di(*p*-aminophenyl) sulphone. Various workers have shown the sulphoxide to be almost without action *in vitro* against micro-organisms including pneumococci, haemolytic streptococci, and *Mycobacterium tuberculosis* H37Rv, in contrast to the considerable activity of dapsone in these species. However, the sulphoxide has definite activity *in vivo* against these same organisms in the mouse and the guinea-pig. These findings have led to the suggestion that the sulphoxide is activated by conversion to the sulphone in the animal body (Jensen and Schmith, 1943; Youmans, Feldman, and Doub, 1946; Wagner and Kimmig, 1946).

In order to evaluate the specific usefulness of the sulphoxide in leprosy it is desirable to know the extent of its conversion to dapsone in the human body. The metabolic fate of sulphoxides varies with different compounds and in different species. A number of instances are known where thioethers are oxidized to sulphoxides and are excreted in this form rather than as sulphones, for example, in the metabolism of phenothiazine (Whitten, Filmer, and Clare, 1947), chlorpromazine (Salzman and Brodie, 1956), and a pyrazolidinedione containing an alkylthio group (Burns, Yü, Ritterband, Perel, Gutman, and Brodie, 1957); the oxidation of di(*p*-aminophenyl) sulphide by a guinea-pig liver microsome preparation is said to go to the sulphoxide but no further (Gillette, Kamm, and Brodie, 1959). On the other hand, sulphides can be oxidized *in vivo* to the sulphone, for example, *p*-methylthioaniline (Rose and Spinks, 1948), and the catabolism of ethanethiol probably follows this route after methylation (Snow, 1957; Lowe, 1960). These oxidations probably involve formation of the sulphoxide as an intermediate step.

A method has been developed for the determination of di(*p*-aminophenyl) sulphoxide and dapsone in the presence of each other. It has been used to investigate metabolism in three animal species and is now available for investigation of metabolism in man.

Bushby and Woiwod (1956) showed that dapsone is largely excreted as the mono-*N*-glucuronoside. Our method therefore includes a preliminary acid treatment to hydrolyse such conjugates, and the results show the sum of free plus acid-labile compounds.

METHODS

Various methods of distinguishing between di(*p*-aminophenyl) sulphoxide and dapsone were considered. The sulphoxide gives a colour reaction with concentrated sulphuric acid (100° for 10 min.) whilst the sulphone does not. However, the intensity of the colour is far too low for convenience ($\varepsilon = 670$ at 520 mμ). A method has now been

developed for separating the sulphoxide from the sulphone by differential extraction from an organic solvent with acids of differing strength; this enables a diazotization method similar to that of Francis and Spinks (1950) to be applied to both components.

Procedure.—Urine samples (5 ml.) are acidified with N HCl (2.5 ml.); they are left for 1 hr. at 20 to 25° to allow for hydrolysis of acid-labile conjugates. After neutralization with N NaOH (2.5 ml.) the samples are diluted to contain 3 to 40 μg./ml. of either sulphoxide or sulphone. For estimation 1 ml. is mixed with 3 ml. 0.2 M phosphate buffer *p*H 7.0. Blood samples (3 ml.) are mixed with 0.8 M phosphate buffer *p*H 7.0 (1 ml.).

For the first extraction, 4 ml. of buffered urine or blood, prepared as above, is shaken with isobutyl methyl ketone (20 ml.). Both sulphoxide and sulphone pass quantitatively into the organic phase.

For the second extraction, a 15 ml. aliquot of the separated ketone layer is extracted with 0.1 N HCl (4 ml.); 88.9% of the sulphoxide and 2.3% of the sulphone pass from the ketone solution into the acid layer. Of the acid layer 2 ml. is mixed with 0.1% $NaNO_2$ solution (1 ml.), and 5 min. later 1% sulphatoethyl-*m*-toluidine solution (0.5 ml.) is added. After 20 min., ethanol (3 ml.) is added and the optical density is read at 520 mμ. A standard curve, which should be rectilinear, is prepared from readings on solutions containing 2 to 20 μg. di(*p*-aminophenyl) sulphoxide in 2 ml. 0.1 N HCl diazotized and coupled as above. The apparent sulphoxide content of the aliquot of 0.1 N HCl from the second extraction is read from the standard curve. Let this value be x μg.

For the third extraction, a 10 ml. aliquot of the ketone layer remaining after the extraction with 0.1 N HCl is further extracted with 2N HCl (4 ml.). The small residue of sulphoxide is quantitatively extracted into the stronger acid, together with 93.8% of the remaining sulphone. Of the acid layer, 3 ml. is taken and mixed with 0.1% $NaNO_2$ solution (0.5 ml.). After 5 min. 5% ammonium sulphamate solution (1 ml.) is added, followed 5 min. later by 0.2% *N*-1-naphthylethylenediamine solution (0.5 ml.). After a further 30 min. the solution is diluted to 6 ml. and the optical density read at 535 mμ. A standard curve (rectilinear) is constructed from readings on solutions containing 2 to 20 μg. of dapsone in 2N HCl (2 ml.) diazotized and coupled as above. The apparent dapsone content of the aliquot of 2N HCl from the third extraction is read from the standard curve. Let this value be y μg.

The amounts of sulphoxide and sulphone in the buffered solution taken for the first extraction are given by the equations:

di(*p*-aminophenyl) sulphoxide content (μg.) ... ... $=3.01x-0.075y$

dapsone content (μg.) ... ... $=2.92y-0.36x$

(The derivation of these equations is shown below.) From these figures the concentrations present in the original blood or urine may be calculated, taking into account the dilutions involved in making the buffered solution for extraction.

General Precautions.—The isobutyl methyl ketone is presaturated with phosphate buffer, and the 0.1 N and 2N HCl solutions with isobutyl methyl ketone, in order to avoid volume changes during the extraction. All extractions are carried out by shaking the solutions for 1 min. in stoppered tubes and centrifuging the mixtures to separate the layers cleanly. Nitrite and *N*-1-naphthylethylenediamine solutions are freshly prepared each day. Stock solutions of the pure drugs contained 1 mg./ml. in dilute acid; the sulphone may be kept several weeks, but the sulphoxide only for three days. Diluted solutions (10 μg./ml.) are prepared as required. Colours formed after coupling fade in strong daylight, and the tubes are kept in the dark except during the actual measurement. Solutions are thoroughly mixed after each addition of reagent. This is especially important after ethanol additions, where faulty mixing can easily occur.

Analysis of the Method.—Coupling of diazotized dapsone with sulphatoethyl-*m*-toluidine does not occur satisfactorily in 2N HCl, and for the third extraction a coupling component which requires this strength of acid was substituted. Di(*p*-aminophenyl) sulphoxide and dapsone at equal concentrations diazotize and couple with sulphatoethyl-*m*-toluidine to produce colours of almost equal intensity at 520 mμ. The same is true of the colours produced with *N*-1-naphthylethylenediamine measured at 535 mμ. Thus no appreciable error arises when measurements are made on mixtures containing both amino compounds.

The formula for calculating the results was derived from a series of experiments in which standard solutions of di(*p*-aminophenyl) sulphoxide and dapsone were extracted under conditions identical to those used in the procedure described above. Extraction into 0.1 N and 2N HCl was determined directly using standard solutions of the sulphoxide and sulphone in isobutyl methyl ketone (saturated with buffer). Extraction into isobutyl methyl ketone was determined indirectly: standard solutions of sulphoxide and sulphone in phosphate buffer *p*H 7.0 were extracted into isobutyl methyl ketone and an aliquot of the ketone layer was extracted with HCl (0.1 N for the sulphoxide, 2N for the sulphone). The efficiency of the first extraction step was calculated from knowledge of the efficiency of the overall extraction and that of the extractions with acid. Each extraction was carried out several times. Mean results are given in Table I.

Using these figures, equations may be derived as follows:

Let the prepared sample of blood or urine (4 ml.) contain A μg. of sulphoxide and B μg. of sulphone. This sample is quantitatively extracted into isobutyl methyl ketone (20 ml.). A 15 ml. aliquot thus contains 0.75 (A+B): it is extracted with 0.1 N HCl (4 ml.), and the measurement (x) is made on a 2 ml.

aliquot of the acid. From the determined extraction efficiencies

$$x = 0.5 \times 0.75\ (0.889A + 0.023B)$$
$$\therefore\ x = 0.334A + 0.0086B \quad \ldots\ldots(1)$$

After this extraction a 10 ml. aliquot of the ketone layer contains 0.5 [$(-0.889)\ A + (1 - 0.023)\ B$]; it is extracted with 2N HCl (4 ml.), and the measurement (y) is made on a 3 ml. aliquot of the acid. From the determined extraction efficiencies

$$y = 0.75 \times 0.5\ (0.111A + 0.938 \times 0.977B)$$
$$\therefore\ y = 0.0415A + 0.344B \quad \ldots\ldots(2)$$

Solving the simultaneous equations (1) and (2) gives the expressions for A and B quoted in the section on procedure.

The complete method was tested by determination of known mixtures of the two components in water and by determinations on blood and urine to which known amounts had been added. The results (Table II) show that some sets of figures have a small bias, but without any consistent trend. The standard deviation was quite similar for each set and could be taken as ±5% for the method as a whole. This is satisfactory for practical requirements.

Animal Experiments.—The animals were fed on normal laboratory diets and food was withdrawn 18 hr. before the administration of the drug. The compound was dosed in aqueous suspension by means of a stomach tube. The urine from the animals in metabolism cages was collected in a container chilled in ice, acidified, and extracted as soon as practicable after excretion. For rats and guinea-pigs the numbers of animals were sufficient to ensure a representative excretion of urine over the periods involved. With rabbits excretion was promoted by giving 15 ml. water at the time of treatment, but even so it was inconveniently erratic. This was partly overcome by repeating the dosing and collection from each individual rabbit after a rest period of three days. The urines from the two collections were combined as far as possible, as shown in Table IV. Where there was an excretion in only one of the pair of experiments over a given period figures are shown in brackets. These figures are based on the assump-

TABLE I

EXTRACTION OF DI(*p*-AMINOPHENYL) SULPHOXIDE AND DAPSONE

	1st Extraction. From *p*H 7·0 Buffer into Isobutyl Methyl Ketone		2nd Extraction. From Isobutyl Methyl Ketone into 0·1 N HCl		3rd Extraction. From Isobutyl Methyl Ketone into 2N HCl	
	Sulphoxide	Sulphone	Sulphoxide	Sulphone	Sulphoxide	Sulphone
Phosphate buffer *p*H 7·0 (ml.)	4	4	—	—	—	—
Isobutyl methyl ketone (ml.)	20	20	15	15	10	10
Hydrochloric acid	—	—	4 ml. 0·1 N	4 ml. 0·1 N	4 ml. 2 N	4 ml. 2 N
Wt. of compound taken (μg.)	10–20	10–20	10–20	125–750	10–20	10–20
No. of determinations ..	9	9	9	12	9	9
Mean % extraction	101·6	99·9	88·9	2·31	101·8	93·8
Standard error on mean ..	0·6	0·8	0·6	0·01	1·6	0·5

TABLE II

RESULTS OF ESTIMATIONS ON SYNTHETIC MIXTURES OF DI(*p*-AMINOPHENYL) SULPHOXIDE AND DAPSONE

	In Water		In Urine		In Blood	
Number of determinations ..	12		10		6	
Component	Sulphoxide	Sulphone	Sulphoxide	Sulphone	Sulphoxide	Sulphone
Range of amounts of components in mixtures (μg.) ..	5·2–48	7·4–37·2	1·6–21·6	15·4–46·2	10·5–101·5	20·8–131
Mean % error on result ..	−2·4	2·7	3·4	−3·3	−5·5	−0·6
Standard deviation of % error	5·0	4·4	3·9	6·2	2·1	4·5

TABLE III

URINARY EXCRETION FROM GUINEA-PIGS AND RATS DOSED WITH DI(*p*-AMINOPHENYL) SULPHOXIDE

Guinea-pigs (wt. 250 g.) each received 2·5 mg. di(*p*-aminophenyl) sulphoxide orally. 'Rats (wt. 300 g.) each received 3 mg. of the drug orally. Figures for both species refer to 2 separate groups of 6 animals. Cumulative % figures for dapsone are based on the sulphoxide equivalent.

Species	Time after Dose hr.	Sulphoxide Excretion				Dapsone Excretion			
		mg.		Cumulative % of Dose		mg.		Cumulative % of Dose	
Guinea-pig	0–3	0·38	0·59	2·5	4·0	0·19	0·21	1·0	1·5
	3–6	1·07	0·58	9·5	8·0	0·68	0·27	5·5	3·0
	6–12	0·40	0·46	12·5	11·0	0·26	0·30	7·0	5·0
	12–24	0·35	0·26	14·5	12·5	0·18	0·10	8·0	5·5
Rat	0–3	1·72	1·61	9·5	9·0	0·61	0·68	3·0	3·5
	3–6	2·12	1·36	21·5	16·5	1·11	0·74	9·0	7·5
	6–12	1·39	1·06	29·0	22·5	0·88	0·64	13·5	10·5
	12–24	0·59	0·51	32·5	25·0	0·37	0·34	15·5	12·5

TABLE IV

URINARY EXCRETION FROM RABBITS DOSED WITH DI(*p*-AMINOPHENYL) SULPHOXIDE

Each animal received 10 mg. per kg. of di(*p*-aminophenyl) sulphoxide orally on two separate occasions. Cumulative % figures for dapsone are based on the sulphoxide equivalent. Values in parentheses refer to a single experiment only.

Collection Period (hr. after Dose)	Expt. No.	Sulphoxide Excretion		Dapsone Excretion	
		mg.	Cumulative % of Dose Excreted	mg.	Cumulative % of Dose Excreted
Rabbit 1. *Dose:* 30 *mg. in each experiment*					
0–2	1+2	20·9	35	0·0	0
2–5	2	3·68	(48)	0·33	(1·0)
2–12	1	2·62	47	0·32	2·0
5–12	2	1·03		0·21	
12–24	1+2	1·15	49	0·28	3·1
Rabbit 2. *Dose:* 20 *mg. in each experiment*					
0–2	3+4	16·1	40	1·69	4·2
2–6	3+4	2·99	48	0·50	5·5
6–12	3	0·49	(50)	0·15	(6·2)
6–24	4	1·77	51·5	0·46	7·3
12–24	3	0·26		0·14	

tion that the rate of excretion was identical in the two experiments with the same animal. Results from two rabbits are presented in Table IV as typical.

RESULTS

The analytical method has been used to investigate the oxidation of di(*p*-aminophenyl) sulphoxide to dapsone in guinea-pigs, rats, and rabbits. The dose of drug (10 mg./kg.) was comparable, on the basis of the surface area of the body, with therapeutic doses used in man. At this dose the blood levels were very low, and only urinary excretion has been studied in detail. Results are given in Tables III and IV.

The total excretion of sulphoxide and sulphone is much less with the guinea-pig than with the rat, but the pattern of excretion is similar in these species. In both, the excretion at 3 hr. is about one third, and at 6 hr. two thirds, of the total 24 hr. excretion. There is considerable oxidation of the compound in the body and, of the detectable excretion products, about one third is dapsone and two thirds sulphoxide. The excretion in rabbits is quite different. Elimination is much more rapid; about 75% of the 24 hr. excretion occurs in the first 2 hr. Also the conversion to dapsone is much less. The fraction of diazotizable material excreted in the sulphone form was 6, 12, 10, and 6% in four different experiments.

DISCUSSION

There are clearly differences in the metabolism of di(*p*-aminophenyl) sulphoxide in different species, though some conversion to dapsone may perhaps be expected in all cases. The smaller extent of oxidation in the rabbit is probably the result of the more rapid excretion of the sulphoxide.

Since this work was carried out a paper has appeared (Jardin, 1958) reporting the chromatographic investigation of urine from patients receiving di(*p*-aminophenyl) sulphoxide. The results show qualitatively that the unchanged drug is probably the principal excretion product; dapsone is found in somewhat smaller amounts along with some relatively minor metabolites. Jardin expresses his opinion that the amount of dapsone excreted is not sufficient to account for the antileprotic activity of the sulphoxide. This point, however, can only be convincingly proved when quantitative information on the blood levels and urinary excretion of the two compounds is obtained. The method described in this paper should enable this to be done.

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DEPLETION OF PITUITARY CORTICOTROPHIN BY RESERPINE AND BY A NITROGEN MUSTARD

BY

M. SAFFRAN* AND MARTHE VOGT

From the Department of Pharmacology, University of Edinburgh

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A single intraperitoneal injection of reserpine (2.5 mg./kg.) into rats produced a fall in the corticotrophin concentration of the pituitary to 30% of the resting value; recovery was not far from complete at 40 hr. A single injection of a nitrogen mustard caused an even greater loss of pituitary corticotrophin; 24 hr. after the injection the concentration was 10% of the resting value. There is no reason to assume that the effect of reserpine is due to an interference with storage of ACTH in the tissue and is not simply due to the fact that the drug acts as a stressing agent. The shape of the curve representing the fall in pituitary ACTH during the early phases of a sudden stress may be very similar to that of the fall in adrenal ascorbic acid produced by the released ACTH. This suggests that, under these circumstances, resynthesis is slow and the diminishing stores of ACTH in the pituitary reflect mainly the release of the hormone.

It is well known that reserpine depletes tissues of their stores of amines, such as the catecholamines and 5-hydroxytryptamine. Does reserpine also deplete stores of peptidic substances, such as the pituitary hormones ? To attempt to answer this question, the content of corticotrophin in the pituitary of the rat was estimated at intervals after the administration of a large dose of reserpine. The effects were compared with those of the severe stress of an injection of a nitrogen mustard. In all animals the adrenal ascorbic acid was also measured.

METHODS

Drug Administration

Young adult female rats, weighing about 200 g. and obtained from a single colony, were used throughout. They were fed on cubes and water and kept at 23°. Rats injected with the same solution were kept together in a cage. Injections were made without previous "training." At different intervals after the injection pairs of rats were removed from each cage, rapidly decapitated, and estimations of pituitary ACTH and adrenal ascorbic acid carried out. The following injections were given:

Reserpine.—A solution containing 2.5 mg. of reserpine per ml. ("Serpasil" ampoules, Ciba Ltd.) was injected intraperitoneally in a dose of 1 ml. per kg.

Vehicle.—The vehicle for the reserpine, supplied by Ciba Ltd., was injected intraperitoneally in volumes of 1 ml./kg.

Saline.—A 0.9% solution of sodium chloride was injected intraperitoneally in volumes of 1 ml./kg.

Nitrogen Mustard.—10 mg. of di(2-chloroethyl) methylamine hydrochloride ("Mustine," Boots) was taken up in 6 ml. of water. 1 ml. (=1.7 mg.) was injected subcutaneously into each rat.

Assay of Corticotrophin

Rats.—Young adult male rats, ranging in weight from 180 to 200 g., were used in the bioassay of corticotrophin. They were obtained from 3 different sources: the animals from one source showed much greater variability of response than the others; the best index of precision (λ) was obtained with rats from Messrs. A. J. Tuck, Rayleigh, Essex.

Extraction of Pituitary Gland.—The glands of the injected animals were carefully dissected from the base of the skull. Usually the posterior lobe separated easily. The anterior lobe was quickly weighed on a torsion balance and was placed into a thick-walled test tube (7 mm. inside diameter × 75 mm.), containing 0.5 ml. glacial acetic acid and a few grains of finely ground washed sand. The tissue was crushed and ground thoroughly with a glass rod, enlarged at the end to fit the bottom of the tube. The rod was drained on the side of the test tube, the tube was covered with a square of parafilm and was heated in a sand bath at 70 to 80° for 30 min. The tubes were centrifuged for 5 min. and aliquots of the supernatan.

*Permanent address: Allan Memorial Institute of Psychiatry, McGill University, Montreal 2, Canada.

fluid dried overnight in a vacuum desiccator over NaOH. The residues were stored in the freezer (−17°). In one experiment, posterior lobes were treated in a similar way, but the tissue of three rats was pooled.

Bioassay.—The *in vitro* bioassay procedure of Saffran and Schally (1955) was used with some modifications. Four, instead of 8, rats were used in each assay, permitting one person to carry out 2 assays per day unaided, with some loss in precision (average λ close to 0.2). Ethylene chloride was used instead of methylene chloride for the extraction of the steroids. This substitution was made necessary by the lack of a cooling system on the lamp housing of the Unicam spectrophotometer. Ethylene chloride is not transparent to ultra-violet light at wavelengths much below 240 mμ, so that it was not possible to examine the shape of the absorption curve of the extract by taking a reading at 230 mμ. Incubation of the adrenal tissue was carried out in 5 ml. vials in a shaking incubator instead of in a conventional Warburg apparatus. The vials were gassed by blowing out the air with a strong stream of 95% oxygen containing 5% carbon dioxide, and quickly stoppering with a rubber stopper while the stream of gas played on the mouth of the vial.

The residues of 0.1 to 0.25 ml. of the original pituitary extracts were taken up in 0.5 ml. of 0.25% aqueous acetic acid, and 0.1 ml. portions of these solutions were used as the U_2 doses in the 4-point bioassay, while 0.1 ml. of 1 in 4 dilutions in 0.25% acetic acid were used as U_1. A solution of a commercial preparation of corticotrophin ("Cortrophin," Organon) in 0.25% acetic acid was used as a laboratory standard, with S_2 = 20 mU. and S_1 = 5 mU. in volumes of 0.1 ml. added per incubation vial.

