Vol. 15. No. 3.

SEPTEMBER, 1960

BRITISH JOURNAL OF PHARMACOLOGY AND CHEMOTHERAPY

LONDON
BRITISH MEDICAL ASSOCIATION
TAVISTOCK SQUARE, W.C.1.

YEARLY SUBSCRIPTION (4 NUMBERS) £4 4s. U.S.A. $13.50. SINGLE NUMBERS £1 5s.

Brit. J. Pharmacol. (1960), **15**, 361.

BRITISH PHARMACOLOGICAL SOCIETY AND SCANDINAVIAN PHARMACOLOGICAL SOCIETY

COPENHAGEN MEETING, 26th, 27th, and 28th JULY, 1960

SYMPOSIUM ON "THE PHARMACOLOGY OF MEMBRANES"

Dr. POUL KRUHØFFER, Institute of Physiology, University of Copenhagen: "Physiology of Membranes."

Dr. KARL ZERAHN, Institute of Biological Chemistry, University of Copenhagen: "The Sodium Pump."

Dr. JENS CHR. SKOU, Institute of Physiology, University of Aarhus: "The Effect of Drugs on the Cell Membrane."

Dr. STEPHEN THESLEFF, Institute of Pharmacology, University of Lund: "The Influence of Innervation of Membrane Properties."

DEMONSTRATIONS

1. **P. Lund Nilsen** (*Pharmacological Laboratory, Meco-Dumex, 37 Prags Boulevard, Copenhagen, S*).

Determination of local anaesthetics by the mouse-tail method.

COMMUNICATIONS

1. **A. L. A. Boura** and **A. F. Green** (*The Wellcome Research Laboratories, Beckenham, Kent*).

New adrenergic neurone blocking agents.

2. **A. L. A. Boura, A. McCoubrey** and **W. G. Duncombe** (*The Wellcome Research Laboratories, Beckenham, Kent*).

The distribution of bretylium and other quaternary ammonium salts in the peripheral sympathetic nervous system.

3. **A. K. Armitage, Janet Boswood** and **B. J. Large** (*May & Baker, Dagenham, Essex*).

Some pharmacological properties of a new xanthine derivative with potent bronchodilator and coronary dilator properties.

4. **A. Herxheimer** (*Department of Pharmacology, The London Hospital Medical College, London, E.1*).

Some interactions of morphine and nalorphine in mice.

5. **D. J. Jenden** (*introduced by* **W. L. M. Perry**) (*Department of Pharmacology, University of California Medical Center, Los Angeles 24, California*).

The effect of ryanodine on relaxing systems in skeletal muscle.

6. **E. M. Vaughan Williams** (*Department of Pharmacology, South Parks Road, Oxford*).

A comparison of the effects of 5-hydroxytryptamine, histamine and anaphylaxis on cardiac intracellular potentials and contractions.

7. **C. Rerup** (*Department of Pharmacology, University of Lund, Sweden*).

Difference parameters in the twin cross-over test (insulin and corticotrophin) and their consequences for assay validity.

8. **K. Martindale, G. F. Somers** and **C. W. M. Wilson** (*University of Liverpool and the Distillers Co. (Biochemicals) Liverpool*).

Some factors affecting the production of gastric ulcers in guinea-pigs and rats.

9. **M. W. Parkes** and **Joan T. Pickens** (*Pharmacological Laboratory, Research Department, Roche Products, Welwyn Garden City, Herts.*).

Conditions governing the assay of analgesics by the writhing response of mice to phenylbenzoquinone.

10. **A. W. Lessin** (*introduced by* **M. W. Parkes**) (*Pharmacological Laboratory, Research Department, Roche Products, Welwyn Garden City, Herts.*).

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11. **H. M. Cabot, R. Caldeyro-Barcia, R. J. Fitzpatrick** and **C. Mendez-Bauer** (*Servicio Fisiologica Obstetrica of the University of Uruguay*).

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13. **F. Hobbiger** (*Department of Pharmacology, Middlesex Hospital Medical School, London, W.*1).

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14. **E. E. Daniel** (*introduced by* **M. Weatherall**) (*Department of Pharmacology, The London Hospital Medical College, London, E.*1).

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15. **D. Elmqvist** and **T. R. Johns** (*introduced by* **S. Thesleff**) (*Department of Pharmacology, University of Lund, Sweden*).