Determination of Adrenal Ascorbic Acid

Extraction of the Tissue.—While the pituitary gland was being removed from the head, the adrenals were excised from the body, freed of adhering fat, weighed in pairs on a torsion balance, and ground in a small test tube (10 × 75 mm.) in 0.3 ml. of 4% trichloroacetic acid with finely ground sand and a glass rod enlarged at one end. The rod was washed down with the trichloroacetic acid solution to a total volume of 1.2 ml. The tubes were then covered with parafilm and were stored in the freezer.

Measurement of Ascorbic Acid.—A modification of the method of Roe and Kuether (1943) was used. The adrenal extracts were thawed and about 50 mg. of acid-washed charcoal was added to each tube. The tubes were again covered with the parafilm and were shaken vigorously for 15 sec. A pellet of cotton-wool, fitting the tube snugly, was slowly pushed down the tube with a glass rod. As the cotton-wool passed through the extract, the particles of charcoal and precipitated protein were trapped by the fibres, and a clear solution passed through to the top of the pellet. An aliquot (usually 0.03 or 0.04 ml.) of the clear solution was removed from each tube to a series of fresh similar tubes.

To these aliquots were added one drop of the thiourea reagent and 0.3 ml. of the dinitrophenylhydrazine reagent. The tubes were covered with parafilm and were placed in boiling water for exactly 15 min. (Guillemin, Fortier, and Lipscomb, 1959) and then cooled in ice water. To each tube was added 1 ml. of ice-cold 85% (v/v) sulphuric acid. The tubes were covered with parafilm and inverted to mix the contents. The colours were read in microcells in the Unicam spectrophotometer at 520 mμ. A calibration curve was contructed with ascorbic acid solutions in 4% trichloroacetic acid taken through the whole procedure.

RESULTS

Saline.—Four hours after the injection of 0.9% saline, the content of corticotrophin in the pituitary had fallen to 50% of the value at zero time, while at 8 hr. the amount had returned nearly to the starting level (Fig. 1a). At 40 hr. the values were again lower.

The ascorbic acid in the adrenals was not significantly lowered in this experiment.

Vehicle.—The administration of the reserpine vehicle produced signs of discomfort in the animals. The corticotrophin decreased to about 45% at 8 hr., then returned to about 60% at 18 hr. and remained there (Fig. 1b).

The adrenal ascorbic acid also fell to a minimum at 4 and 8 hr., with subsequent recovery. There is no doubt that the vehicle acted as a stress but that recovery was rapid.

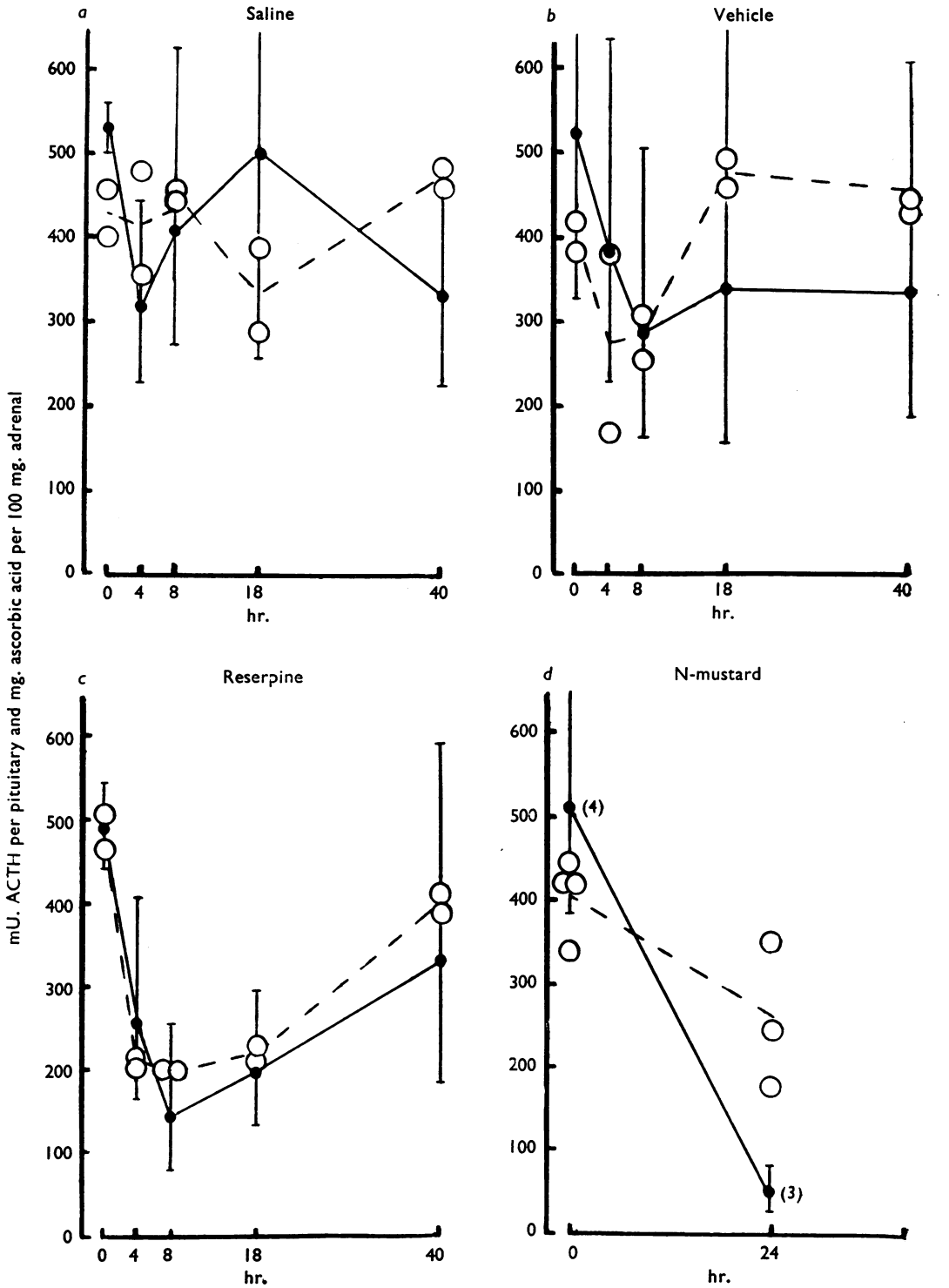
Reserpine.—After reserpine, the corticotrophin decreased to a minimum of 30% at 8 hr., thereafter slowly returning to about 75% at 40 hr. (Fig. 1c).

The adrenal ascorbic acid fell to a minimum of 40% at 4 hr. and 8 hr., then gradually returned to 80% at 40 hr., the time course of the changes closely resembling that of the changes in corticotrophin concentrations.

The losses in ACTH were of a degree not previously encountered after the injection of drugs, but of the magnitude reported (Fortier, 1959;

FIG. 1.—a, b and c: ●——● mU. ACTH per anterior lobe, each dot being the mean for a pair of rats, the vertical strokes representing the confidence limits of the estimation (P=0·95). – – – – mean adrenal ascorbic acid concentrations for the same pairs of rats. **O** individual ascorbic acid values.

FIG. 1.—d: ●——● mU. ACTH per anterior lobe, means and confidence limits (P=0·95) of 4 controls and 3 experimental animals. – – – – mean adrenal ascorbic acid concentrations for the same rats. **O** individual ascorbic acid values.


a
Saline
b
Vehicle
c
Reserpine
d
N-mustard
mU. ACTH per pituitary and mg. ascorbic acid per 100 mg. adrenal
0
100
200
300
400
500
600
0 4 8 18 40
hr.
0 24
(4)
(3)

Fortier and DeGroot, 1959) after adrenalectomy (fall to 31%) or after removal of one and enucleation of the other adrenal (fall to 23%). In order to help in the interpretation of the results, it was thought desirable to find out what response would follow a single injection of a severely toxic drug. Thus the next experiment was carried out with a nitrogen mustard.

Nitrogen Mustard. — At 8 hr. after the administration of the drug there were few signs of toxicity, and the five animals were therefore left for a total of 24 hr. By that time two of the rats had died, and the survivors exhibited oedema and weakness. The corticotrophin in the pituitary of the survivors had fallen to 10% (Fig. 1d), and the adrenal ascorbic acid was at 70% of the starting value. The values for ascorbic acid at zero time were lower in this group of rats than in the groups used for the other treatments, and therefore the fall to 70% (or 260 mg./100 g. adrenal) represented a concentration of ascorbic acid which approached the minimum figures observed in stress.

One experiment of longer duration was carried out, in which the action of reserpine was compared with that of an unspecific stressing agent—the reserpine vehicle—and in which the behaviour of the stores of ACTH in the posterior lobe of the pituitary was also examined. The posterior lobe contains an appreciable amount of ACTH which has been shown to be released in certain forms of stress (Mialhe-Voloss, 1956, 1958; Rochefort and Saffran, 1957). Reserpine (2.5 mg./kg.) was injected intraperitoneally on 4 consecutive days, and the ACTH estimated separately in anterior and posterior lobe 24 hr. after the last injection. Rats injected with the same volume of vehicle served as controls. There was no difference in the concentration of ACTH in pituitaries of rats injected with reserpine and injected with the vehicle, and this was true for the ACTH in the anterior lobe as well as for that in the posterior lobe. The absolute amount in both lobes was about one-half of the figures for unstressed rats. After prolonged treatment, therefore, there was also no sign of specific interference of reserpine with storage of ACTH.

Discussion

Reserpine is known to interfere with the transport and storing mechanism of catecholamines and 5-hydroxytryptamine, but not with that of histamine. The reason for this difference is unknown, as is the exact nature of the processes transporting and storing amines in certain cells against a concentration gradient. The present work was done with the purpose of finding out whether the storage of pituitary hormones, for example, ACTH, was interfered with by reserpine. The results showed a large drop of ACTH in pituitaries after a single injection of reserpine. The interpretation was complicated by the necessity of assessing the extent to which reserpine would lower ACTH concentration simply because it acts as a stressing agent. The conclusion reached was that the release of ACTH after reserpine could be explained entirely on the basis of its stressing action; one reason for this conclusion was the observation that a very toxic substance, a nitrogen mustard, caused an ever greater loss of ACTH; the second was the short duration of the effect: practically normal concentrations were found 40 hr. after a single injection, whereas reserpine-depleted stores of 5-hydroxytryptamine take many days to fill up again. Since formation of 5-hydroxytryptamine is a very fast process (Brodie, Spector, Kuntzman, and Shore, 1958), the delay can only be accounted for by a prolonged action of the reserpine; such action should affect all substances, the storage of which is interfered with, for the same length of time. Lastly, repeated injections of reserpine lowered the stores of ACTH to the same extent as repeated injections of the solvent, and this finding applied equally to the stores in the anterior as to those in the posterior lobe, where the ACTH might be held by a different chemical process. At neither site was there any indication that reserpine produced effects not also obtained by other noxious agents.

At least one other polypeptide is known, on which reserpine has no depleting action, namely substance P. Studies of its concentration in brain (Paasonen and Vogt, 1956) have shown that it remains unchanged after the injection of doses of reserpine which cause a severe fall in the content of 5-hydroxytryptamine.

The simultaneous estimation of the concentrations of adrenal ascorbic acid and pituitary corticotrophin shows that, in short-term experiments, the general trend of the changes is often similar. Thus, during periods of a sudden increase in demand on ACTH, release of hormone appears not to be balanced by resynthesis, so that the resulting fall in pituitary stores can be used as an index of increased secretion.

Reserpine ("Serpasil") and its vehicle were kindly supplied by Ciba Laboratories Ltd., and ACTH ("Cortrophin") by Organon Laboratories Ltd.

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THE EFFECTS OF INTRACISTERNAL SARIN AND PYRIDINE-2-ALDOXIME METHYL METHANESULPHONATE IN ANAESTHETIZED DOGS

BY

R. V. BROWN*

From the Chemical Defence Experimental Establishment, Porton Down, Wiltshire

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Dogs poisoned by the anticholinesterase sarin could be saved by intravenous administration of atropine sulphate together with a suitable oxime. The central effects of intracisternal sarin were respiratory paralysis and vasomotor stimulation. The problem arose as to whether the oxime, being a quaternary nitrogen compound, could enter the brain from the blood, and could have a central action on the paralysed respiration. The methyl methanesulphonate of pyridine-2-aldoxime administered intracisternally, after sarin poisoning by the same route, was ineffective; atropine, given intravenously, was effective. The central and peripheral effects of sarin were thus reversed by the atropine-oxime therapy, the central effects by atropine, the peripheral by the oxime.

Dogs given many times the LD50 of the potent anticholinesterase sarin (isopropyl methyl-phosphonofluoridate) can be saved by the intravenous injection of atropine sulphate and the methiodide of pyridine-2-aldoxime. Atropine alone, while re-starting respiration, is incapable of maintaining it (Brown, Kunkel, Somers and Wills, 1957; Wills, Kunkel, Brown and Groblewski, 1957). The oxime alone is ineffective in re-establishing respiration. If the oxime is given 5 min. after the atropine, respiration improves within 0.5 min. which might be compatible with a central effect although quaternary nitrogen compounds penetrate the blood-brain barrier with difficulty (see Koelle and Steiner, 1956).

The experiments were undertaken to assess the central effects of sarin and of pyridine-2-aldoxime and the relative importance of central and peripheral paralysis of respiration in poisoning with sarin.

METHODS

The sarin was diluted with saline immediately before use and the volume injected was always less than 0.12 ml. Because of its greater solubility and equal efficacy (Davies and Willey, 1958), the methyl methanesulphonate of pyridine 2-aldoxime was used in the present work instead of the methiodide and was administered in aqueous solution containing 100 μg. or 1,000 μg./0.1 ml.; both freshly made and older solutions wer used, and were often tested by the copper acetate-benzidine acetate test for the presence of cyanide. The atropine sulphate solution contained 2 mg./ml., and, except as noted, was always given intravenously, 0.5 mg./kg.

The dogs were anaesthetized by pentobarbitone sodium given intravenously, enough being administered to abolish the palpebral reflex, thus giving deep surgical anaesthesia. Additional small doses were given as needed. Blood pressure was recorded on a smoked paper by a mercury manometer from a femoral or common carotid artery. The trachea was cannulated. Respirations were recorded by a Marey tambour connected with the tracheal cannula. Artificial respiration (not recorded) was given by a Starling Ideal pump. An external saphenous vein was used for intravenous injections.

The dog was placed on its belly and the neck sharply flexed. A midline incision was made with a diathermy knife from the occiput to the second spinous process. When the atlanto-occipital membrane was felt, a long needle (22 gauge) was thrust through the membrane at an acute angle. The blood pressure was observed so that any inadvertent damage to the medulla could be detected and such preparations were discarded. The penetration into the cistern was confirmed by the appearance of cerebrospinal fluid. The needle was clamped in place with towel clips and used for all intracisternal injections.

RESULTS

Effects of Pyridine-2-aldoxime Alone

Nine dogs were given pyridine-2-aldoxime, 100 or 1,000 μg. intracisternally. Only three animals

*Present address: Chemical Warfare Laboratories, Army Chemical Center, Maryland, U.S.A.

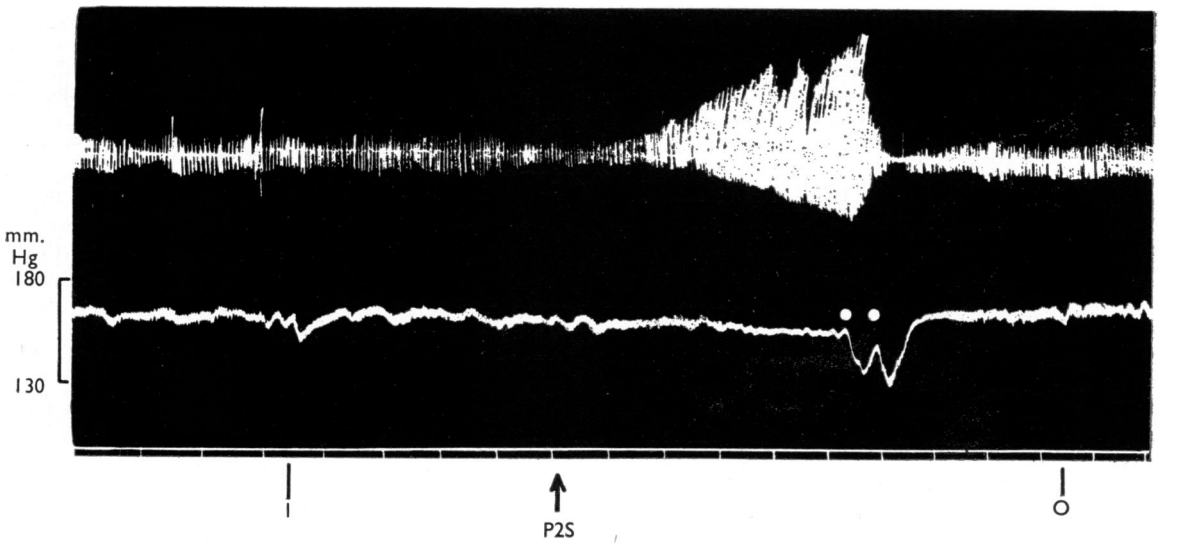

FIG. 1.—Dog, 8 kg. The effects on respiration and blood pressure of pyridine-2-aldoxime (at **P2S**) when 1 mg. was given intracisternally. The point of insertion of the needle into the cistern is marked **I**; the syringe was removed from the indwelling needle at **O**, to test fluid pressure. Upper tracing, respiration; middle tracing, blood pressure; bottom tracing, time in min. The fluctuations in blood pressure after each white dot followed injections of pentobarbitone given intravenously.

received the smaller dose, but it was repeated several times in each. At varying intervals after the first injection, slight changes of doubtful significance in respiratory rate or amplitude occurred. Subsequent doses had no visible effects, and there were no obvious changes in blood pressure. Of the six dogs which were given 1,000 μg. of pyridine-2-aldoxime, five showed some lightening of anaesthesia. The palpebral reflex returned within 1 to 8 min. after the injection; this was accompanied by severe hyperpnoea, and additional anaesthetic had to be given (Fig. 1). A second intracisternal injection of 1,000 μg. of pyridine-2-aldoxime was without effect. Intravenous or intracisternal injection of atropine after the oxime also had little effect as compared with the change observed in animals which had respiratory failure after sarin. This was to be expected, since atropine is known to have little effect on normal breathing.

Since Askew, Davies, Green and Holmes (1956) have demonstrated that many of the oximes liberate cyanide in the body and also on ageing outside the body, all the pyridine-2-aldoxime solutions used in these experiments were tested for free cyanide. None was found, nor was any detected in the cerebrospinal fluid.

After the main experiment was over, 100 to 500 μg. of sodium cyanide was injected into the cistern of several dogs. Cyanide was detected in the cerebrospinal fluid for 4 to 6 min. after this injection, and the respiratory responses were entirely different from those following pyridine-2-aldoxime injected intracisternally.

Effects of Sarin Treated with Pyridine-2-aldoxime and Atropine

The intracisternal injection of 100 μg. (average 8.62 μg./kg.) of sarin into six dogs caused a reduction in the amplitude of respiration, sometimes preceded by an increase in amplitude for 2 to 3 min.; the rate usually increased. Ultimately respiration failed. Artificial respiration was required in 10.5 ± 5.8 min. and was stopped 18 min. after the injection, but still none of the dogs breathed spontaneously. At 20 min., injection of atropine promptly restored adequate respiration. The injection of 100 μg. of pyridine-2-aldoxime intracisternally 10 min. later produced no clear respiratory response. After these two injections, respiration remained satisfactory for the remaining 3 hr. of the experiment.

The administration of sarin was also followed by a progressively rising blood pressure, which reached its peak about the time of respiratory failure (Fig. 2). Cardiovascular collapse then set in, but almost as soon as artificial respiration was begun the blood pressure returned to the previous

high level, or an even higher one. Thereafter there was a slow progressive decline in blood pressure. At 20 min., the pressure was still above the initial control level, but injection of atropine brought it at first below this level, and then quite rapidly back to about that seen initially. Subsequent injection of pyridine-2-aldoxime had little effect on the blood pressure. During the remaining 3 hr., the pressure remained at about the control level, although slow changes in pressure were present in most dogs from time to time.

There were no noteworthy differences between the responses of six dogs which received 100 μg. of sarin intracisternally, followed by artificial respiration and atropine only, and those of the dogs already considered which were given pyridine-2-aldoxime as well. The dogs in the former group were smaller and hence had a higher dose of sarin/kg. This was reflected in the shorter time to respiratory failure, 6.2 ± 4.0 min. In both groups, salivation commonly followed the intracisternal injection of sarin within 1 to 5 min. and persisted until injection of atropine.

Effects of Large Doses of Sarin and of Subsequent Treatment

Six dogs were given 25 μg./kg. of sarin intracisternally. The change in breathing was like that in the dogs which received 8.6 μg./kg., but respiratory failure occurred more rapidly in 6.0 ± 2.12 min.

The blood pressure changes were a little different. There was a rise in pressure and then some extrasystoles began to appear before the peak blood pressure was reached. Shortly thereafter severe bradycardia developed in five dogs (Fig. 3). The maximum pressure recorded by the mercury manometer was 217 mm. Hg before artificial respiration was started and 236 mm. Hg thereafter. The bradycardia continued through periods when artificial respiration was not given, with the mean blood pressure showing a rise and then a decline. The intracisternal injection of 100 μg. of pyridine-2-aldoxime 20 min. after sarin in dogs with bradycardia abolished the bradycardia completely in one dog, slightly increased the heart rate in one dog, and had no effect in three dogs. Bilateral vagotomy (2 dogs) did not relieve the bradycardia.

Eighteen min. after the sarin injection (usually 12 min. after artificial respiration began) the pump was stopped to test for the appearance of spontaneous respiration. Two min. later the pump was started and pyridine-2-aldoxime was injected intracisternally. Only two dogs showed any return of spontaneous breathing; in one of these breathing was adequate but poor, in the other failure occurred very quickly. In five dogs in this group, the intravenous injection of atropine

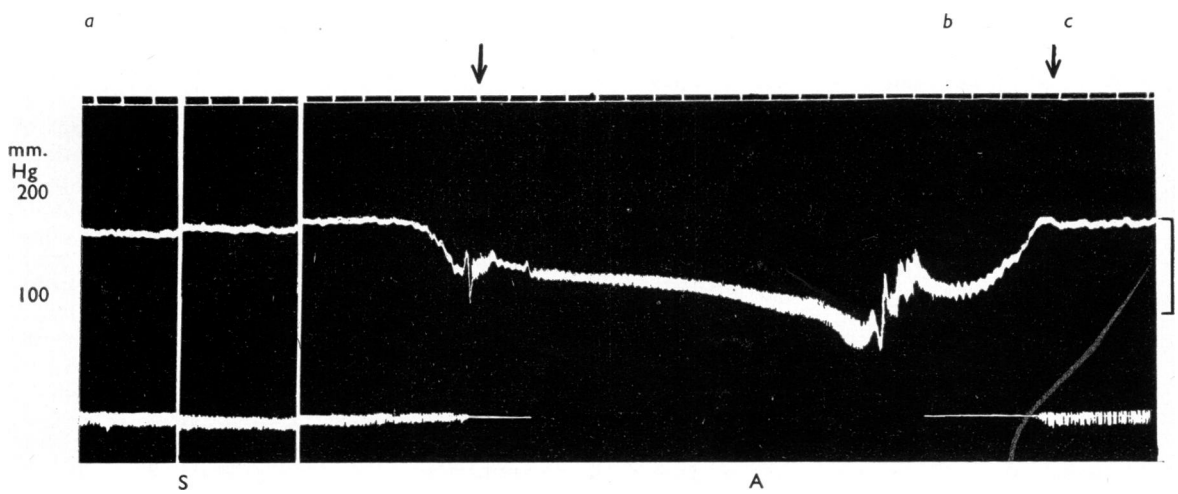

FIG. 2.—Female dog, 7.5 kg. The effects of 100 μg. of sarin, injected intracisternally at S, on respiration (top trace) and blood pressure (middle trace). Time in min. (lower trace). The break in the record of respiration denotes the period of artificial respiration. Atropine sulphate (0.5 mg./kg.) was given intravenously at A. 30 min. after the injection of sarin, 100 μg. of pyridine-2-aldoxime was injected intracisternally (between **a** and **b**). It had little or no effect on respiration or blood pressure. Tracing **b** was made 46 min. and tracing **c** 175 min. after the sarin injection.

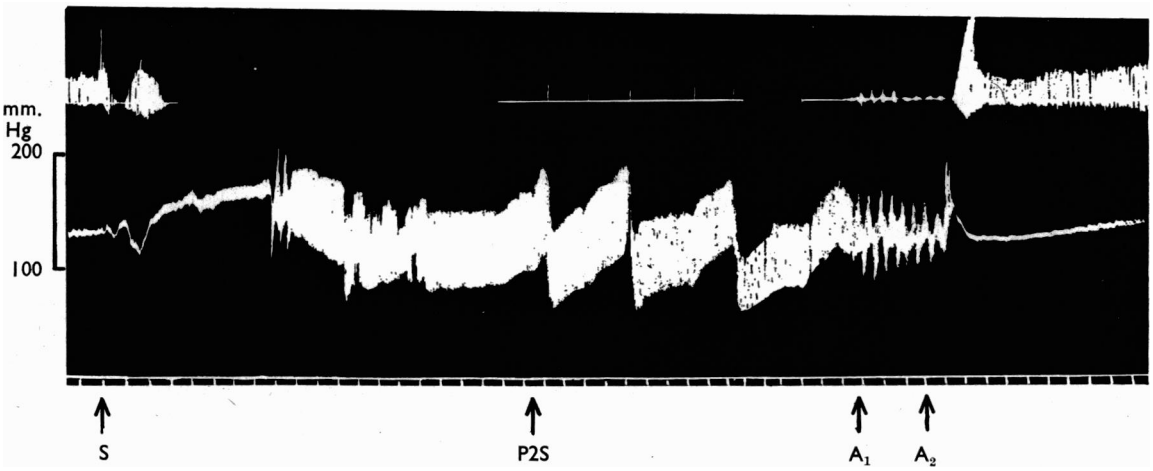

FIG. 3.—Dog, 8.5 kg. Records as in Fig. 2. An intracisternal injection of 25 μg./kg. of sarin was given at S. Note onset of extreme bradycardia about 8 min. later when salivation began. 100 μg./kg. of pyridine-2-aldoxime (at **P2S**) was ineffective. Atropine sulphate (20 μg./kg.) given intracisternally (at A_1) was partially effective. When 0.5 mg./kg. of atropine was given intravenously (at A_2) it was completely effective. The breaks in the records of respiration denote artificial respiration.

started adequate spontaneous respiration; the sixth, which was already breathing spontaneously but feebly after pyridine-2-aldoxime and artificial respiration, showed great respiratory improvement after the atropine injection. After some 30 to 60 min. when the experiments were over, no cyanide was found either in the pyridine-2-aldoxime solutions or in the cerebrospinal fluid of any of these dogs.

DISCUSSION

The doses of sarin given were all certain fatal doses, and, pending therapy, artificial respiration was essential in all dogs. Heymans, Pochet, and Van Houtte (1956) have shown that sarin does not influence the chemoreceptors. Somers and Brown (unpublished observation) have shown that doses of 10 μg./kg. of sarin intravenously had little deleterious effect on neuromuscular transmission. Hence the respiratory failure encountered after 100 μg. of sarin intracisternally must have been central in origin, as the mean dose for all dogs receiving that dose was 9.37 ± 1.97 μg./kg.

Even the larger dose of sarin, 25 μg./kg. intracisternally, produced only two signs which might have been due to peripheral action, salivation and bradycardia. The bradycardia was presumably due to some sarin being carried back with the venous blood to the heart, where it acted on the pacemaker. This effect was unaffected by bilateral vagotomy but was abolished by atropine. The salivation was probably central in origin, as it occurred in almost all the dogs which received sarin and was the only "muscarinic" effect seen in most. No other satisfactory explanation of so limited an effect is forthcoming.

Polet and de Schaepdryver (1959) found that the intracarotid injection of small doses of sarin (2 μg./kg.) stimulated, and large doses (>10 μg./kg.) depressed, respiration in dogs connected to donor-perfused heads by nervous tissue only. In the present experiments in which sarin was given intracisternally, there was always total respiratory paralysis of central origin. The conclusion of Polet and de Schaepdryver (1959) that the lethal action of sarin is not due to a primary and direct paralysis of the respiratory centre is not, therefore, tenable.

Whether given to the isolated head by intracarotid injection (Polet and de Schaepdryver, 1959) or by intracisternal injection, sarin causes a large rise of blood pressure, as reported by Dirnhuber and Cullumbine (1955). Atropine promptly abolishes this stimulation, so that the blood pressure returns to its initial level. The cardio-inhibitory centre in the medulla was not stimulated by sarin intracisternally, which confirms the results of Polet and de Schaepdryver (1959).

Pyridine-2-aldoxime, administered intracisternally, did not alter the central effects produced by sarin, but atropine appears to be an entirely adequate antidote to the central effects of sarin and its action is not altered by administration of the oxime. The failure of the oxime, injected

intracisternally, to improve respiration after sarin is difficult to explain. It is possible that pyridine-2-aldoxime does not penetrate the brain substance or the ependymal lining of the cisterna, but the lightening of anaesthesia from the larger dose raises some doubt on this point.

There are many observations which indicate that intrathecal anticholinesterases increase the quantity of acetylcholine in the brain (eserine: Bhattacharya and Feldberg, 1958; Bhawe, 1958; ethyl pyrophosphate: Metz, 1958; dyflos (diisopropylfluorophosphate): Michaelis, Finesinger, Verster, and Erickson, 1954). The role of normally-released acetylcholine in the central nervous system remains to be clarified. Whatever its role, its effects on the respiratory centre are abolished by atropine. Michaelis *et al.* (1954) concluded that atropine did not prevent the accumulation of acetylcholine, it only abolished the effects of that which was present. On other cholinergic endings, apart from those in skeletal muscle, atropine is equally effective in blocking the action of acetylcholine (see Brown *et al.*, 1957).

As de Candole, Douglas, Lovatt Evans, Holmes, Spencer, Torrance and Wilson (1953) have pointed out, respiratory failure after systemic sarin poisoning is due partly to central paralysis and partly to paralysis of neuromuscular transmission. It appears from the present results that atropine was fully effective against the central component, but it is well known that atropine in therapeutic doses has no effect on neuromuscular transmission (see tum Suden, 1958).

It is well established that pyridine-2-aldoxime leads to regeneration of cholinesterase and rapidly restores neuromuscular transmission (Brown *et al.*, 1957; Wills *et al.*, 1957; Hobbiger, 1957; Askew, 1957; Kewitz and Nachmansohn, 1957; Koelle, 1957). In the intact animal atropine can be expected to fail therapeutically against any dose of sarin sufficient to produce practically complete neuromuscular block, and any oxime known at present will fail therapeutically against any dose of sarin sufficient to produce central respiratory paralysis. Together, they are therapeutically successful against many times the LD50 of sarin (Brown *et al.*, 1957; Wills *et al.*, 1957).

Hobbiger (1957), Kewitz and Nachmansohn (1957), and Rutland (1958) have reported that therapeutically effective oximes restore the acetylcholinesterase content of the blood towards the normal level after its depression by sarin; they were unable to demonstrate a similar restoration of the brain cholinesterase. The usual explanation has been that there was an effective barrier between blood and brain. In the above experiments, it has been shown that pyridine-2-aldoxime was not effective even when an attempt was made to by-pass this barrier.

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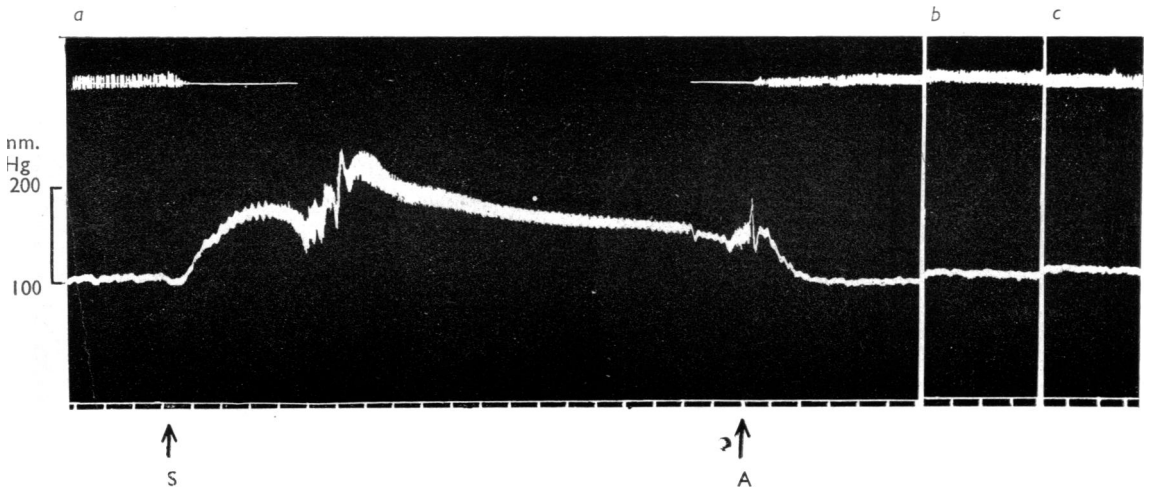
ERRATUM

In a paper by R. V. Brown in the March 1960 issue, Fig. 2 on p. 172 was printed upside down. The figure is correctly reproduced here for the convenience of readers who may wish to paste it into their copies.



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EFFECTS OF RESERPINE, CHLORPROMAZINE AND SODIUM SALICYLATE ON THE ENZYMIC ACTIVITY OF RAT LIVER

BY

S. M. KIRPEKAR* AND J. J. LEWIS

From the Department of Experimental Pharmacology, Institute of Physiology, University of Glasgow

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The effects of reserpine, chlorpromazine and sodium salicylate on oxidative phosphorylation, stimulation of glutamic acid oxidation and adenosinetriphosphatase activity have been compared *in vitro* with those of 2,4-dinitrophenol using rat liver preparations. All three drugs inhibit oxidative phosphorylation, slightly stimulate glutamic acid oxidation and stimulate adenosinetriphosphatase activity at high concentrations. Reserpine has no effect on adenosinetriphosphatase activity. It is suggested that the characteristic depressant effects of reserpine on isolated tissues *in vitro* may be due to interference with production of energy by oxidative phosphorylation.

Substances which depress the formation of high energy phosphate bonds without markedly affecting oxygen consumption are known as uncoupling agents. 2,4-Dinitrophenol is a typical uncoupling agent and has been extensively used in studies on the mechanism of oxidative phosphorylation. A number of compounds chemically unrelated to one another and to dinitrophenol dissociate oxidation from phosphorylation and have been claimed to exert their biological actions through this property (Brody, 1955). Thus the antipsychotic agents, chlorpromazine and reserpine, inhibit oxidative phosphorylation in rat brain mitochondria (Abood and Romanchek, 1957) and chlorpromazine exerts a similar action in rat liver mitochondria (Berger, Strecker, and Waelsch, 1957). It has also been shown (Brody, 1956) that salicylates act as uncoupling agents, and reserpine, after *in vivo* administration, causes a significant fall in the concentrations of high energy phosphate in rat liver (Kirpekar and Lewis, 1959a).

Dinitrophenol inhibits oxidative phosphorylation (Loomis and Lipmann, 1948), stimulates the oxidation of various substrates such as glutamate, α-ketoglutarate, succinate and pyruvate + malate (Potter and Recknagel, 1951), and stimulates adenosinetriphosphatase activity (Lardy and Wellman, 1953; Myers and Slater, 1957). In the present experiments a comparison has been made between the effects of reserpine, chlorpromazine, sodium salicylate and the effects of dinitrophenol on these three systems in rat liver preparations *in vitro*.

*Present address: Department of Pharmacology, State University of New York, 450, Clarkson Avenue, Brooklyn 3, N.Y., U.S.A.

METHODS

A solution of reserpine (2.5 mg./ml.) and the corresponding control solvent solution (Ciba) were used and diluted as required. Aqueous solutions of chlorpromazine hydrochloride (Smith, Kline and French), sodium salicylate and 2,4-dinitrophenol were used, and diluted to give the required concentrations.

Preparation of Mitochondria

All operations were carried out at 0°. The method used for the isolation of mitochondria was essentially that described by Schneider (1948) and Schneider and Hogeboom (1950). The animals were fasted overnight to reduce the glycogen content of the liver. The liver from a freshly killed albino rat was quickly excised, washed in ice-cold 0.25 M-sucrose, blotted gently with filter paper and weighed on a torsion balance. The liver was disintegrated for 2 min. at 1,500 rev./min. in 9 volumes of cold 0.25 M-sucrose in a Potter-Elvehjem homogenizer using a plastic pestle. The mitochondrial pellet was finally suspended in 0.25 M-sucrose, so that 2.5 ml. of suspension contained the mitochondria derived from 1 g. of fresh liver. Mitochondria obtained in this manner showed a large increase in oxygen uptake if exposed to dinitrophenol or with glucose and hexokinase (Aldridge, 1957).

Measurement of Oxidative Phosphorylation

All incubations were carried out in standard Warburg flasks using the medium described by Parker

(1958) to suspend the mitochondria. Each flask contained 0.5 ml. of mitochondrial suspension, representing 200 mg. wet wt. of liver, 0.00115 mole sodium adenosine triphosphate, 0.00115 mole adenosine monophosphate, 0.125 mole potassium chloride, 0.014 mole magnesium chloride hexahydrate, 0.001 mole disodium ethylenediamine tetra-acetic acid, 0.0154 mole potassium phosphate, 0.0166 mole glycylglycine (*p*H 6.75), 0.01 mole glutamic acid as substrate, 0.06 mole glucose, 400 units of hexokinase (a preparation of type II hexokinase [Sigma Chemical Company] containing 70,000 units/g. was used) and 0.1 ml. of aqueous drug solution: for reserpine controls, 0.1 ml. of the diluted control solution was used. In the absence of drug 0.1 ml. of distilled water was used. The final flask volume was 3.0 ml., the centre well containing 0.2 ml. of 20% (w/v) KOH and a filter paper strip. The *p*H of the medium was 6.8 and the incubations were carried out at 37° in an atmosphere of air. In these experiments, glucose and hexokinase were not added to the medium in the main chamber but placed separately in the side arm. After equilibration for 10 min., the contents of the side arm were tipped and the oxygen uptake recorded for a further period of 10 min. The flasks were then immediately removed from the manometers and the reaction terminated by addition of 0.5 ml. of 60% (w/w) perchloric acid. In each experiment, the initial inorganic phosphorus content was determined in a blank flask at zero-time by deproteinizing with 0.5 ml. of 60% (w/w) perchloric acid before adding the mitochondria. The blank flask was taken through the procedure in the same manner as the others. After deproteinizing with 60% (w/w) perchloric acid the solutions were centrifuged and the inorganic phosphorus content of the supernatant fluid determined. In this way both oxygen and phosphorus uptake were measured over a period of 10 min.

Measurement of Stimulation of Oxidation

The oxidation of glutamic acid was measured using the Warburg method. The contents and the final concentration of each flask were as described in the previous section, but glucose and hexokinase were omitted. The mitochondrial suspension (0.25 ml.) added to each flask was equivalent to 100 mg. wet wt. of liver. The final volume was 3.0 ml., the *p*H 6.8 and the bath temperature 37°. The contents of the flasks were equilibrated for 10 min. and readings taken at 10 min. intervals for 1 hr.

Measurement of Adenosinetriphosphatase Activity

Adenosinetriphosphatase activity of whole liver homogenates was measured using the method of Lardy and Wellman (1953). The amount of inorganic phosphorus liberated in 10 min. from adenosine triphosphate in the presence of a liver suspension was taken as a measure of activity. Experiments were carried out in Warburg flasks at 25° in an atmosphere of air. Each flask contained 0.06 mole sodium adenosine triphosphate, 0.15 mole potassium chloride, 0.1 ml. water, reserpine control solution or the drug solution, and 0.3 ml. of liver homogenate in 0.25 M sucrose, equivalent to 100 mg. wet wt. of liver in the side arm. The total volume after tipping was 1.0 ml., the *p*H of the solution 7.2. The flasks were placed in the Warburg bath and shaken for 5 min. for temperature equilibration and the reaction started by tipping in the enzyme source. After shaking for 10 min. the flasks were immediately taken out and the reaction stopped by adding 1.0 ml. of 10% (w/w) perchloric acid. In each experiment the inorganic phosphate formed in a blank flask was estimated by deproteinizing with 1.0 ml. of 10% (w/w) perchloric acid and this value subtracted from all readings. The solutions were centrifuged and the inorganic phosphorus content of the supernatant fluid determined. Phosphate determinations were made by the method of Allen (1940).

Results

Table I shows the effects of reserpine, chlorpromazine and sodium salicylate on oxidative phosphorylation in rat liver mitochondria. In the presence of glucose and hexokinase the rate of oxygen uptake was about 2.5 times that without the acceptors. All three drugs inhibited phosphorylation without simultaneously affecting oxygen uptake. At 10^{-6} M dinitrophenol had very little effect on oxidative phosphorylation: it caused almost 50% inhibition at 10^{-5} M and 100% at 10^{-4} M. Chlorpromazine at 10^{-5} M had practically no effect on the P/O ratio (uncoupling only 5%) but began to exert its effect at 10^{-4} M, when uncoupling was about 30%, while at 5×10^{-4} M the phosphorus uptake was completely inhibited. At this same concentration chlorpromazine depressed oxygen uptake. Sodium salicylate had no uncoupling effect at 10^{-5} M, but caused 35% depression at 10^{-3} M and there was complete inhibition at 10^{-2} M. At 0.8×10^{-4} M reserpine caused 36% inhibition of phosphorylation while at 1.5 times this concentration the depression was 61%. A volume of the reserpine control solution equivalent to higher concentrations of reserpine slightly depressed the P/O ratio. Reserpine had a smaller effect on oxygen uptake, but a greater effect on phosphorus uptake than chlorpromazine. When compared with dinitrophenol, neither reserpine nor chlorpromazine nor sodium salicylate was a strong uncoupling agent. Chlorpromazine and reserpine appeared to be equipotent, but salicylate was much less potent than either.

Table II shows stimulation of the oxidation of glutamic acid in an acceptor-deficient medium. Dinitrophenol slightly stimulated it at 10^{-6} M: the effect was doubled at 10^{-5} M and increased more

TABLE I

THE EFFECT OF DINITROPHENOL, CHLORPROMAZINE, SODIUM SALICYLATE, AND RESERPINE ON OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA

All concentrations are molar.