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16. **Eva M. Kovacs** and **G. A. H. Buttle** (*Department of Pharmacology, The School of Pharmacy,* 29-39 *Brunswick Square, London, W.C.*1).

Immunological aspects of transplanted tumours.

17. **L. C. Blaber** and **W. C. Bowman** (*Department of Pharmacology, The School of Pharmacy,* 29-39 *Brunswick Square, London, W.C.*1).

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18. **P. S. J. Spencer** (*introduced by* **G. B. West**) (*Department of Pharmacology, The School of Pharmacy,* 29-39 *Brunswick Square, London, W.C.*1).

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19. **A. W. Cuthbert** (*introduced by* **G. B. West**) (*Department of Pharmacology, The School of Pharmacy,* 29-39 *Brunswick Square, London, W.C.*1).

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21. **Shirley A. P. Price** and **G. B. West** (*Department of Pharmacology, The School of Pharmacy,* 29-39 *Brunswick Square, London, W.C.*1).

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22. **D. F. Elliott, E. W. Horton** and **G. P. Lewis** (*National Institute for Medical Research, Mill Hill, London, N.W.*7).

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23. **G. P. Lewis** (*National Institute for Medical Research, Mill Hill, London, N.W.*7).

The possible role of bradykinin in the inflammatory response.

24. **F. M. Sullivan** (*Department of Pharmacology, Guy's Hospital Medical School, London, S.E.*1).

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26. **B. W. Payton** (*introduced by* **J. P. Quilliam**) (*Department of Pharmacology, The Medical College of St. Bartholomew's Hospital, London, E.C.*1).

The effect of some central nervous system depressants and hexamethonium on neuromuscular transmission.

27. **D. F. J. Mason** (*Department of Pharmacology, The Medical College of St. Bartholomew's Hospital, London, E.C.*1).

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28. **Sybil Lloyd** and **Mary Pickford** (*Physiology Department, University of Edinburgh*).

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29. **D. H. Sproull** (*National Institute for Medical Research, Mill Hill, London, N.W.*7).

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The influence of some psychotropic drugs on the social behaviour of male sticklebacks.

33. **P. Lund Nilsen** (*Pharmacological Laboratory, Meco-Dumex, 37 Prags Boulevard, Copenhagen, S*).

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34. **P. J. Nicholls** (*introduced by* **J. D. P. Graham**) (*Department of Materia Medica and Pharmacology, Welsh National School of Medicine, Cardiff*).

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38. **M. M. Airaksinen** (*Department of Pharmacology, University of Helsinki*).

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39. **Anja Tissari** (*Department of Pharmacology, University of Helsinki*).

5-Hydroxytryptamine in the foetal and young guinea-pig.

40. **R. S. Stacey** (*Department of Pharmacology and Therapeutics, St. Thomas's Hospital Medical School, London, S.E.1*).

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41. **J. G. A. Pedersen** (*Aktieselskabet Pharmacia, 48 Lindealle, Copenhagen*).

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43. **R. W. Foster** (*introduced by* **G. Brownlee**) (*Department of Pharmacology, King's College, London, W.C.2*).

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The effect of caffeine and depolarizing drugs on the membrane potential and the contracture induced in mammalian muscle.

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51. **P. F. D'Arcy** and **J. J. Grimshaw** (*Research Division, Allen & Hanburys, Ware, Hertfordshire*).

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52. **P. F. D'Arcy, J. W. Fairbairn** and **J. J. Grimshaw** (*Research Division, Allen & Hanburys, Ware, Hertfordshire*).

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53. **F. G. Sulman** (*introduced by* **E. Jacobsen**) (*Department of Applied Pharmacology, The Hebrew University-Hadassah Medical School, Jerusalem*).

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54. **I. Møller Nielsen** and **I. Huus** (*Department of Pharmacology, Lundbeck & Co. A/S, Copenhagen*).

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Brit. J. Pharmacol. (1960), **15**, 365.