Drug	Conc.	ΔPμ Mole (±S.E.)	ΔOμ Atoms (±S.E.)	P/O (±S.E.)	% Uncoupling
Control	—	25·82 (±0·52)	9·5 (±0·28)	2·76 (±0·05)	—
Dinitrophenol ..	1×10^{-6}	19·39 (±0·16)	7·94 (±0·10)	2·43 (±0·05)	12
	1×10^{-5}	12·28 (±0·46)	8·80 (±0·47)	1·40 (±0·11)	49
	1×10^{-4}	0·00	6·90 (±0·12)	0·00	100
Chlorpromazine ..	1×10^{-5}	20·25 (±0·27)	7·74 (±0·11)	2·61 (±0·00)	5
	1×10^{-4}	17·22 (±1·60)	8·67 (±0·60)	1·96 (±0·06)	29
	5×10^{-4}	0·00	2·14 (±0·05)	0·00	100
Sodium salicylate ..	1×10^{-5}	27·52 (±1·10)	10·05 (±0·02)	2·71 (±0·10)	2
	1×10^{-4}	16·44 (±0·86)	7·63 (±0·20)	2·16 (±0·17)	22
	1×10^{-3}	12·46 (±1·40)	6·87 (±0·14)	1·79 (±0·17)	35
	1×10^{-2}	0·00	5·96 (±0·07)	0·00	100
Reserpine control ..		17·91 (±1·6)	7·94 (±0·58)	2·25 (±0·10)	—
Reserpine	$1·6\times10^{-5}$	22·51 (±0·16)	11·25 (±0·18)	2·00 (±0·04)	11
	$0·8\times10^{-4}$	12·44 (±0·29)	8·68 (±0·58)	1·44 (±0·09)	36
	$1·2\times10^{-4}$	4·51 (±1·27)	4·88 (±0·31)	0·87 (±0·23)	61

TABLE II

EFFECTS OF DINITROPHENOL, RESERPINE, CHLORPROMAZINE, AND SODIUM SALICYLATE ON OXYGEN UPTAKE IN AN ACCEPTOR-DEFICIENT MEDIUM

Control values (1 hr.) are taken as 100%. All concentrations are molar.

Drug	Conc.	% Oxygen Uptake
Dinitrophenol ..	1×10^{-6}	115
,, ..	1×10^{-5}	200
,, ..	1×10^{-4}	265
Reserpine ..	$1·6\times10^{-5}$	115
,, ..	$0·8\times10^{-4}$	117
,, ..	$1·2\times10^{-4}$	83
Chlorpromazine	1×10^{-5}	107
,,	5×10^{-5}	113
,,	1×10^{-4}	124
,,	5×10^{-4}	6
Sodium salicylate	1×10^{-5}	104
,, ,,	1×10^{-4}	119
,, ,,	1×10^{-3}	108
,, ,,	5×10^{-3}	100
,, ,,	1×10^{-2}	100

than 2.5 times at 10^{-4} M. Reserpine at 1.6×10^{-5} M and 0.8×10^{-4} M stimulated oxygen uptake 15%. The reserpine control solution had virtually no effect on glutamic acid oxidation. When used with liver slices, the control solution significantly stimulated oxygen uptake (Kirpekar and Lewis, 1959a). Chlorpromazine had no effect at 10^{-5} M, caused approximately 25% stimulation at 10^{-4} M and almost complete inhibition at 5×10^{-4} M. Sodium salicylate caused slight stimulation at 10^{-4} M, but even at very high concentrations (10^{-3} to 10^{-2} M) it had very little effect. None of the drugs tested had so marked an effect on glutamic acid oxidation as dinitrophenol. Dinitrophenol apparently compensates for the deficiency of glucose and hexokinase, since in its presence oxygen uptake was even greater than in the presence of such an acceptor.

Table III shows the effects of chlorpromazine, reserpine, sodium salicylate and dinitrophenol on liver adenosinetriphosphatase activity. Dinitrophenol had a very high stimulant activity at 3×10^{-4} M, but at lower concentrations it was less effective and at 10^{-6} M inactive. Chlorpromazine had no effect at 10^{-5} M and 10^{-4} M, but at 10^{-3} M had about half the effect of dinitrophenol (3×10^{-4} M). Reserpine at the concentrations tested had no stimulant effect upon adenosine-

TABLE III

EFFECTS OF DINITROPHENOL CHLORPROMAZINE, RESERPINE, AND SODIUM SALICYLATE ON LIVER ADENOSINETRIPHOSPHATASE ACTIVITY

All concentrations are molar.

Drug	Conc.	μmole Inorganic Phosphate Liberated ±(S.E.)
Control ..	—	0·87 (±0·05)
Dinitrophenol ..	3×10^{-6}	1·10 (±0·07)
,, ..	3×10^{-5}	2·59 (±0·12)
,, ..	3×10^{-4}	8·35 (±0·10)
Chlorpromazine	1×10^{-5}	1·04 (±0·16)
,, ..	1×10^{-4}	1·04 (±0·08)
,, ..	1×10^{-3}	3·67 (±0·02)
Reserpine ..	$1{\cdot}6\times10^{-5}$	1·04 (±0·14)
,, ..	$0{\cdot}8\times10^{-4}$	0·37 (±0·13)
,, ..	$1{\cdot}2\times10^{-4}$	0·22 (±0·09)
Sodium salicylate	1×10^{-4}	1·32 (±0·00)
,, ,,	1×10^{-3}	2·15 (±0·21)
,, ,,	1×10^{-2}	5·97 (±0·19)

triphosphatase activity, but caused slight inhibition at higher concentrations. Sodium salicylate enhanced adenosinetriphosphatase activity (6.00 μ mole of phosphate at 10^{-2} M); had little stimulant effect at 10^{-3} M and none at 10^{-4} M. It was interesting to note that the concentrations of dinitrophenol which produced maximum stimulation of oxygen uptake and of adenosinetriphosphatase action were similar. This does not, however, appear to be true of the other compounds investigated.

Table IV summarizes the results and gives comparative data on the four compounds studied.

DISCUSSION

Dinitrophenol, which is a typical uncoupling agent, powerfully stimulates adenosinetriphosphatase activity and glutamic acid oxidation, and causes marked inhibition of phosphorylation without affecting oxygen uptake. Reserpine, chlorpromazine and sodium salicylate all inhibit oxidative phosphorylation, and with higher concentrations both oxygen and phosphorus uptake are depressed. There is some difference of opinion regarding the action of chlorpromazine on rat brain mitochondria. Abood and Romanchek (1957) have shown that chlorpromazine depresses oxidative phosphorylation, while Berger, Strecker and Waelsch (1957) did not confirm this but reported depression of P/O ratios in rat liver mitochondria and thereby stressed the difference in the metabolic behaviour of mitochondria from the brain and liver. Our observations support their finding that chlorpromazine inhibits phosphorylation in rat liver mitochondria. Reserpine has been reported by Abood and Romanchek (1957) to be an effective uncoupling agent in rat brain mitochrondria, and our results show that it is an uncoupling reagent in rat liver mitochondria. It appears therefore that, as far as reserpine is concerned, brain and liver mitochondria behave similarly. It is also possible that reserpine, which has marked effects on the central nervous system, may possess a preferentially higher affinity for brain than for liver cells. Reserpine has also been reported by the same authors in *in vivo* studies to have an uncoupling effect in preparations of mitochondria

TABLE IV

COMPARATIVE CONCENTRATIONS OF DINITROPHENOL, RESERPINE, CHLORPROMAZINE, AND SODIUM SALICYLATE AFFECTING THE P/O RATIO, OXIDATION AND ADENOSINETRIPHOSPHATASE ACTIVITY IN RAT LIVER

All concentrations are molar.

Experiment	Dinitrophenol	Reserpine	Chlorpromazine	Sodium Salicylate
50% inhibition of P/O ratio	1×10^{-5}	1×10^{-4}	2×10^{-4}	$2{\cdot}5\times10^{-3}$
Maximum stimulation of oxidation ..	1×10^{-4}	$0{\cdot}8\times10^{-4}$	1×10^{-4}	1×10^{-4}
Maximum stimulation of adenosinetriphosphatase activity	3×10^{-4}	None	1×10^{-3}	1×10^{-2}

from the region of the basal ganglia. It has been shown recently by Desci and Mehes (1959) that the tranquillizing agents benactyzine and meprobamate also have an uncoupling effect which is exerted more effectively on preparations from the same regions.

At a concentration of 10^{-4} M dinitrophenol markedly stimulates the oxidation of glutamic acid. The other drugs are inactive, and although they inhibit oxidative phosphorylation in certain concentrations they do not possess the property of stimulating oxidation. Dinitrophenol stimulates oxidation markedly and at the same time inhibits phosphorylation. Chlorpromazine (10^{-3} M) and sodium salicylate (10^{-2} M) stimulate adenosinetriphosphatase activity considerably, but there appears to be no relation between the dose causing maximum stimulation of adenosinetriphosphatase activity and the effect on oxygen uptake. Reserpine, on the other hand, has little stimulant effect on oxidation (10^{-4} M) but at the same concentration has a slight inhibitory effect on adenosinetriphosphatase activity.

The effects of chlorpromazine and reserpine, like those of thyroxine, may be due to intracellular physical processes such as the swelling of the mitochondria. Thyroxine causes the mitochondria to swell (Dickens and Salmony, 1956; Tapley, Cooper, and Lehninger, 1955) and the uncoupling effect that it produces is attributed to this property rather than to its interaction with the enzymes concerned with oxidative phosphorylation. It would be interesting to see whether chlorpromazine or reserpine cause swelling of mitochondria although the general pharmacological properties of reserpine and chlorpromazine are quite different from those of thyroxine.

Chlorpromazine and salicylates react with ferric salts. Chlorpromazine when mixed with ferric chloride solution is oxidized and produces a brilliant red colour. Our recent observations (Kirpekar and Lewis, 1959b) of the apparent formation of a hydrallazine-iron complex and the observation of Fels and Kaufmann (1959) that chlorpromazine possesses a specific affinity for iron make it possible that chlorpromazine influences phosphorylation at the cytochrome-cytochrome oxidase level. But a few experiments carried out with crystalline catalase (an iron containing enzyme) have not shown chlorpromazine to have an inhibiting effect even when used at very high concentrations (1.8×10^{-2} M). Salicylates are chelating agents, but reserpine has not so far been reported to form chelation complexes.

In intact animals reserpine is known to exert its characteristic effects rather slowly. Gillis and Lewis (1956) and Kirpekar and Lewis (1958) have shown that reserpine depresses skeletal muscle, the smooth muscles of intestine, uterus and arteries, and that it also depresses heart muscle. This effect may be due to its uncoupling action (Kirpekar and Lewis, 1959a). Born and Bülbring (1955) have shown that dinitrophenol in high concentrations decreases the tension and spontaneous movements of the taenia coli muscle. Moreover, when the dinitrophenol-treated muscle is stimulated with histamine, the tension increases but is not maintained although histamine is still present. Anoxia also depresses tissue activity. There is thus some evidence that, under the conditions of our experiments, reserpine and chlorpromazine act like dinitrophenol, but the absence of a reserpine effect on adenosinetriphosphatase activity shows that chlorpromazine and reserpine have different modes of action from one another and from dinitrophenol, a view supported by pharmacological observations. Sodium salicylate has no antipsychotic action of the type exerted by reserpine and chlorpromazine, but it has some marked effects on the central nervous system: it can, for example, cause nausea and vomiting, analgesia, antipyresis and respiratory stimulation. It has also been shown to inhibit catalase. The effects of sodium salicylate on the metabolic rate (Cochran, 1952) and in causing depletion of liver glycogen (Sproull, 1954) and hyperglycaemia (Bornstein, Meade, and Smith, 1952) may have some bearing on its effect on oxidative phosphorylation, but in man even large doses of salicylate do not produce the effects typical of an uncoupling agent.

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A COMPARISON OF THE KALLIKREIN–KININ SYSTEM IN SHEEP AND DOGS

BY

J. H. GADDUM AND P. S. GUTH*

From the A.R.C. Institute of Animal Physiology, Babraham, Cambridge

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Three preparations of kallikrein, made from the urine of man, dog and sheep, all formed active kinins when incubated with the plasma of sheep or dogs. The six kinins could not be distinguished from one another by parallel assays on guinea-pig ileum, rat uterus and rat duodenum; they are thought to be identical, or nearly so. As judged by the amount of kinin produced, a sample of dog plasma appeared to contain about 4·3 times as much substrate as a sample of sheep plasma. This ratio did not depend on the kallikrein used. Attempts to compare the three kallikreins gave inconsistent results which may be due to the presence in the preparations of kallikrein of other enzymes acting on the same substrate. Sheep plasma does not form kinins on contact with glass.

Kallikrein is a non-dialysable substance extractable from urine, pancreas, pancreatic juice, salivary glands and saliva of many animals (Frey, Kraut, and Werle, 1950). Its actions depend, partly at least, on its ability to react with plasma proteins to produce a smooth muscle stimulating substance called kallidin (Frey *et al.*, 1950 ; Werle, 1955) or plasma kinin (Horton, 1958 ; Horton, 1959a ; Margolis, 1958 ; Lewis, 1958 ; Hilton and Lewis, 1958).

The reaction is complicated by the presence in plasma of substances, kallikreinase and kininase, capable of destroying kallikrein and kinin. This report is concerned primarily with a comparison of the entire system, urinary kallikrein, plasma kallikrein substrate, kallikreinase and kininase in two mammalian species.

METHODS

Rat Uterus.—The uteri from virgin rats (150 to 250 g.) were suspended in de Jalon solution in a 3 ml. bath at 30°.

Guinea-pig Ileum.—The terminal segments of ileum of guinea-pigs (180 to 300 g.) were suspended in Tyrode solution in a 3 ml. bath at 37°. Atropine sulphate (1 mg./l.) was used when the spontaneous movement was not otherwise controllable.

Rat Duodenum. — The proximal segments of duodenum of rats (150 to 250 g.) were suspended in de Jalon solution in a 3 ml. bath at 30 to 37°. The tissue was stored at 4° for 2 to 3 hr. before use. The dose interval was usually 3 min. The bath was washed out by continuous upward displacement and overflow. The preparations seemed most responsive 30 sec. after this slow flow was stopped, and the substances were injected at this time. The inflow was started 15 sec. after injection in the experiments with the duodenum, and 1 min. with the ileum and uterus preparations.

Preparations of Human, Sheep and Dog Urinary Kallikreins.—Urine from male humans, a Clun Forest wether sheep and mongrel dogs of both sexes was collected under toluene and frozen until use. The procedure for preparing crude kallikrein was that described by Gaddum and Horton (1959) with the exception that the urine was concentrated by placing it in dialysis bags and hanging them before a fan and heat source, rather than by the use of a climbing film evaporator. The concentrated urine was dialysed and the kallikrein precipitated with acetone.

Preparation of Kinins.—The procedure for making the six kinins used in this study was that described by Horton (1958) and Gaddum and Horton (1959). Urinary kallikrein from three sources (human, sheep and dog) (1 mg. kallikrein per 10 ml. plasma) was incubated with acid-treated plasma from two sources (sheep and dog) for 2 hr. at 37°. This acid-treatment consisted in incubation at *p*H 2 and 37° for 10 min. ; this was found by Horton (1958, 1959b) to inhibit kallikreinase and kininase without affecting kinin formation. After incubation and neutralization the mixture was poured into boiling ethanol, and the kinin prepared by drying the alcoholic extract.

The first letter of the kinin notation system used in this paper refers to the source of kallikrein, while the second letter refers to the source of the substrate. Thus HD is the kinin produced when human urinary

*Present address: Department of Pharmacology, Hahnemann Medical College, Philadelphia, Pa., U.S.A.

kallikrein reacts with dog plasma substrate; this was the preparation used as a standard.

Kallikrein Substrate Assay.—The procedure outlined for the preparation of the kinins was designed for maximum kinin formation. Excess kallikrein and an incubation period about twice as long as necessary for the reaction to go to completion were used. The limiting factor in the reaction was the substrate concentration of the plasma. By using the same kallikrein with different substrates, preparing the kinins thus formed and assaying them against a standard kinin (HD), a measure of the relative substrate concentration was obtained.

Results

The experiments illustrated in Fig. 1 showed, by a new method, that sheep resemble other animals in that their urine contains kallikrein and their plasma contains both kallikrein and a kallikreinase which is destroyed by incubation at *p*H 2 and 37° for 10 min. as recommended by Horton (1958, 1959b). Three samples of a preparation of sheep urinary kallikrein (2 mg./10 ml. of the mixture) were incubated at 40° for 24 hr. in dialysis bags with (*A*) de Jalon solution, (*B*) normal sheep plasma and (*C*) acid-treated sheep plasma; and allowed to dialyse for 24 hr. against 10 volumes of de Jalon solution, which was changed once during the dialysis. Kinin was presumably formed during this process and mostly dialysed away; the contents of each bag was then incubated at room temperature with 10 to 25 ml. of dialysed acid-treated sheep plasma, and serial samples were taken from each incubation mixture and tested every 9 min. on an isolated rat uterus preparation. The tracing shows the effects of samples at 45 and 54 min. after mixing.

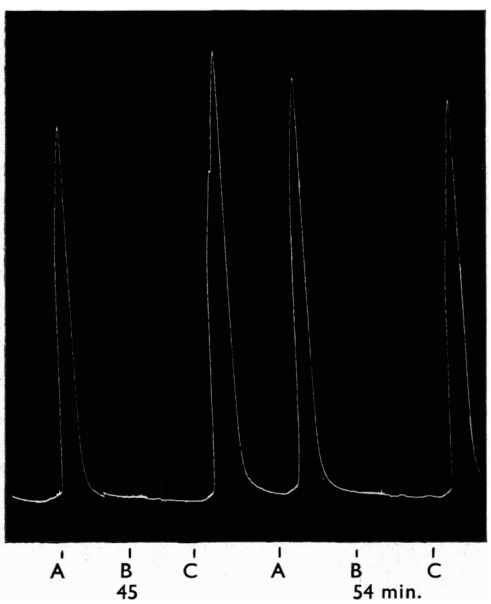

FIG. 1.—Isolated rat uterus. Presence of kallikreinase in sheep plasma and its inhibition by acid treatment. Three samples of sheep kallikrein were incubated with (A) de Jalon solution, (B) normal sheep plasma and (C) acid-treated sheep plasma, and the kinin dialysed away. They were then all incubated with acid-treated plasma and tested at intervals as indicated. Sample B now contained no kallikrein.

The kallikrein incubated with normal sheep plasma (*B*) was completely inactivated, presumably by kallikreinase. The kallikrein incubated with acid-treated sheep plasma (*C*) was spared and formed kinin when added to fresh plasma. This demonstrates that acid-treatment inhibits kallikreinase, and provides evidence that kallikrein is an enzyme. The kallikrein had presumably formed kinin during the dialysis, but had not itself been inactivated in the process; it behaved like a catalyst.

In another experiment, kallikrein, which had been incubated overnight at room temperature with acid-treated plasma in a dialysis bag, was compared with kallikrein which had been incubated with de Jalon solution. After these preliminary incubations they were both incubated with acid-treated dog plasma for 2 hr. and kinin was then extracted with boiling alcohol. The amount of kinin formed by the sample exposed to plasma was estimated to be 80% of the amount formed by the other sample. This result indicates that the acid treatment had not completely destroyed both kallikreinase and kininase.

Fig. 2 is the tracing of an experiment designed to illustrate the presence of kininase in sheep plasma and its destruction by acid treatment. Three samples of kinin made from sheep kallikrein and sheep plasma were incubated at room temperature with (*A*) de Jalon solution, (*B*) normal sheep plasma, and (*C*) acid treated sheep plasma. Serial samples were taken every 3 min. and tested on isolated rat uterus. The tracing shows the progressive destruction of kinin by normal sheep plasma, but not in the other two samples.

Table I gives the results of parallel assays against HD kinin of the kinins produced by allowing three urinary kallikreins (human, sheep and dog) to react with two substrate sources (sheep and dog). The index of discrimination (Gaddum, 1955) is a measure of the dissimilarity of two substances. It is the ratio of estimates of the activity of one substance when compared with

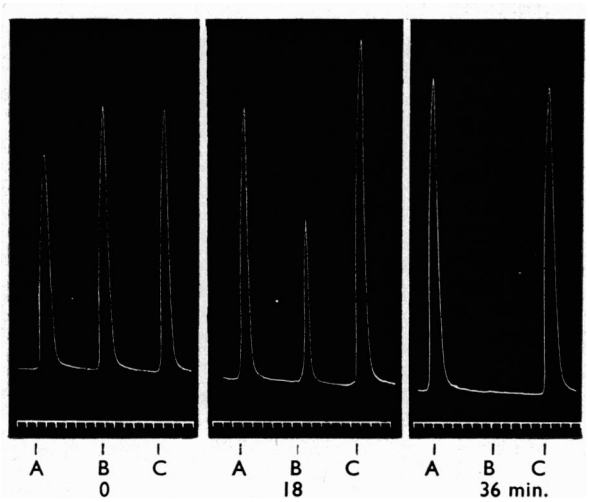

FIG. 2.—Presence of kininase in sheep plasma and its inhibition by acid treatment. Kinin incubated with normal sheep plasma is destroyed (B), while kinin incubated with de Jalon solution (A) or acid-treated plasma (C) is preserved.

TABLE I

COMPARATIVE ACTIVITY OF THE KININS

Activity of 1 mg. of various kinins in terms of mg. HD kinin. Means and ranges of 4 six-point assays. The first letter in the first column denotes the source of kallikrein and the second letter the source of substrate. S—sheep, H—human, D—dog.

Kinin	Guinea-pig Ileum	Rat Uterus	Rat Duodenum	Index of Discrimination	
				RU/GPI	RD/RU
SD	1·9 (1·3–2·8)	2·1 (1·5–3)	1·9 (1·4–2·5)	1·1	0·9
HD	1·0	1·0	1·0	1·0	1·0
DD	0·33 (0·33–0·33)	0·31 (0·29–0·35)	0·38 (0·33–0·5)	0·9	1·2
SS	0·46 (0·33–0·53)	0·66 (0·33–1·2)	0·59 (0·25–1·3)	1·4	0·9
HS	0·14 (0·083–0·25)	0·23 (0·20–0·3)	0·25 (0·22–0·28)	1·6	1·1
DS	0·095 (0·062–0·17)	0·056 (0·04–0·067)	0·095 (0·071–0·17)	0·6	1·7

another on two different smooth muscle preparations. An index near unity indicates that the preparations used are unable to discriminate between the two agents being tested. This method clearly discriminates between kinins on the one hand, and substance P or angiotensin on the other (Gomes, 1955).

In the case of the kinins, they have all been assayed against HD kinin on three smooth muscle preparations. The indices of discrimination calculated from the parallel assay figures indicated that no qualitative differences between the various kinins could be detected.

In these experiments there was excess of kallikrein, and incubation was continued until the reaction was complete. In these circumstances it would be expected that the amount of kinin produced would be directly proportional to the concentration of substrate. Table II shows 9 estimates, based on this assumption, of the ratio of the concentration of substrate in the sample of dog plasma to that in the sample of sheep plasma. These ratios were calculated from the figures given in Table I. The differences between these 9 estimates are within the error of the methods used. There is no evidence that the ratio depends either

TABLE II

SUBSTRATE IN THE PLASMA OF SHEEP, HUMAN AND DOG

Nine estimates of the ratio of the concentration of kallikrein substrate in a sample of dog plasma to that in a sample of sheep plasma calculated from the results shown in Table I. Thus 4·1 = 1·9/0·46.

Kallikrein	Test Organ		
	Guinea-pig Ileum	Rat Uterus	Rat Duodenum
Sheep ..	4·1	3·2	3·2
Human ..	7·1	4·3	4·0
Dog ..	3·5	5·5	4·0

on the kallikrein or on the method of assay. The simplest interpretation of these results is that dog plasma contains the same substrate as sheep plasma, but in 4.3 times the concentration.

Similar calculations of ratios can be made from the figures in Table I to compare the different kallikreins with one another. In this case it would not be expected that the ratio would give a direct estimate of the concentration of kallikrein, since there was always excess of kallikrein. On the simplest assumptions the amount of kinin produced would be independent of the kallikrein used. All the figures in the top half of Table I would then be equal to one another, and all the

figures in the bottom half would also be equal. This is clearly not the case; the figures show consistently that sheep kallikrein produced more kinin than human kallikrein, and that dog kallikrein produced less.

Attempts were made to compare the three kallikreins by the method of Horton (1958, 1959a), in which the kinin formed by small doses of kallikrein in a short time is measured, but the results were difficult to interpret. When acid treated dog plasma was incubated for 10 min. with 10 to 100 μg. of each of the three kallikreins, the results seemed to show that the kallikrein made from dog urine was the most active. In a similar experiment with dog plasma diluted 1:10, the human kallikrein appeared to be the most active. These results were both unexpected, since the experiments shown in Table I had suggested that sheep kallikrein was the most active of the three.

It was thought that these results might depend on the presence of kininase in the preparation of kallikrein. This would not be surprising since there is evidence that urine may contain kininase (Frey, Kraut, and Werle, 1950; Jensen, 1958; Horton, 1959a). In order to test this possibility, the standard preparation of kinin (HD) was incubated with the three kallikreins and the fluid tested on rat uterus. This experiment showed that the preparation of sheep kallikrein caused a gradual destruction of kinin, and that the other two kallikreins had a smaller effect of the same kind. This finding does not account for the observed difference in the yields of kinin, but it does draw attention to a possible source of error in experiments of this kind.

Armstrong, Jepson, Keele, and Stewart (1955) reported that dog plasma is not activated by contact with glass to produce a smooth muscle stimulating substance. In the course of these experiments we have noted that sheep plasma similarly does not appear to be activated by contact with glass.

Discussion

These results suggest that the same kinin is formed by the action of human, dog or sheep kallikrein on either dog or sheep substrate, and that there was no difference between these substrates except in concentration. There were clear differences in the amount of activity formed by the different kallikreins. This might be taken as evidence that the kallikreins are different, but there was no evidence that kallikreins have a different action on the substrate obtained from the same species from that which they have on substrates obtained from other species.

A possible explanation of the results depends on the assumption that the preparations of kallikrein contain another enzyme which competes with kallikrein for the same substrate, but does not form a peptide which stimulates plain muscle. Another explanation is suggested by the evidence of Lewis (1959) that urine may contain two different kinin-forming enzymes. There are other possible explanations. It is clear that more evidence is needed.

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UNSPECIFIC DRUG ACTION. THE EFFECTS OF A HOMOLOGOUS SERIES OF PRIMARY ALCOHOLS

BY

H. P. RANG

From the Department of Pharmacology, University College, London

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Experimental results relating to the unspecific depressant action of normal primary alcohols from methanol to octanol on four separate biological systems are presented. It was found that the log-concentration action curves of alcohols on all four systems were straight over most of their range and, for any one system, parallel throughout the series. With arithmetic increase in the alcohol chainlength the concentration required to produce a given effect diminished logarithmically. The rate of this decrease varied in different biological systems, and was always less than the rate of decrease of solubility with chainlength. In two of the systems investigated alcohols beyond octanol failed to show any activity (cut-off phenomenon). The implications of these findings are discussed, with reference to the mechanism of action of unspecific depressants. Ferguson's principle of using thermodynamic activity instead of concentration as an index of activity was applied to the present results. In an appendix, the results are compared with predictions according to Mullins' hypothesis of narcotic action, and found not to agree well.

A wide range of simple organic compounds can exert qualitatively identical depressant actions on many different organisms. The striking absence of chemical specificity in the compounds tested led to the theory that physical rather than chemical properties govern the activity of the compounds. This approach resulted in the formulation of two theories of narcotic action, namely the Overton-Meyer theory and the Traube theory.

Meyer, in 1899, proposed that narcosis results from the drug dissolving in a fatty phase within the cell. This theory predicts that the narcotic potency of a drug will depend on its fat/water partition coefficient, and also that lipoid-rich cells will be most readily affected. Overton (1901) published data showing a striking correlation between narcotic potency (in tadpoles) and olive-oil/water partition coefficient for a wide range of compounds. In 1935 K. H. Meyer and Hemmi found a correlation between narcotic potency of vapours in mice and the oleic-acid/water partition coefficient of the compound. This was held to support the Overton-Meyer theory. Traube (1904) correlated narcotic potency with the activity of lowering surface tension at an air/water interface. Meyer and Hemmi (1935) found that this correlation does not hold with vapours. On the other hand, Warburg (1921) considered that Traube's air/water interface was a rather unlikely model of any cell component, and measured the adsorption of narcotics at a charcoal/water interface, and found a good correlation with narcotic activity. He postulated inhibition of an enzyme by adsorption of narcotic at some cellular interface as a mechanism of narcosis.

Concurrently with these investigations of narcosis, investigations of the bactericidal activity of organic compounds were carried out, mainly by Tilley and Schaffer (1926, 1928), who investigated the relationship between bactericidal potency and chainlength in homologous series. They found that with an arithmetic increase in chainlength, the equitoxic concentration fell logarithmically up to a certain chainlength (the cut-off point) at which it rose rapidly and very soon reached the solubility limit. The logarithmic fall in equitoxic concentration ran approximately parallel to the fall in solubility with chainlength, but was always slightly less steep. Tilley and Schaffer did not seek to correlate potency with any particular physical property.

Also at this time, new insecticides were being sought. Holt (1916) and Moore (1917) related insecticidal potency with boiling point, and Moore put forward the generalization that potency increases with boiling point up to a certain boiling point, above which compounds cease to be toxic. Boiling point is a way of expressing the distribution of a compound between its liquid and vapour phases.

Ferguson (1939) suggested that the use of distribution coefficients be avoided by measuring potency in terms of the thermodynamic activity of the depressant agent in the solution, instead of its concentration. Since the thermodynamic activity of a substance is by definition equal in all phases of a system at equilibrium, the problem of distribution coefficients between numerous different phases within a cell, any of which might be the biophase in which the drug exerts its pharmacological effects, is by-passed. Using this new approach, Ferguson recalculated the data of earlier workers, and also measured the insecticidal potency of many different vapours. A number of generalizations emerged. For nearly all the compounds tested the thermodynamic activity required to produce a narcotic or toxic effect was 0.1 or more. In a few striking exceptions the activity required was much lower (e.g., ammonia, hydrocyanic acid), and the pharmacological action was also quite different. Hence these compounds may be excluded from the wide group of unspecific depressants. Ferguson also found that within an homologous series the thermodynamic activity required to produce a given effect increases steadily with chainlength until it reaches unity (the activity of a saturated solution). Beyond this point the effective thermodynamic activity is unattainable and the cut-off noticed by earlier workers is thereby explained without having to postulate a sudden change of properties at a particular chainlength.

On the other hand Brink and Posternak (1948), investigating depression of conduction in isolated nerve preparations by homologous series of narcotics, found that the equipotent thermodynamic activity showed no progressive rise throughout a homologous series, but rose sharply to unity at a certain chainlength.

Mullins (1954) devised a more direct approach to the problem. He started with the hypothesis that narcotics act by dissolving in some particular phase of the cell (probably the membrane), and that equal narcotic effects are produced when an equal volume fraction of this phase is occupied by narcotic molecules. By making a number of assumptions and approximations concerning the physico-chemical properties of the biophase and its interaction with the narcotic, Mullins was able to predict equipotent thermodynamic activities within a homologous series, and he presents experimental results which show reasonable agreement with these predictions.

The present work was undertaken with the following aims in view.

1. To gain comparative data on the action of a single homologous series (normal primary alcohols) on several different biological systems. This is the converse of the approach usually adopted in the past, and it was interesting to see whether the slight differences between homologous series patterns were also evident when the same series was used on different systems.

2. To throw more light on the mechanism of cut-off, as argued by Ferguson on the one hand and Brink and Posternak on the other.

3. To try to determine the level of organization at which alcohols exert their action, by investigating a sequence of phenomena ranging from mobility of whole organisms to oxygen consumption of isolated tissues.

4. To provide further material on which to test Mullins' hypothesis.

Methods

Paramecium Immobilization

The concentration of different alcohols required to produce the same degree of immobilization in a population of paramecia was measured.

0.2 ml. of a suspension of paramecia in water (containing 100 to 200 organisms) was placed in a small flat perspex cup. Six such cups fitted into the revolving stage of a low-power binocular microscope, so that each could be swung rapidly into the field of view. At a noted time a volume of alcohol solution (0.05 to 0.2 ml.) calculated to give a suitable final concentration was added from a syringe. At known intervals after addition of alcohol, the cups were examined in turn, and counts of the number of immobile organisms (which sink conveniently to the bottom and are quite easily distinguishable) were made. Counts of each cup were made at 5 min. intervals, until the count became steady. When such an equilibrium had been reached (usually after about 15 min.) a drop of strong phenol solution was added to each cup. This immobilized all the organisms and the total number in each cup was counted. The fraction of the total number of organisms immobilized at equilibrium was taken as the response to a particular alcohol concentration.

Gut Contractility

Experiments were carried out on the guinea-pig ileum, suspended in oxygenated Tyrode solution in a 1 ml. organ bath maintained at 32 to 34°.

Contractions elicited by additions of acetylcholine to the bath were recorded by means of a light frontal-writing lever exerting a tension of about 0.3 g. on the ileum. Alcohols dissolved in the Tyrode washing fluid were applied to the preparation and the assays carried out with an automatic assay apparatus (Boura, Mongar and Schild, 1954).

In one set of experiments the ileum was stimulated electrically. The stimulus was supplied from a square-

pulse stimulator, and applied to the preparation between its attachments, which were made with fine wire in place of thread.

Tissue Oxygen Consumption

Experiments were carried out on chopped guinea-pig lung. The animal was killed by a blow on the back of the neck, and the lungs removed and washed in Tyrode solution. The lungs were then chopped into particles 1 mm. square in cross section, using a MacIlwain tissue chopper. 0.2 ml. samples were prepared, using the sampler described by Mongar and Schild (1957).

A few samples were dried and weighed. Their dry weight was about 3 mg. and varied very little between samples from the same animal.

Oxygen consumption was measured by Warburg's direct method. The main compartment of each flask contained 3.0 ml. of Tyrode solution (containing phosphate buffer in place of bicarbonate) and the lung particles, and, in most of the experiments, the alcohol. The central well contained 0.4 ml. of 10% sodium hydroxide solution absorbed in a filter paper roll. In some experiments with water-miscible alcohols, the alcohol was placed in the side-arm, and added only after the control rate of oxygen consumption had been established. In most cases, however, it was necessary to add the alcohol to the main compartment right from the start, and to compare the oxygen uptake with that of separate control flasks.

Anaphylactic Histamine Release

The method described by Mongar and Schild (1957) was used.

Guinea-pigs were sensitized by injecting a solution of 2% egg albumen and 0.5% phenol in normal saline, 1 ml. being given subcutaneously and 1 ml. intraperitoneally. The animals were used 3 to 10 weeks later.

The lungs were chopped as before, and washed for at least 20 min. in 500 ml. of Tyrode solution, in order to remove as much as possible of the histamine released from damaged cells. 0.2 ml. samples were prepared as before, and each sample was placed in a 5 ml. beaker containing 1 ml. of Tyrode solution, or Tyrode plus inhibitor, and a round glass bead was added to help stirring. The beakers were fitted into a shaking rack in a water-bath at 37°, and left for 15 min. 0.1 ml. of 1% egg albumen solution was then added to each (with the exception of the blanks, to which Tyrode was added) and after a further 15 min. shaking the supernatant was sucked off with a filter-tipped pipette. The supernatant solutions were placed in a boiling water-bath for 2 min. in order to destroy histaminase and acetylcholine.

Histamine was assayed on the guinea-pig ileum, using an automatic assay apparatus (Boura, Mongar and Schild, 1954).

Calculation of Thermodynamic Activity

The activities of alcohols in aqueous solution were calculated from the activity coefficients given by

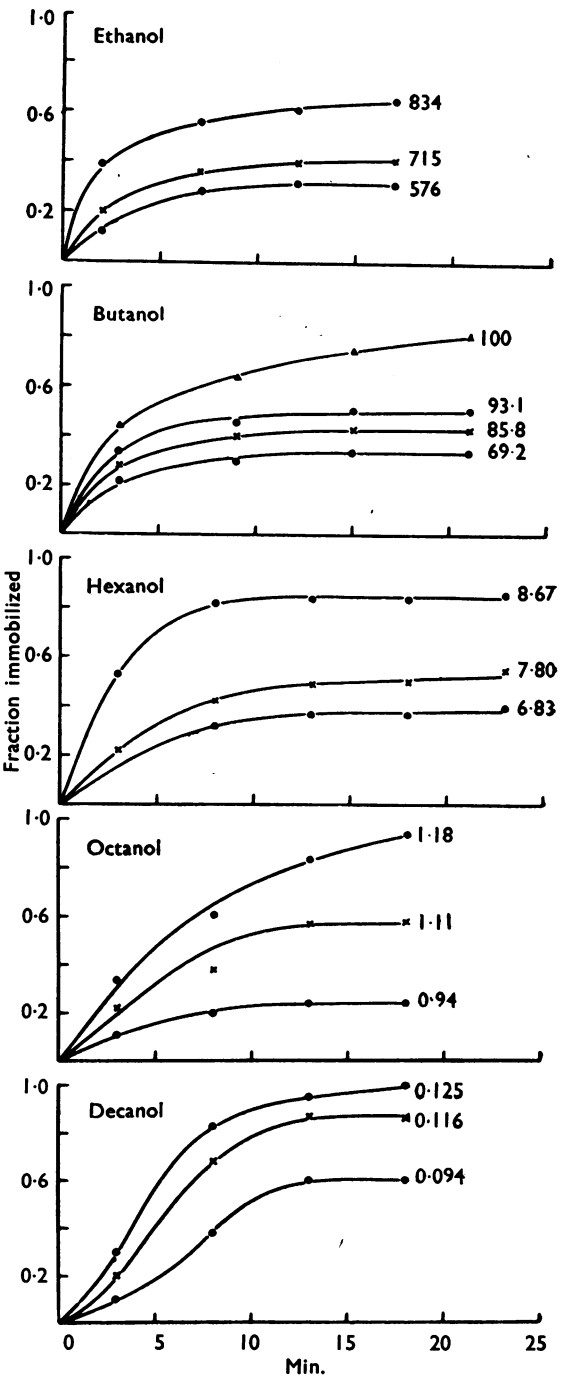

FIG. 1.—Time-action curves of alcohols on paramecium mobility. The figures appended to the curves show the alcohol concentration (mM). Each point represents the mean of three or four observations.

Butler, Thomson and MacLennan (1933), and the mole fraction of alcohol in solution, using the relation:

Thermodynamic activity = Activity coefficient × Mole fraction

For alcohols dissolved in Tyrode solution, no measured activity coefficients are available and certain approximations were necessary. Lindenberg (1948) found that the activity of ketones in sodium chloride solution behaved according to the equation:

$\log (\gamma_s/\gamma_0)/\mu = K = cN$

where γ_s, γ_0 = activity coefficients in salt solution and water respectively.

μ = ionic strength of salt solution.

K = displacement constant, peculiar to salt and organic solute.

c = proportionality constant, peculiar to organic solute.

N = molecular refraction of organic solute.

For ketones c = 6·3.

In the present measurements, this value of c was used and the ionic strength of Tyrode solution calculated to be 0.159. The correction was, in fact, small (10% for octanol, progressively less for lower alcohols).

Results

Paramecium Immobilization

The qualitative effects of alcohols on paramecium followed a fixed pattern. Paramecia normally move smoothly, rotating continuously about a longitudinal axis and occasionally changing direction abruptly. The first effect of alcohols is to cause their path to become spiral (coinciding with the rotation). The forward movement then becomes progressively slower and eventually stops. At this stage the anterior end is stationary while the posterior end moves in a circle. This movement gradually subsides.

These stages were clearer and the sequence took place more slowly with higher than with lower alcohols.

Such a sequence is unlikely to be the result of overall depression of the cell, and more probably represents a depression of ciliary action passing over the membrane in a fixed pattern.

Fig. 1 shows time-action curves of alcohols on paramecia for alternate members of the homologous series tested. In most cases a clear equilibrium was reached, the time taken for equilibration varying from about 8 min. with ethanol to about 13 min. with decanol. In a few cases (for instance, with the highest concentration of butanol and octanol) no clear equilibrium was established. Where this complication arose the percentage immobilization at the time of equilibrium with lower alcohol concentrations was used.

In other similar toxicity studies, various measures have been used as criteria of activity, such as percentage mortality after a standard time, time taken to kill 50%, etc. In view of the steadily increasing time taken for equilibration

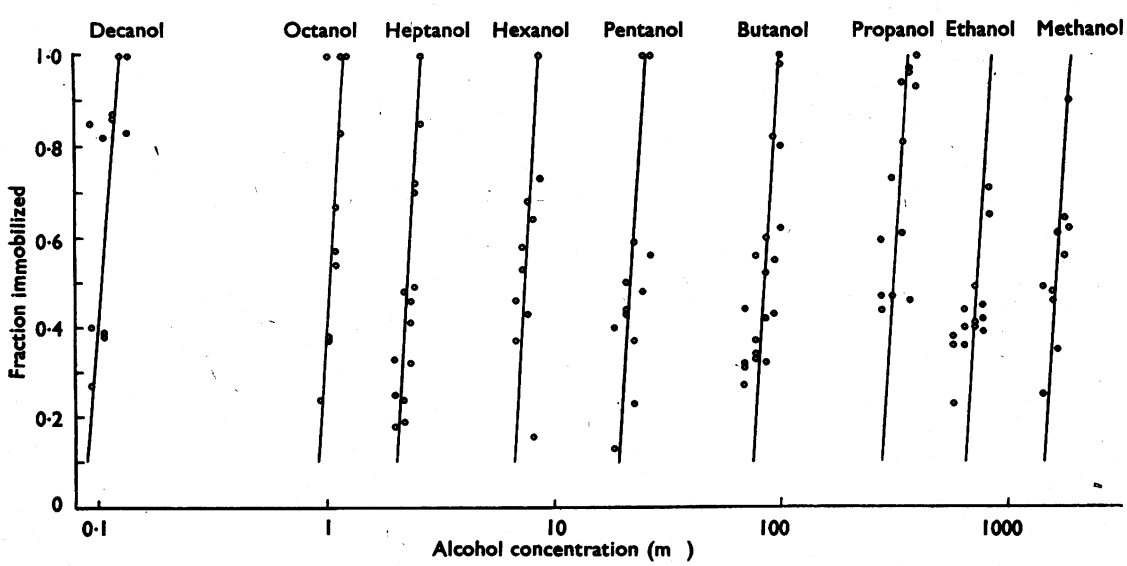

FIG. 2.—Concentration-action curves of alcohols on paramecium mobility. Each point represents a single observation. Parallel regression lines are drawn through the points.

observed in the homologous series of alcohols, it was felt that the criterion of activity must be independent of time relations. Hence percentage immobilization at equilibrium was used as a measure of pharmacological activity.