THE RESPONSES OF THE *VENUS* HEART TO CATECHOL AMINES AND HIGH CONCENTRATIONS OF 5-HYDROXYTRYPTAMINE

BY

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(RECEIVED JANUARY 28, 1960)

The catechol amines excite the isolated heart of *Venus mercenaria* in a characteristic manner. This response was not obtained with phenethylamine, tyramine, ephedrine, or mescaline, nor with histamine, nor with the basic n-alkylamines. 5-Hydroxytryptamine had a distinctive effect at high concentrations (above 3×10^{-6} M) different from that at lower doses. The response to high concentrations was dominated by an increase in muscle tone. Hearts exposed to high concentrations of 5-hydroxytryptamine and other tryptamine analogues for long periods became tachyphylactic to low doses of these substances. However, high doses of 5-hydroxytryptamine (about 2×10^{-5} M) still excited the tachyphylactic heart, but the response was then like that to the catechol amines. When high bath temperatures rendered the heart insensitive to 5-hydroxytryptamine, high concentrations of this compound again had the catechol amine effect. The possibility of a physiological role for the catechol amines or high 5-hydroxytryptamine concentrations is discussed.

While studying structure-activity relations on the heart of *Venus mercenaria* (Greenberg, 1960), attention was drawn to the effects of some catechol amines and high concentrations of 5-hydroxytryptamine.

The responses of lamellibranch hearts to adrenaline and noradrenaline are varied (Krijgsman and Divaris, 1955; Welsh, 1953; Fänge, 1955; Gaddum and Paasonen, 1955). For example, most hearts are excited by adrenaline ; when treated with high concentrations they are arrested in systole. On the other hand, the oyster (Jullien, 1936) and *Anodonta cygnea* (Fänge, 1955) show negative inotropic effects when treated with adrenaline. Again, *Amblema peruviana* (Motley, 1934) and *Cardium edule* (Gaddum and Paasonen (1955) are arrested in diastole by high concentrations of catechol amines.

Only a few workers have tested both catechol amines and 5-hydroxytryptamine on the lamellibranch heart (Welsh, 1953 ; Fänge, 1955 ; Gaddum and Paasonen, 1955). In the species tested, the hearts have always been found to be more sensitive to 5-hydroxytryptamine than to adrenaline or noradrenaline. In only three species was there a notable qualitative difference between the two excitatory actions.

High concentrations of 5-hydroxytryptamine have never been studied, probably for the good reason that the physiological role of this substance as excitatory neurohumour (Welsh, 1957) is likely to be associated with near-threshold concentrations.

Tachyphylaxis to 5-hydroxytryptamine was demonstrated by Gaddum on guinea-pig ileum in 1953. It has since been observed in a number of mammalian preparations (Gaddum and Hameed, 1954), but has never been produced in molluscan preparations.

In the present study on the *Venus* heart the mode of excitation by catechol amines is examined over the entire range of action. High concentrations of 5-hydroxytryptamine are also tested and it is found that they can induce tachyphylaxis to moderate concentrations of this substance. When such desensitization has been established, however, high 5-hydroxytryptamine doses evoke responses similar to those of the catechol amines. The structure-activity relations of this response are briefly studied. The possibility that there is a physiological role for the catechol amines in *Venus* is suggested.

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METHODS

Preparation.—Large specimens of *Venus mercenaria* (clams; quahogs), obtained fresh from Narragansett Bay, were employed. The hearts were removed in the manner described by Welsh and Taub (1948). The excised ventricles were immersed in a jacketed, aerated 10 ml. bath and stretched between a stainless steel hook and an isotonic lever balanced either at 500 or 1,000 mg. The perfusion fluid in the bath proper was filtered sea water obtained from Woods Hole, Mass. Water from a reservoir, thermostatically maintained at 15°, was circulated through the bath jackets. Responses were recorded on a kymograph with a smoked drum.

Drugs were made up in standard solutions of 10^{-1} M to 10^{-3} M. These were serially diluted with distilled or sea water. The dose in a volume of 0.1 to 1.0 ml. was added directly to the bath fluid with a hypodermic syringe. Proper mixing was provided by the stream of aerating bubbles. The addition of 1.0 ml. of distilled water alone has no effect on the clam heart. Most doses are expressed as moles per litre, but concentrations of Brom LSD and Mytolon in the bath are given as g./ml.

The length of exposure of a heart to a particular dose varied between a min. and over an hr. depending upon the compound and the concentration used. At least 5 min. were always allowed between washing and the addition of the succeeding dose.