Fig. 2 shows the fraction of organisms immobilized at equilibrium plotted against alcohol concentration (on a logarithmic scale) for the series of alcohols from methanol to decanol (nonanol excluded). Each point represents a single determination. Since straight lines fit the points well the use of a probit transformation was considered unnecessary. The lines have all been plotted in accordance with a common regression coefficient, judged by eye. In no case does the regression line conflict with the trend of the points.

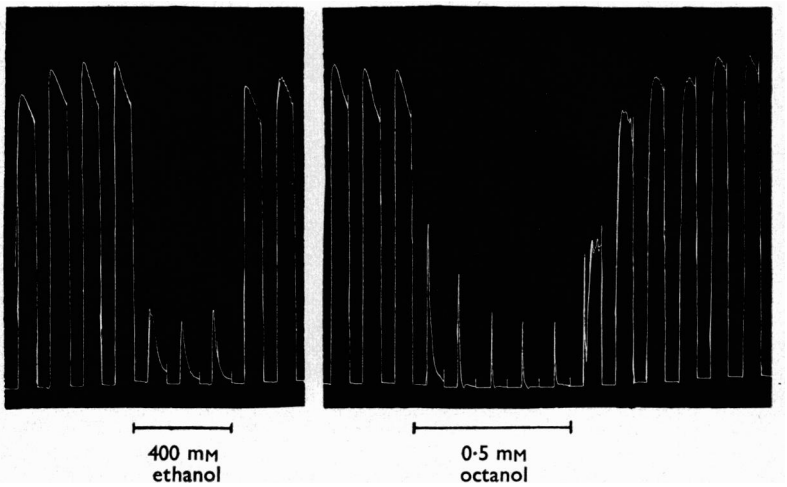

FIG. 4.—Effect of ethanol and octanol on maximal responses of the guinea-pig ileum to 1 μg./ml. acetylcholine.

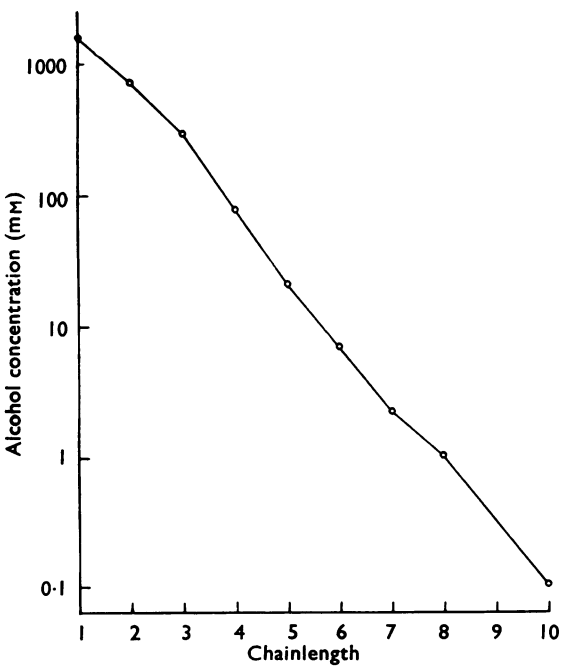

FIG. 3.—Relationship between alcohol chainlength (that is, the number of carbon atoms) and the concentration required to immobilize 50% of a population of paramecia. The concentrations are taken from the regression lines of Fig. 2.

Since these logarithmically-plotted regression lines are parallel, the ratio of concentrations of different alcohols required to immobilize a given fraction of organisms is independent of the fraction selected. Hence it is valid to use equiactive concentrations as a measure of the relative potencies of different alcohols.

The concentration of alcohol causing 50% immobilization at equilibrium has been used as a criterion for comparison. This concentration may be obtained from the regression lines in Fig. 2. The precision of these estimates may be expressed by the coefficient of variation, $\lambda(=0.039)$.

Fig. 3 shows the concentration of alcohol required for 50% immobilization plotted logarithmically against the number of carbon atoms in the chain. It shows a linear logarithmic decrease in equiactive concentration with increasing chainlength. Certain deviations from linearity are seen, namely a flattening at the top of the graph, and a flattening between heptanol and octanol. The flattening at the top has been found also in other experiments in the present series. The kink between heptanol and octanol, however, has not been observed elsewhere.

Depression of Gut Contractility

The action of alcohols is rapidly and completely reversible. This is shown in Fig. 4. The rate of recovery is somewhat less than the rate of development of the effect. If a series of equal doses of agonist are given, and then alcohol is added, the response declines in an exponential

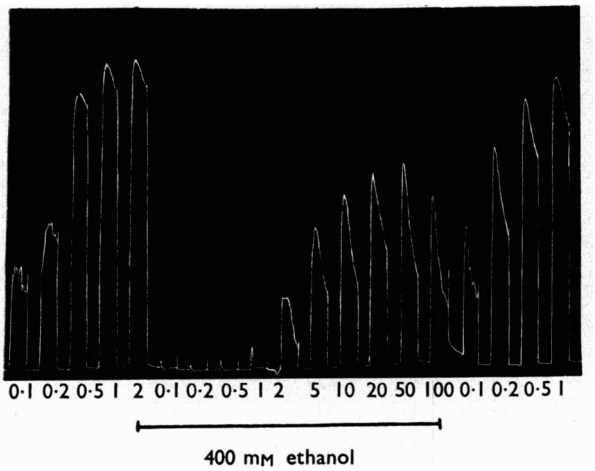

FIG. 5.—Effect of ethanol on the responses of the guinea-pig ileum to acetylcholine. Acetylcholine concentration in μg./ml. is shown under each contraction.

manner, eventually flattening off to a steady lower value. Recovery after removal of alcohol is similarly exponential. The time required for equilibration with alcohols increased with chain-length from about 1 min. for methanol to about 5 min. for octanol. Decanol was found not to reach equilibrium after 20 min. exposure and recovery of the response after its removal was incomplete.

The specificity of the action of alcohols was tested by comparing their effects on contractions produced by histamine, acetylcholine, and electrical stimulation. In one experiment, equivactive doses of histamine and acetylcholine were given alternately, and alcohol was added. Alcohol affected both responses equally and simultaneously. In another experiment, maximal doses of histamine were alternated with maximal electrically-induced contractions (which were slightly smaller than the histamine contractions). Alcohol affected both these responses equally, the electrically-induced contractions remaining smaller than the histamine contractions by the same fraction. Hence the action of alcohols shows no specificity.

It was found that alcohols diminished both the gradient and the maximum of agonist log-concentration action curves. Fig. 4 shows that there is a qualitative modification of the contraction by alcohols. The normal response of smooth muscle to addition of acetylcholine is an immediate rapid contraction, followed by a slower phase which reaches a peak and is followed by gradual relaxation in the presence of acetylcholine. In the presence of a strongly depressant concentration of alcohol, relaxation sets in immediately after the rapid phase of contraction. There is no slow phase of contraction, and relaxation is often complete before the stimulant is washed out. This strongly suggests that alcohols interfere with the contractile mechanism itself rather than with the receptors. The simplest

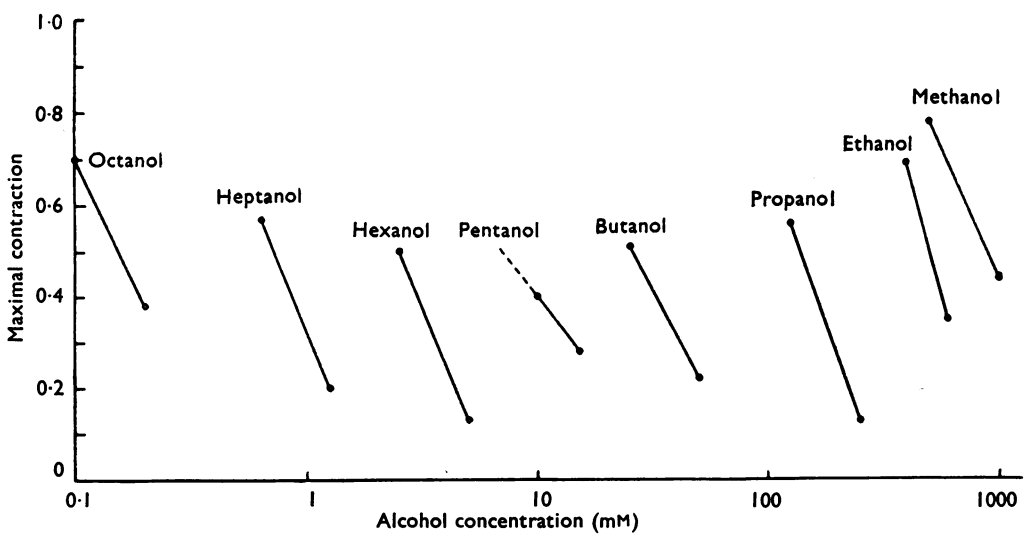

FIG. 6.—Concentration-action curves of alcohols on the responses of the guinea-pig ileum to acetylcholine. The ordinates represent the ratio of the maximal contractions in the presence and in the absence of alcohol. Each point represents a single determination and all were obtained on a single preparation.

way to measure such effects is by diminution of the maximal contraction (Arunlakshana and Schild, 1959).

In practice this was made difficult by the fact that, in the presence of depressants, the maximal contraction tends to diminish with successive doses of stimulant, as shown in Fig. 5. The acetylcholine dose was successively doubled in the presence of alcohol. The contractions increased in size up to a dose of 50 μg., but the response to the following dose of 100 μg. was considerably smaller. Increasing the dose interval reduced this effect, but considerably increased the error. The procedure adopted was to use a standard dose interval of just over one minute, and to take the largest response in a successively doubling series as the maximal response in the presence of depressant.

Preliminary experiments with alcohols showed that the relation between log-concentration of depressant and action in depressing the maximal contraction followed a sigmoid form, approximately linear between 20% and 80% effect, and tailing off symmetrically at either end. In the quantitative tests, therefore, each alcohol was applied in two concentrations, selected to give effects larger and smaller than 50% and to lie within the 20–80% range. The concentration required for a 50% effect, which was used as a criterion for comparison of potency, was obtained by linear interpolation.

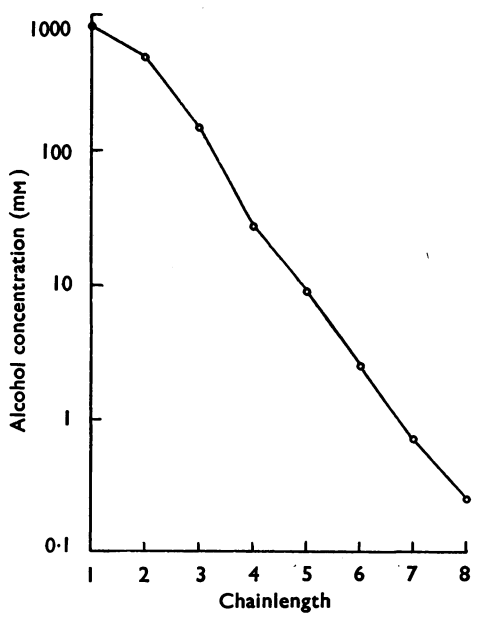

FIG. 7.—Relationship between alcohol chainlength and the concentration required to cause 50% depression of the maximal contraction of guinea-pig ileum to acetylcholine. The points plotted are the means of all the determinations.

Fig. 6 shows a series of two-point comparisons of the alcohols. Each point represents the mean of three or four observations on different ileum preparations. It was found that different preparations varied considerably in their sensitivity to alcohols, but the relative potencies of different alcohols varied much less. The coefficient of variation, λ, was 0.252, indicating an error about nine times that of the paramecium test. The parallel nature of the regression lines and their regular spacing in Fig. 6 is obvious. Fig. 7 shows the linear logarithmic decrease in the concentration required for 50% depression of maximal contraction (taken from the regression lines in Fig. 6) with chainlength. As observed with the paramecium immobilization experiments, there is a slight flattening between methanol and ethanol, but no other marked irregularity.

Depression of Lung Oxygen Consumption

Under the conditions of the experiment, oxygen uptake by lung particles in Tyrode solution continued at a steady rate for about 2½ hr. after placing the flasks in the Warburg apparatus. The rate of uptake in Tyrode solution, expressed as qO_2 (μl. absorbed per min. per mg. dry weight of tissue) varied from 2.5 to 5 in different animals, but samples from one animal gave consistent values.

In the presence of strongly depressant alcohol concentrations, the oxygen uptake frequently slowed and stopped after about 1½ hr. This was, however, always preceded by a period of steady uptake, from which the qO_2 was calculated. This cessation of oxygen uptake was probably due to the death of the tissue resulting from profound depression of its function.

As in the experiments on gut contractility, preliminary experiments showed that the log-concentration action curve was sigmoid in form, with a linear middle region, and two-point comparisons were again used. Fig. 8 shows the series of results obtained on the alcohol series. Each point represents a single experiment, and the straight lines in cases where more than two concentrations were used were drawn by eye. In the lower part of the series, from methanol to hexanol, the results are very similar to those obtained in the previous experiments in that the lines are all parallel and fairly regularly spaced,

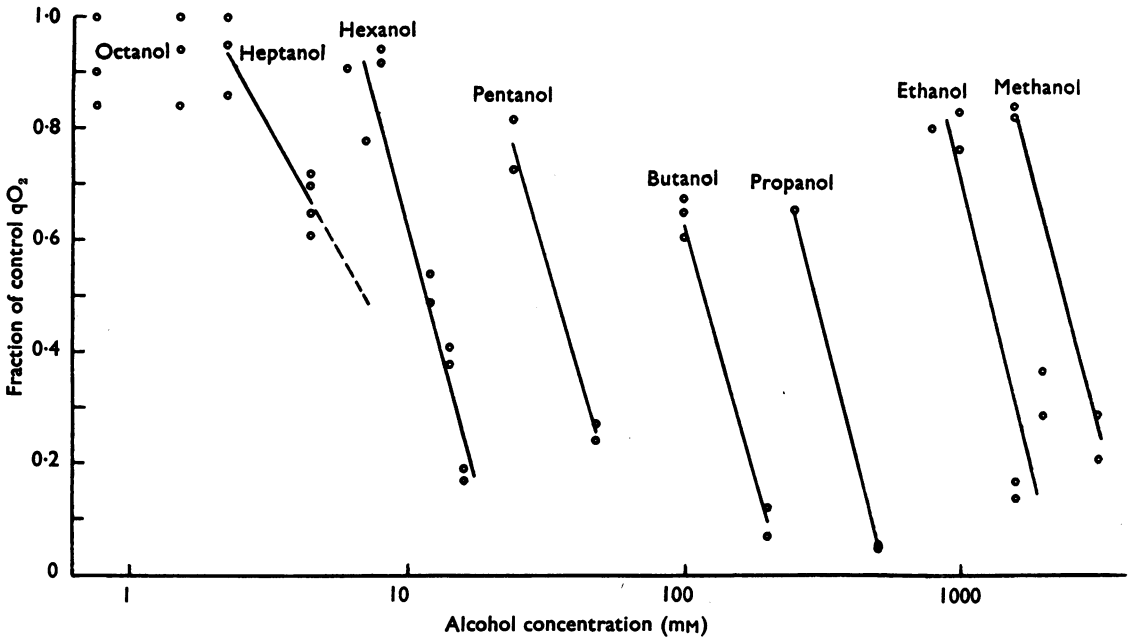

FIG. 8.—Concentration-action curves of alcohols on lung oxygen consumption. The ordinates represent the ratio of oxygen consumptions in the presence and in the absence of alcohol. Each point represents a single determination.

with the exception of methanol and ethanol. Above hexanol, however, the pattern changes completely. Heptanol shows a regression line less steep than the lower members, while octanol showed no significant activity at all. With both these alcohols the highest concentration used was close to saturation.

For the lower members of the series (hexanol downwards) the coefficient of variation, λ, was 0.182. Thus the variability was about six times that encountered in the paramecium tests, due partly to the lower gradient of the regression lines and partly to the greater scatter of the points.

The alcohol concentration required for 50% depression of oxygen consumption is shown plotted against chainlength in Fig. 9. This shows that, as far as hexanol, the pattern is the same as that found in the previous tests. The point for heptanol is taken from the extrapolated regression line of Fig. 8 and represents an unattainable concentration.

Throughout the series, the concentration required for 50% depression of oxygen consumption was approximately three times that required for 50% immobilization of paramecia and approximately 1.5 times that required for 50% depression of gut contractility.

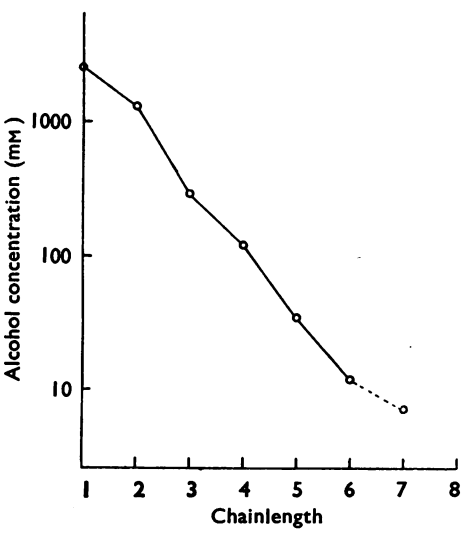

FIG. 9.—Relationship between alcohol chainlength and the concentration required to cause 50% depression of lung oxygen consumption. The value for heptanol was obtained by extrapolation of the regression line in Fig. 8, and represents an unattainable concentration.

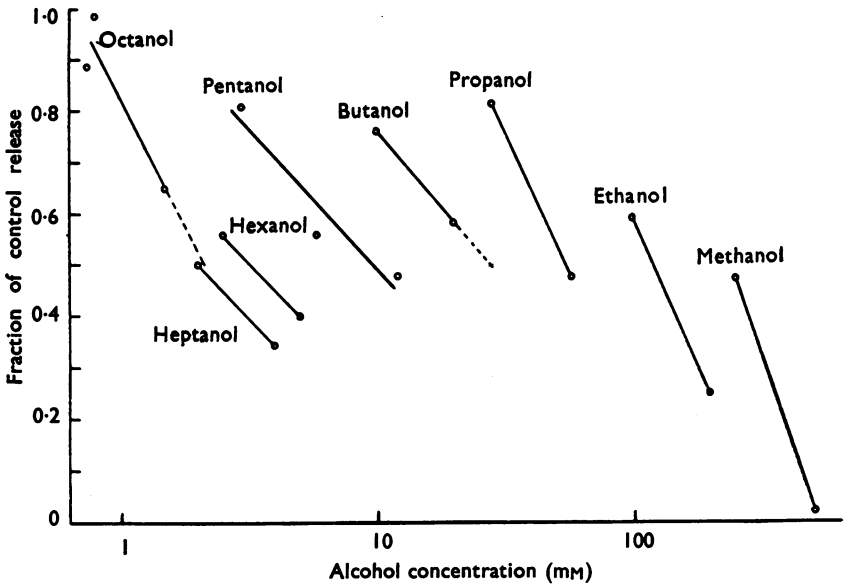

FIG. 10.—Concentration-action curves of alcohols on anaphylactic histamine release. Each point is the mean of all determinations at a particular alcohol concentration.

Depression of Lung Histamine Release

The amount of histamine released by antigen from chopped lung particles in the absence of any inhibitor varied considerably between preparations from different animals. Different samples from the same animal also showed some variation. The mean histamine release from fifty-three 0.2 ml. lung samples obtained from 18 animals was 0.96 μg. Expressed as a percentage of the total histamine, the variation was much less, and the mean value was 26%. In the absence of antigen under the same conditions the mean release was 3%.

As in the previous experiments, it was found that the log-concentration action curves for all these types of compounds were sigmoid in shape, with a middle linear region, and 2-point comparisons were again used.

The results obtained with the alcohol series are shown in Fig. 10. The deviations from parallelism are not significant. The coefficient of variation, λ, was 0.321, that is, the variability, was approximately ten times as great as that encountered in the paramecium experiments.

The spacing of the regression lines is fairly regular as far as hexanol, but the interval between hexanol and heptanol is less, and between heptanol and octanol there is no interval. The highest concentration of octanol used was close to saturation, and did not produce 50% inhibition. Thus, just as in the experiments on oxygen consumption, a cut-off point is reached, beyond which chainlength compounds show no activity. However, in the oxygen consumption experiments, heptanol showed a reduced gradient and an anomalously high equiactive concentration, and octanol showed no activity, while in these experiments both heptanol and octanol show the same gradient as the lower alcohols although their equiactive concentrations are anomalously high.

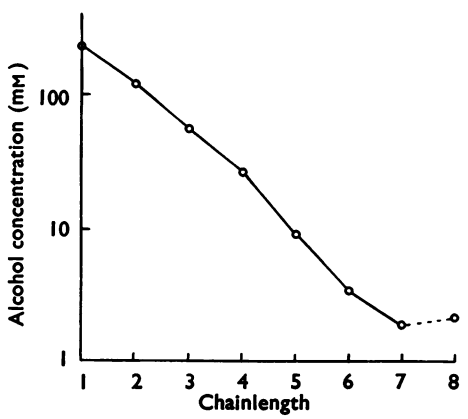

FIG. 11.—Relationship between alcohol chainlength and the concentration required to cause 50% depression of anaphylactic histamine release. The value plotted for octanol was obtained by extrapolation of the regression line in Fig. 10, and represents an unattainable concentration.

Fig. 11 shows alcohol concentration required for 50% inhibition of histamine release plotted against chainlength. Up to hexanol, the graph is approximately linear (no flattening between methanol and ethanol is apparent). The cut-off with octanol is clearly shown.

Discussion

The Log-Concentration Action Relationship

For each system investigated, a set of log-concentration action curves for the series of alcohols has been presented, and it is interesting to compare these figures. In each case the regression lines plotted were parallel over most of the range of alcohols investigated, but the gradient differs between different systems. Thus, in the tests on paramecium toxicity, the regression coefficient was 720, in the tests on gut contractility 90, in the tests on lung oxygen consumption 174, and in the tests on lung histamine release 59. In the paramecium toxicity test, the measurement was quantal, whereas in the other experiments it was almost certainly quantitative (one cannot be absolutely certain that these other effects were not due to the all-or-nothing depression of a certain proportion of cells, but this seems most unlikely). The coefficient given for the tests on paramecium toxicity is therefore not comparable with the other coefficients since it represents only the variability of the population, whereas the regression coefficient in quantitative experiments has other significance.

The constancy of gradient among different members of the homologous series indicates that the order of the reaction is constant. The difference in gradient between different biological systems may be interpreted in two ways. First, the reaction order may vary, which would suggest that the biophase shows fundamentally different properties in the different systems, and that the mode of action of alcohols in the different systems is different. In view of the characteristic pattern of activity in different members of the homologous series, it seems unlikely that the mode of action in different systems is as unrelated as this theory would suggest. Second, the discrepancies of gradient may be explained on the basis of a common mode of action if one supposes that the dependence of the different biological phenomena investigated on the single system which is affected by alcohols shows different quantitative relationships. Thus, the lung oxygen consumption (which has the steepest gradient) may show the complete range of inhibition, from 0 to 100%, over a narrow range of inhibition of the system which is the primary site of action of alcohols, whereas inhibition of histamine release takes place over a much wider range. This explanation appears to be more plausible than the first, since it preserves the unity of the action of unspecific depressants, which is the most striking feature evident in these results.

The differences which are observed in the higher and lower members of the homologous series are more difficult to explain than those of gradient between different systems. Methanol in most of the systems investigated showed a steeper gradient than the succeeding members of the series. It also showed an anomalous potency, greater than would be expected from its chainlength. Ferguson (1939) disposes of the anomalies of methanol by assuming that its physical toxicity is complicated by its chemical reactivity. This is a convenient explanation, but, without definite evidence that its mode of action differs from that of higher alcohols, it does not seem entirely justified. The fact that methanol shows similar anomalies in many of its physical properties (density, refractive index, etc.) does not dispose of the need to explain the anomalies in its biological action. Indeed, it would seem that if its biological distinctiveness could be explained in terms of its physical distinctiveness this would be a very useful step in the elucidation of the mechanism of action of unspecific depressants.

In the two systems investigated in which a cut-off in activity occurred in alcohols lower than octanol, an interesting discrepancy is evident. In the tests on depression of histamine release, no change in gradient of log-concentration action curves was evident before the cut-off activity. In contrast, the tests on depression of oxygen consumption showed that heptanol gave a regression line which was markedly less steep than that of the lower alcohols. Octanol showed no activity whatever. At present, no explanation is available to account for this.

Validity of the Concept of Unspecific Drug Action

The term " unspecific " implies that drugs of this class exert actions on a wide range of biological phenomena, and also that the relative potency of two different drugs is independent of the system on which they are tested. The investigations have verified the first implication, but the second is found not to hold. Fig. 12 is a composite diagram showing the effect of chainlength on equiactive alcohol concentration for the living

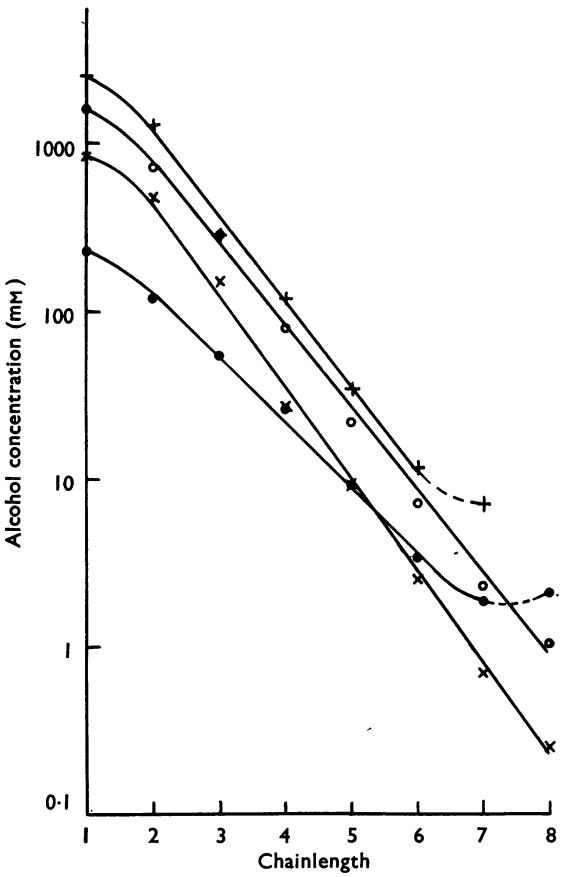

FIG. 12.—The relationship between alcohol chainlength and the concentration required for 50% depression of gut contractility (X), paramecium mobility (O), lung oxygen consumption (+), and lung histamine release (●). The curves have been smoothed out for clarity.

systems investigated. These lines are clearly not parallel. Thus equal concentrations of hexanol are required to cause 50% depression of both gut contractility and anaphylactic histamine release, while with ethanol the concentration required to depress gut contractility is about four times that required to inhibit histamine release. In this strict sense, therefore, the action is not truly unspecific, as ethanol might be said to be specific in depressing histamine release. Similarly, the phenomenon of cut-off means that alcohols completely lose their activity on one system, while retaining it on another. This again confers a type of specificity in certain higher alcohols. It seems, therefore, that the concept of completely unspecific drug action is untenable.

Ferguson's distinction of physical and chemical drug action seems to describe better the obvious differences between these classes of drugs. However, certain obstacles arise even with this distinction. There is the difficulty of deciding exactly what is meant by physical and chemical action. Ferguson and Hawkins (1949) have endeavoured to resolve this difficulty by investigating the toxic actions of very simple inorganic gases, including some inert gases, which are incapable of entering into chemical reactions. They found that these compounds exert the same actions as other narcotics acting by the alleged physical mechanism, and the thermodynamic activities required to produce a certain effect are in accordance with those found for other substances. Thus it is clear that no chemical process involving electron transfer takes place, but it would be difficult to demonstrate such a process taking place in the action of alleged chemically-acting drugs, whose action is entirely reversible. Indeed, it would seem quite possible that drugs acting at thermodynamic activities less than those compatible with Ferguson's criterion for physical activity might be doing so, not on account of any electron-transferring chemical process, but by virtue of some special molecular configuration which renders their physical interaction with the biophase much more disturbing than that produced by the wide range of undistinguished molecules which form Ferguson's physically-acting group.

We therefore have to turn to less direct criteria, of which the most illuminating is the thermodynamic activity of the compound required to produce an effect. Ferguson and Pirie (1948) found that the activities of compounds required to kill grain weevils fell into two clear groups. Those acting at activities between 0.1 and 1 they termed physical agents, and those acting at lower activities (usually very much lower) were termed chemical agents. Such a distinction separates the two groups very nicely if a single biological activity is considered, but different biological phenomena show quite wide variations in the thermodynamic activities of physically-acting drugs required to produce a given effect. Thus in the present investigation the thermodynamic activities of lower alcohols required to depress anaphylaxis were below 0.01, and in no case was an activity greater than 0.1 required with ethanol. This is obviously explained by the smaller disturbance of function which was used as a

TABLE I

CONCENTRATIONS (mM) AND THERMODYNAMIC ACTIVITIES OF ALCOHOLS REQUIRED FOR 50% DEPRESSION OF FUNCTION IN THE BIOLOGICAL SYSTEMS INVESTIGATED

Alcohol	Paramecium Mobility		Gut Contractility		Lung Oxygen Consumption		Lung Histamine Release	
	Concentration	Thermodynamic Activity	Concentration	Thermodynamic Activity	Concentration	Thermodynamic Activity	Concentration	Thermodynamic Activity
Methanol	1,600	0·0466	821	0·023	2,500	0·071	230	0·0065
Ethanol	730	0·0588	476	0·032	1,320	0·085	120	0·0077
Propanol	300	0·0694	149	0·035	298	0·070	56	0·0132
Butanol	81	0·0838	26·6	0·024	121	0·117	26·5	0·0234
Pentanol	21·8	0·105	9·0	0·038	34·4	0·145	9·5	0·0400
Hexanol	7·6	0·150	2·5	0·044	11·9	0·208	3·5	0·0613
Heptanol	2·3	0·178	0·71	0·049	7·1	0·497	1·9	0·133
Octanol	1·05	0·279	0·25	0·061			2·2	0·537

criterion of potency. In Ferguson and Pirie's investigation, the response observed was death.

Gavaudon, Dodé, and Poussel (1944), investigating the thermodynamic activities of compounds required to narcotize aquatic animals, found, similarly to Ferguson, that the activities required fell into two groups, but they suggested that the upper limit of activity for a chemically-acting compound was about 0.04. Clearly this limit cannot be set at any fixed value, since it will depend on the system investigated. The distinction between the activities of the two types of drug on any one system is, however, a useful criterion, and is good evidence that separate mechanisms of action are involved.

Relation Between Chainlength and Thermodynamic Toxicity

The term "thermodynamic toxicity" will be used to denote the inverse of the thermodynamic activity of a compound required to produce a certain effect. No theoretical significance attaches to the phrase.

Ferguson (1939) found, in general, a decrease of thermodynamic toxicity with chainlength within a homologous series, but Brink and Posternak (1948) found that it was constant. This is a question of some theoretical interest, and one which should have a definite answer.

Table I shows the thermodynamic activities of alcohols required for 50% depression of function in the four living systems investigated, and Fig. 13

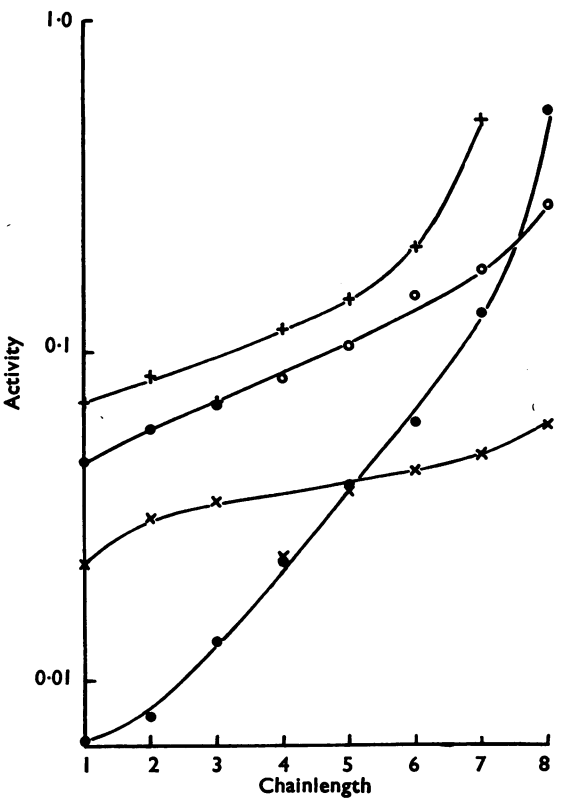

FIG. 13.—Relationship between alcohol chainlength and the thermodynamic activity required for 50% depression of gut contractility (X), paramecium mobility (O), lung oxygen consumption (+), and lung histamine release (●).

shows this plotted logarithmically against chain-length.

In the curves in Fig. 13, it is noticeable that the two cases in which no cut-off occurred, namely, in the tests on paramecium and gut contractility, the curves approximate to a straight line (a large deviation at butanol is evident in the gut contractility curve; this is probably error), while the other two curves in which cut-off was observed show a marked steepening at the top. It must be remembered that both of those tests were carried out in Tyrode solution, and are subject to approximations in the calculation of thermodynamic activity. It is not felt, however, that this can account entirely for the difference.

According to Ferguson's hypothesis, cut-off results from a steady decrease in thermodynamic toxicity with chainlength. Thus at a certain chain-length the required thermodynamic activity exceeds unity and is unattainable. According to this view, there is no reason to anticipate a sharp increase in the required thermodynamic activity immediately preceding the cut-off. However, the present results show that just before the cut-off the thermodynamic activity required is greater than would be predicted from the trend of the thermodynamic toxicity of preceding members of the series. Hence these results agree with Ferguson in that they show a decrease of thermodynamic toxicity with chainlength, and with Brink and Posternak in that they indicate a more rapid decrease of thermodynamic toxicity immediately preceding the cut-off. This conclusion is in agreement with most of the previous quantitative investigations of homologous series, in which it is found that complete cut-off is preceded by reduced thermodynamic toxicity.

It is thus necessary to explain not only the steady fall in thermodynamic toxicity with chainlength but also the more rapid fall which precedes the cut-off. At present no explanation is available for either of these observations, and an explanation will probably be found only when it is possible to test a concrete hypothesis of the mechanism of action of physically-acting drugs by comparing predictions with experimental results.

One feature of the results of Clark (1930) on heart muscle which defies explanation by Ferguson's hypothesis is that in some experiments the higher alcohols were applied in a concentration exceeding that of a saturated solution, in the form of what Clark calls a "colloidal suspension," obtained by prolonged shaking. Clark found that the concentration-action relationship showed no anomaly when such suspensions were used, yet their thermodynamic activity cannot exceed that of a saturated solution.

Mechanism of Action

Mullins (1954) suggested the cell membrane as the site of action, envisaging that the interstices in the lattice structure, which normally confer properties of permeability on it, become blocked up by inert foreign molecules, and the resulting decrease in permeability is responsible for the depression of function. It is indeed difficult to formulate any other mechanism by which the mere presence of inert molecules in a cell can depress its function, and in spite of the inadequate experimental proof of this hypothesis (see appendix) it seems the most probable explanation. It is perhaps an over-simplification to assume that the external cell membrane is the seat of this action: it seems just as likely that some internal barrier, such as the membrane enveloping the mitochondria or other cytoplasmic inclusions, is the site of action, or possibly many different membranes may be involved.

It seems likely that physically-acting depressants in sufficient concentration will depress any aspect of cell function. The question thus arises whether all the functions are depressed in turn as a result of progressive depression of one single system (possibly an enzyme system or decreased permeability of the outer cell membrane), or whether they are depressed individually by these drugs. This question cannot at present be answered. Experiments on the action of unspecific depressants on viable sub-units of the cell, such as mitochondria, might prove illuminating. If it were found that mitochondria were depressed by alcohols in the same way as whole cells, this would be evidence of independent depression of the different units of a living cell, but, if the action on mitochondria differed from that characteristic action on whole cells, then one might assume that depression of some single unit of the cell was responsible for the secondary depression of its many different functions.

Another line of research which might possibly prove constructive in elucidating the mechanism of action is the investigation of the action of alcohols on some synthetic non-living membrane. Thus it would probably prove possible to produce a monolayer of orientated protein molecules, and to study its permeability and the effect of alcohols on it. In this way it might be possible to test Mullins' theory on a system in which it is known that there are no enzymes, and whose physical chemistry should be much easier to work out than that of a cell membrane.

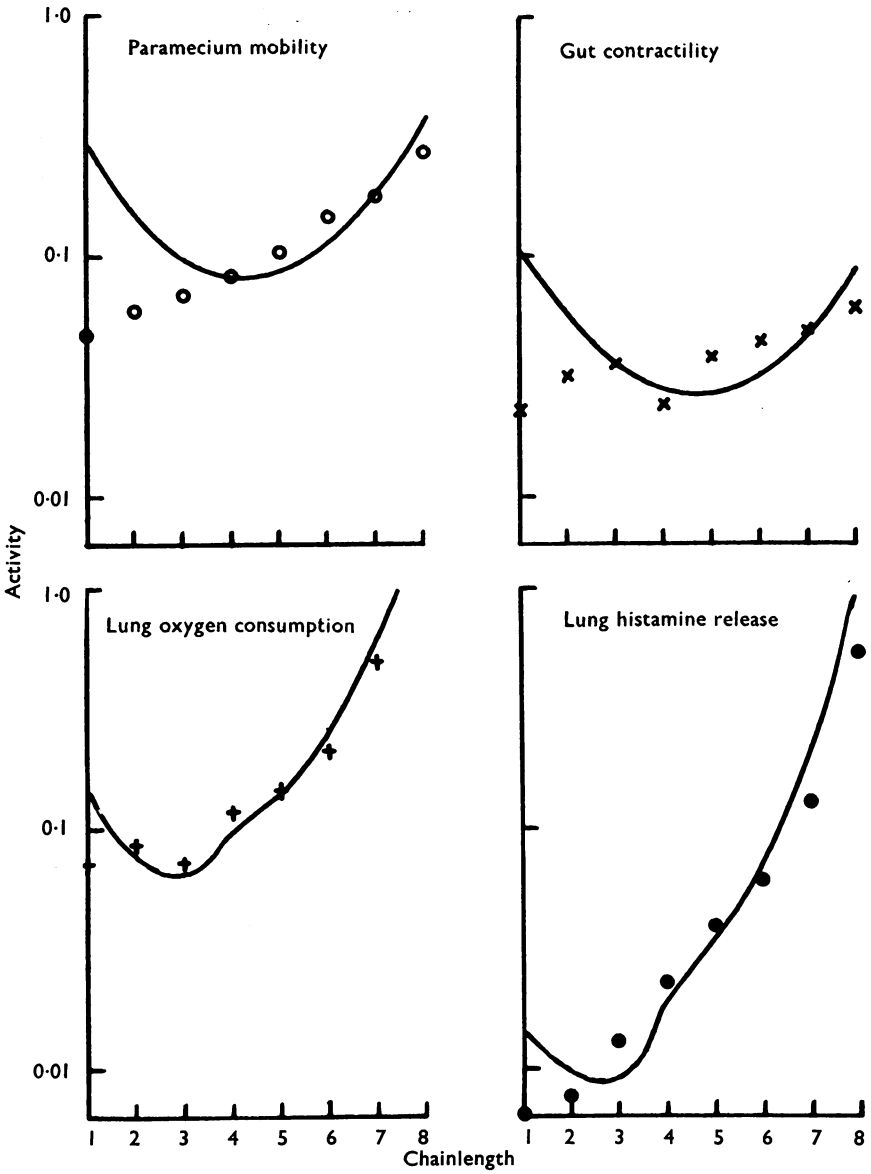

FIG. 14.—Comparison of experimental results with predictions based on Mullins' theory of narcosis. The points are experimental results. The curves are the best-fitting obtainable from Mullins' hypothesis. The constants used in constructing the curves are given in the Appendix.

APPENDIX

Application of Mullins' Hypothesis

Mullins (1954) suggested that narcotics act by dissolving in some phase within the cell (probably a membrane) and that equal narcotic action occurs when an equal volume fraction of the membrane is occupied by narcotic molecules, regardless of their chemical structure.

In order to test this hypothesis, it is necessary to estimate the concentration of narcotic in the biophase. Mullins attacked this problem by estimating the activity coefficient of narcotic dissolved in the biophase, using principles put forward by Hildebrand and Scott (1950). He thus derived the following relationship:

$$\ln\gamma_{nar} = \frac{V_m(\delta_{nar}-\delta_{mem})^2}{RT} \quad \ldots\ldots\ldots\ldots(1)$$

where γ_{nar} = activity coefficient of narcotic in membrane.

V_m = molar volume of narcotic.

δ_{nar}, δ_{mem} = solubility parameter of narcotic and membrane respectively.

This equation is based on theories applying to mixtures of non-polar solvents. A large error may be incurred by applying it in the present circumstances.

The volume fraction of narcotic in the membrane is given by the expression, $X_{nar}V_m$, where X_{nar} is the mole fraction of narcotic in the membrane.

$\gamma_{nar}X_{nar}$ = A_{nar}, the thermodynamic activity required for narcosis.

$\therefore A_{nar}V_m = \gamma_{nar}X_{nar}V_m$.

According to Mullins' hypothesis, $X_{nar}V_m$ is constant.

$\therefore A_{nar}V_m \propto \gamma_{nar}$ .....(2)

In order to calculate γ_{nar} from equation (1), δ_{mem} must be known. This cannot be measured directly. A series of curves of log γ_{nar} against chainlength for different values of δ_{mem} were plotted according to equation (1) using values of V_m and δ_{nar} given by Mullins. According to Mullins' hypothesis (equation 2), one of these curves should be the same shape as the experimental plot of log $A_{nar}V_m$ against chainlength. Thus from each of the four biological systems investigated a value of δ_{mem} giving the best fit between experimental data and theoretical prediction was selected. Fig. 14 shows the experimental data (points) compared with the best fitting theoretical curves. The values of δ_{mem} and $X_{nar}V_m$ used in fitting the curves are as follows:

	$X_{nar}V_m$	δ_{mem}
Paramecium mobility	7·34	11·8
Gut contractility	2·52	11·5
Lung oxygen consumption ..	5·25	12·9
Lung histamine release ..	0·63	13·5

With the paramecium and gut contractility experiments the fit is very poor, but it is much better in the experiments on histamine release and oxygen consumption. However, the striking feature of the theoretical predictions—the minimum at a certain chainlength—is entirely lacking in the experimental results. Some of Mullins' results, relating to depression of nervous tissue, do in fact show this minimum, and the fit is much more convincing. Mullins found that the membrane solubility parameter giving the best fit was about 11, while in the present case it was about 13 in the only cases in which a possible fit was obtained. A parameter of 11 corresponds to a molecule with approximately the same ratio of hydrophilic and hydrophobic groups as butanol, which corresponds roughly with what is known of the cell membrane. A parameter of 13 corresponds to the hydrophilic/hydrophobic ratio of ethanol, which is most unlikely to correspond with the cell membrane.

The present results do not, therefore, support Mullins' hypothesis. However, the theoretical predictions are based on doubtful assumptions, and allow no firm conclusions to be drawn.

My sincere thanks are due to Dr. H. O. Schild, whose advice and encouragement were invaluable.

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THE ACTION OF LOCAL ANAESTHETICS ON THE SPINAL CORD OF THE CAT

BY

D. TAVERNER

From the Department of Medicine, University of Leeds

(RECEIVED DECEMBER 12, 1959)

The effects of intravenously injected lignocaine, procaine and amethocaine on the monosynaptic and polysynaptic reflexes and the cord dorsum potentials elicited by selective electrical stimulation of appropriate afferent fibres have been investigated in decerebrate cats before and after strychnine. The three drugs depress the amplitude of the reflex responses in the basal preparation for 40 to 50 min. Smaller doses eliminate the reflex augmentation produced by strychnine for comparable periods. Very small doses selectively abolish the augmentation of the P component of the cord dorsum response induced by strychnine. The drugs have no effect on post-tetanic potentiation of the monosynaptic reflex response evoked from the gastrocnemius muscle in the cat. Their activity on the spinal cord is roughly proportional to their local anaesthetic potency. The ratios of the doses which produce the same central activity are amethocaine 3: lignocaine 10: procaine 20.

It is known that, in addition to their peripheral effects, local anaesthetics when injected intravenously can modify the activity of the central nervous system in animals and man. Bernhard and Bohm (1954, 1955) showed that intravenous injections of local anaesthetics shortened or abolished the cortical after-discharge evoked by electrical stimulation of the cerebral cortex in monkeys. Bernhard, Bohm, Höjeberg, and Melin (1956) found that intravenous lignocaine would prevent the electroencephalographic appearance of cortical seizure activity on photic stimulation in epileptic patients. Evidence was presented by Bernhard, Bohm, and Höjeberg (1955) which suggested that intravenous lignocaine would prevent or stop both focal and generalized epilepsy, and Taverner and Bain (1958), using a "double-blind" trial technique, showed that this drug was an effective anticonvulsant in patients with status epilepticus.

In both animals and humans Bernhard *et al.* (1954, 1955) observed that local anaesthetics had little effect on the normal activity of the central nervous system, in contrast to their striking effect upon over-active neurones. The present investigation was undertaken to study the effect of local anaesthetics on the spinal cord of the cat under basal experimental conditions and after over-activity had been induced. The results show that intravenous local anaesthetics have effects on the spinal cord in cats which are comparable to those they exert on the brain, and that the central activity of the drugs tested is proportional to their local anaesthetic potency.

METHODS

The experiments were performed upon cats. Decerebration or spinal section was carried out under ether anaesthesia 2 to 3 hr. before the experiments began. The details of the electro-physiological technique have been reported (Bernhard and Taverner, 1951; Taverner, 1952, 1954). The rate of stimulation was once every 4 sec. The height of the main component of the polysynaptic reflex response was measured in preference to measurement of its total area. Previous personal studies by the present author, confirmed in the present work, have shown that this method provides an acceptable, though slightly less accurate, measure of the drug induced changes in a particular polysynaptic reflex evoked under standard conditions of stimulation and recording. The drugs used were amethocaine hydrochloride (0.1%), tubocurarine chloride (0.1%), lignocaine hydrochloride (0.1%), procaine hydrochloride (0.1%), and strychnine hydrochloride (0.01%), and amounts given in the text refer to weights of the salts in each case. They were injected slowly through a polythene tube lying in the brachial vein. The arterial blood pressure was recorded from the common carotid artery.

RESULTS

Electrical stimulation of the group I low-threshold afferent fibres in the nerve to the

gastrocnemius muscle is followed after an interval of 2.5 to 3 msec. by a synchronous reflex discharge in the S1 ventral root. It is usually accepted that this discharge represents activity in two-neuronal monosynaptic reflex arcs, and the efferent nerve impulses run in fibres which pass to the muscles from which the afferent fibres arise (Lorente de Nó, 1935; Renshaw, 1940; Lloyd, 1943). This electrical activity is probably equivalent to the myotatic reflexes or tendon jerks of clinical medicine. Electrical stimulation of a cutaneous nerve such as the sural results in an asynchronous, irregular discharge which appears in the S1 ventral root about 4 to 5 msec. later and lasts for 10 msec. or more. Such impulses have been shown by Lloyd (1943) to represent activity in more complex reflex pathways with several interpolated, internuncial neurones (polysynaptic reflexes). It is believed that the flexor and crossed extensor reflexes depend upon such polysynaptic chains.

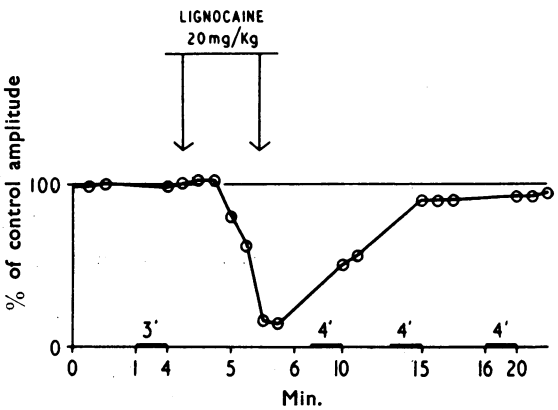

FIG. 1.—Effect of 20 mg./kg. lignocaine on the monosynaptic reflex evoked by stimulation of the nerve to the gastrocnemius muscle. Decerebrate cat. Ordinate: Reflex response as percentage of pre-injection amplitude. Abscissa: Time in min. Bars on base line represent suspension of recording.

Action of Local Anaesthetics on the Spinal Reflexes in the Decerebrate Preparation

Lignocaine.—The intravenous injection of doses of less than 5 mg./kg. lignocaine had no effect on the spinal reflexes. In about half the preparations 5 mg./kg. lignocaine intravenously caused a reduction in amplitude of the polysynaptic reflex to 50% of its pre-injection level, but the monosynaptic reflex was only reduced by about 20% of its pre-injection level. These reductions in amplitude were transient and the reflexes had returned to their pre-injection level within 20 min. Larger doses of lignocaine gave greater and more persistent reductions in the amplitude of the two reflexes.

Fig. 1 shows the effect of injecting 20 mg./kg. lignocaine intravenously in a decerebrate preparation. The injection was given slowly and no significant change in the blood pressure was observed. 30 sec. after the start of the injection the amplitude of the monosynaptic reflex began to fall, and by the end of the injection it was only 15% of the pre-injection amplitude. The reflex was tested at intervals, and it was found that recovery began about 5 min. after the injection and was complete within 10 min. The effect of the same dose of lignocaine (20 mg./kg.) on the polysynaptic reflex is shown in Fig. 2. The reflex began to fall in amplitude soon after the beginning of the injection, and had fallen to 10% of the pre-injection level before the injection was finished. Recovery began about 10 min. later but was slow. 20 min. later the reflex was still only 50% of the pre-injection amplitude and it did not recover until almost 60 min. after the injection. Doses of lignocaine larger than 20 mg./kg. were not given.

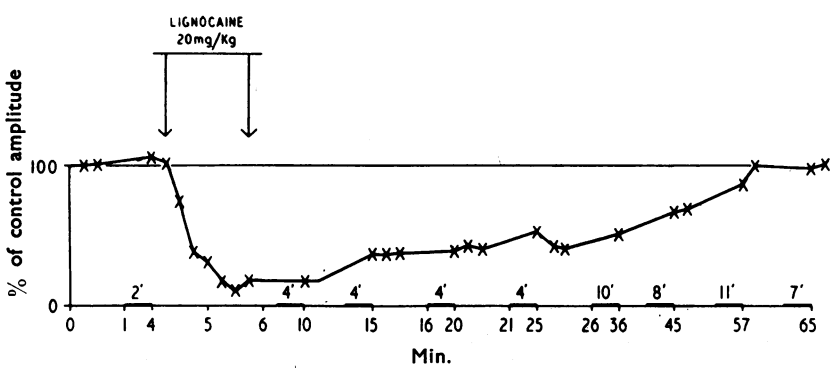

FIG. 2.—Effect of 20 mg./kg. lignocaine on the polysynaptic reflex evoked by stimulation of the sural nerve. Details as Fig. 1.

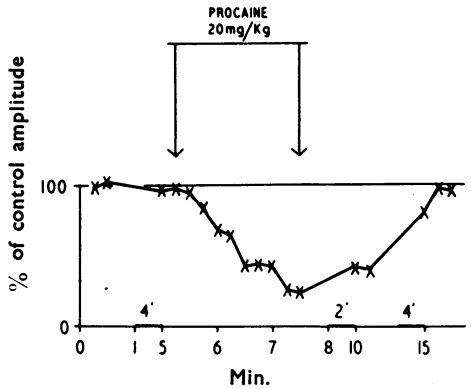

FIG. 3.—Effect of 20 mg./kg. procaine on the polysynaptic reflex evoked by stimulating the sural nerve. Details as Fig. 1.

Procaine.—Intravenous procaine had similar but much less marked effects on these reflexes. Fig. 3 shows the effect of 20 mg./kg. procaine on the polysynaptic reflex in a decerebrate preparation. The amplitude of the reflex fell to 25% of the pre-injection level at the end of the injection but had returned to normal within 15 min. There was no definite effect on the monosynaptic reflex with this dose of procaine. Larger doses of procaine were not given.

Amethocaine.—Amethocaine was found to have effects similar to those of lignocaine but at much lower dose levels. Fig. 4 shows the effect of the injection of 3 mg./kg. amethocaine on the spinal reflexes of a decerebrate preparation. The monosynaptic reflex fell to 50% of the pre-injection level and began to recover about 15 min. later. The reflex had returned to its pre-injection amplitude after 25 min. Similar but more marked changes were observed in the amplitude of the polysynaptic reflex after the same dose of amethocaine. The amplitude fell to 30% of the pre-injection level, recovery began after about 15 min. and the reflex had returned to its pre-injection amplitude 30 min. after the injection. Larger doses of amethocaine produced more profound and longer lasting changes in both reflexes, but with larger doses there was difficulty in maintaining the blood pressure in the preparations. In all experiments it was found that the polysynaptic reflex was affected more than the monosynaptic reflex by intravenous local anaesthetics and that both the extent of the depression and its duration were related to the dose given. Amethocaine was approximately 4 to 5 times as active as lignocaine in its effect on the spinal reflexes.

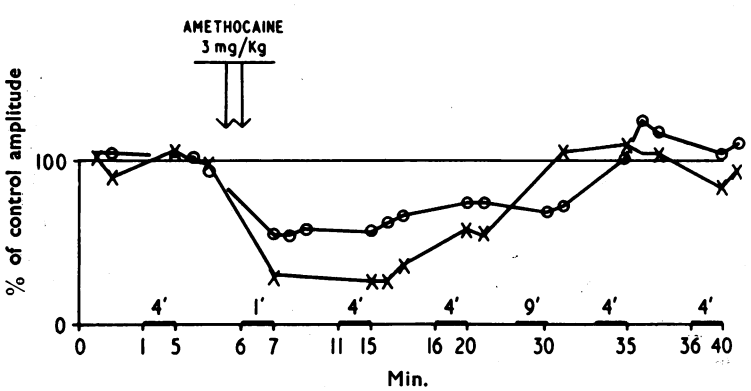

FIG. 4.—Effect of 3 mg./kg. amethocaine on the monosynaptic (O——O) and polysynaptic reflexes (X——X). Details as Fig. 1.

Action of Intravenous Local Anaesthetics on Strychninized Decerebrate Preparations

Strychnine causes a marked increase in the amplitude of the polysynaptic reflex and an increase in the P wave of the cord dorsum potential evoked by stimulating a cutaneous nerve such as the sural. In these experiments the cord dorsum potentials were recorded by a monopolar electrode placed on the dorsal surface of the first sacral spinal segment. It has been suggested by Bernhard and Widén (1953) that the N1 potential represents activity in monosynaptically activated cell bodies in the dorsal grey matter which do not participate in reflex transmission. The N1 deflection is followed by an inconstant positivity (P deflexion) which originates from propriospinal interneurones extending over several segments of the spinal cord. Taverner (1952) showed that strychnine increased the amplitude of the P deflexion in all types of preparation and increased the amplitude of the N1 deflexion in preparations with low spinal cord section.

Action on Polysynaptic Reflex Responses.—It was found that intravenously injected local anaesthetics were able to reverse the effect of strychnine in much smaller doses than those which modified the activity of preparations which had not received strychnine. Fig. 5*a* shows the changes in amplitude of the

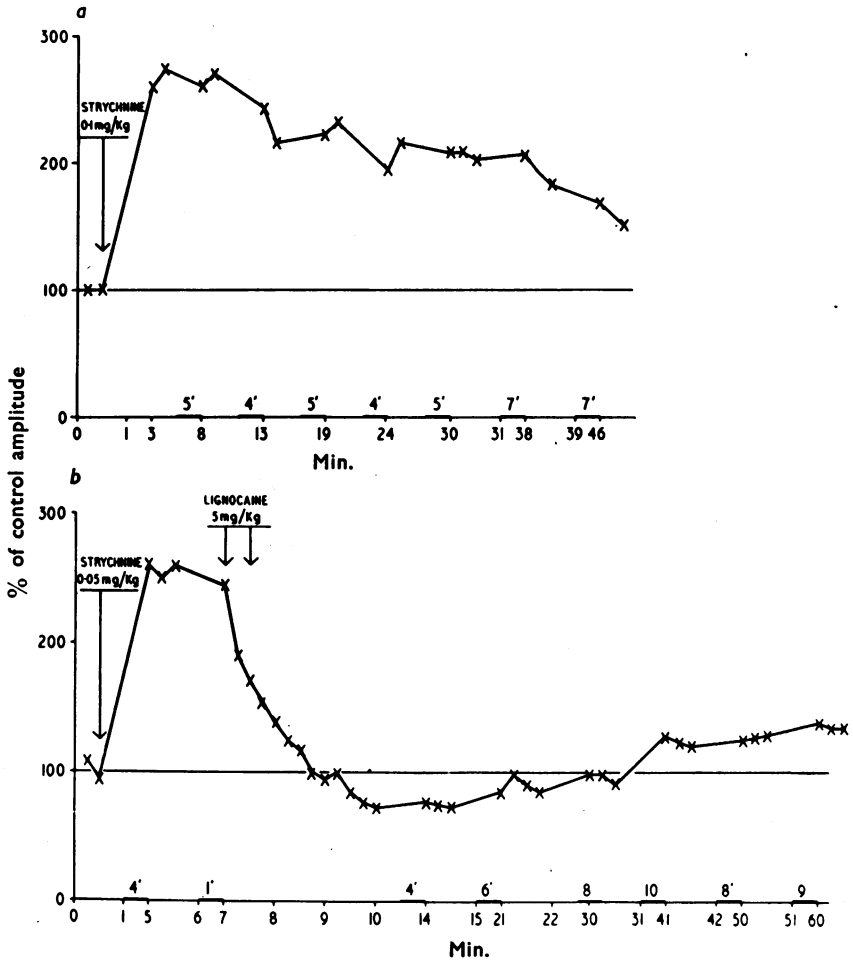

FIG. 5.—*a*, Effect of 0.1 mg./kg. strychnine hydrochloride on the polysynaptic reflex evoked by stimulation of the sural nerve. *b*, Same preparation. Effect of 5 mg./kg. lignocaine on the polysynaptic reflex after the injection of a further 0.05 mg./kg. strychnine hydrochloride. Details as Fig. 1.

polysynaptic reflex immediately after the injection of 0.1 mg./kg. strychnine hydrochloride intravenously in a curarized decerebrate preparation. The reflex increased in amplitude to about 250% of the pre-injection level and then declined slowly in amplitude but did not reach the pre-injection level 60 min. after the injection. Fig. 5*b* shows the effect of a further injection of 0.05 mg./kg. strychnine hydrochloride in the same preparation. The polysynaptic reflex again increased to 250% of the pre-injection level and the same gradual fall in amplitude began. 7 min. after the strychnine was injected, 5 mg./kg. lignocaine was injected intravenously. There was an immediate fall in the amplitude of the polysynaptic reflex to below the pre-strychnine level. 3 min. later the reflex was 75% of the pre-strychnine level, and 20 min. later it had only reached its normal pre-strychnine level. 30 min. after the injection the polysynaptic reflex had begun to increase in amplitude again and was then about 130% of its pre-strychnine value. It remained at this level for a further 30 min., but eventually fell to the pre-strychnine level again.

Similar results were obtained after the injection of procaine in doses of more than 10 mg./kg., but doses of less than 5 mg./kg. had no effect. 2 mg./kg. amethocaine gave comparable reduc-

tions in the amplitude of the polysynaptic reflex after augmentation by strychnine.

Action on Cord Dorsum Potentials.—The action of lignocaine on the cord dorsum potentials is illustrated in Fig. 6. The injection of 1.3 mg./kg. strychnine in a decerebrate preparation with low spinal section at the junction of the dorsal and lumbar segments of the spinal cord caused an increase in the amplitude of the N1 potential to 130% of the pre-injection level. At the same time a P deflexion appeared and its maximum amplitude has been taken as 100%. These changes persisted for 20 min. when an injection of 1 mg./kg. lignocaine was given. The N1 potential was unchanged, but there was an immediate fall in the amplitude of the P deflexion, which rapidly disappeared and did not return.

Similar results in other preparations were obtained with 0.4 mg./kg. amethocaine and with 2 mg./kg. procaine.

Action of Local Anaesthetics on Post-tetanic Potentiation

The amplitude of a monosynaptic reflex response can be increased two- or three-fold by tetanic stimulation of its afferent pathway (Lloyd, 1949). This effect persists for 1 to 2 min., and it has been suggested that it results from temporary physical enlargement of the synaptic endings of the afferent nerve fibres (Eccles, 1953). Tetanic stimulation of polysynaptic reflex pathways has little effect (Lloyd, 1949).

Post-tetanic potentiation of the monosynaptic reflex response evoked by stimulation of the nerve supplying one head of the gastrocnemius muscle was studied before and after the intravenous injection of the three local anaesthetics. Doses comparable to those producing changes in normal spinal cord reflex activity were shown not to have any significant effect on the degree or duration of the post-tetanic potentiation of the monosynaptic reflex response.

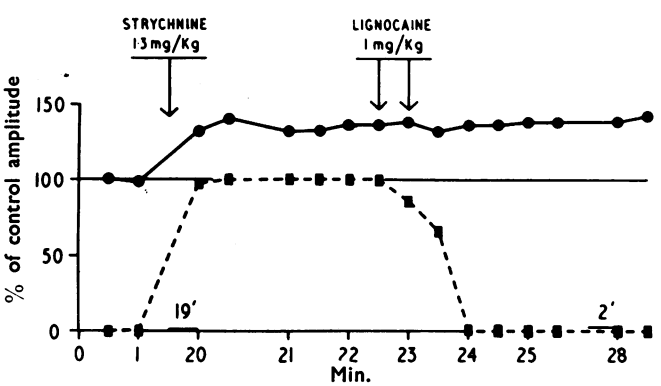

FIG. 6.—Effect of 1.3 mg./kg. strychnine hydrochloride followed by 1 mg./kg. lignocaine on the N1 (●——●) and P (■— — —■) components of the cord dorsum potentials evoked by stimulating the sural nerve. Details as Fig. 1.

DISCUSSION

In this investigation the observations of Bernhard and Bohm (1954, 1955) on the actions of systemic local anaesthetics on the cerebral cortex of the monkey have been extended to the spinal cord of the cat. The reduction in spinal cord activity after the injection of these drugs is of the same order and duration as that found in the brain. The mechanism of this effect is unknown.

It was found that much smaller doses of these agents were required to reverse the effects of strychnine on the spinal cord reflexes and on the cord dorsum potentials than were necessary to depress them in the absence of strychnine. The duration of action, however, was generally the same. In spite of the similarity of this action to the abolition by local anaesthetics of cortical over-activity after electrical stimulation and in clinical epilepsy, it is doubtful if the two forms of over-activity are comparable. There is now strong evidence that the central excitatory action of strychnine is due to the selective depression of both direct and indirect inhibition, and the mechanism may be one of competition with the inhibitory transmitter for the steric configurations on the inhibitory post-synaptic membrane (Bradley, Easton, and Eccles, 1953). The failure of local anaesthetics to influence the amplitude and duration of the post-tetanic accentuation of the monosynaptic reflex response is perhaps suggestive that local anaesthetics exert their action upon the inhibitory post-synaptic membrane, but there is no direct evidence at present.

The relative potency of the three drugs studied was found to be related to their local anaesthetic activity. There was some variation in different experiments, but in general amethocaine was more effective than lignocaine, which was itself more potent than procaine. 3 mg./kg. amethocaine intravenously produced the same reduction in amplitude of evoked

spinal cord activity as 10 mg./kg. lignocaine and 20 mg./kg. procaine. A similar relationship holds for the activity of these drugs in preparations which had received strychnine. These proportions correspond well with the relative doses of the three drugs needed to produce the same local anaesthetic effect, which are approximately 1 : 5 : 10 (Goodman and Gilman, 1955).

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