Benzoquinonium chloride (Mytolon; 2:5-bis (3′ diethylaminopropylamino)-benzoquinone bis benzyl chloride) (10^{-5} g./ml.) was maintained in the bath except during the washing process. This drug is an effective antagonist of acetylcholine in the *Venus* heart (Luduena and Brown, 1952). In these experiments benzoquinonium was used to improve the regularity of the beat by preventing the depression of the heart probably caused by endogenous acetylcholine. Benzoquinonium has no effect on the response of the heart to 5-hydroxytryptamine.

The Effect of High Bath Temperature on the 5-Hydroxytryptamine Effect.—By increasing the temperature of the water in the reservoir, that of the circulating water in the jackets could be raised to 30° or 35°. Temperature equilibrium between the jacket water and the sea water in the bath occurred in about 5 min. Ten min. were always allowed.

The Effect of pH Change on the Performance of the Isolated Venus Heart Preparation.—Aliquots of sea water were adjusted with hydrochloric acid or sodium hydroxide so that the *p*H varied from 4.2 to 9.0. In testing the effects of *p*H on the heart, normal sea water in the bath was rapidly withdrawn and replaced by the altered sea water. The hearts were equilibrated at the new hydrogen ion concentration for 15 min.

Specificity.—Brom LSD (2-brom-(+)-lysergic acid diethylamide) (10^{-5} g./ml.) is an antagonist of 5-hydroxytryptamine on the *Venus* heart (Welsh and McCoy, 1957). This antagonism is believed to be specific. In the present study Brom LSD is used to test the specificity, relative to 5-hydroxytryptamine, of the various exciter agents employed.

Drugs Used.—The following compounds were used in the course of this study: Tryptamine hydrochloride, 3,4-dihydroxyphenylethylamine (dopamine) hydrochloride (Mann Research Laboratories); 5-hydroxytryptamine creatinine sulphate (Nutritional Biochemicals); 2-brom-(+)-lysergic acid diethylamide (Sandoz, Inc.); phenethylamine, heptylamine, *n*-hexylamine, *n*-amylamine, *n*-butylamine, ethylamine (Eastman Kodak); tyramine (Abbott Laboratories); (−)-epinephrine bitartrate, levarterenol bitartrate monohydrate, benzoquinonium chloride (Sterling-Winthrop); ephedrine hydrochloride, mescaline hydrochloride (Hoffman-LaRoche); histamine diphosphate (General Biochemicals); bufotenine, 5-hydroxy-α-methyltryptamine, N′N′-dimethyltryptamine (Upjohn).

RESULTS

The Catechol Amine Effect.—The effects of adrenaline, noradrenaline and dopamine on the *Venus* heart were similar, but differed from those of 5-hydroxytryptamine.

The catechol amines produced a series of responses which varied qualitatively with concentration. There were two sorts of effects in the range of action. At the low-concentration end there occurred a decrease in amplitude with no chronotropic effect. Since these experiments were done in the presence of benzoquinonium this response could not have been due to a stimulation of the acetylcholine receptors of the heart. At higher concentrations an increase in tone, concomitant with an increase in frequency, predominated. At the extreme high end of the concentration range, the response was immediate systolic arrest.

The effects of adrenaline and noradrenaline were almost identical (Fig. 1). The pure negative inotropic effect was a relatively small part of the response occurring just at threshold doses (10^{-5} to 5×10^{-5} M) (Fig. 1a and c). A slight increase of the threshold dose by $1–2 \times 10^{-5}$ M resulted in a response which was a mixture of the negative inotropic and positive chronotropic effects as well as an increase in tone ($2–7 \times 10^{-5}$ M). A dose of adrenaline or noradrenaline 5 to 10 times larger than that which just increases the tone caused systolic arrest ($7–12 \times 10^{-5}$ M) (Fig. 1a).

The action of dopamine differed from that of adrenaline and noradrenaline in two respects. Firstly, the potency of dopamine, with regard to the increase of tone and frequency, was about 10 times that of adrenaline or noradrenaline (Fig. 1c). Secondly, the decrease in amplitude played a more prominent part in the response to dopamine. There was a good deal of variation, but a negative inotropic effect was always obtainable at 2 to 3×10^{-6} M (Fig. 1c). A decrease in amplitude of more than 50% was not unusual. Increases of tone

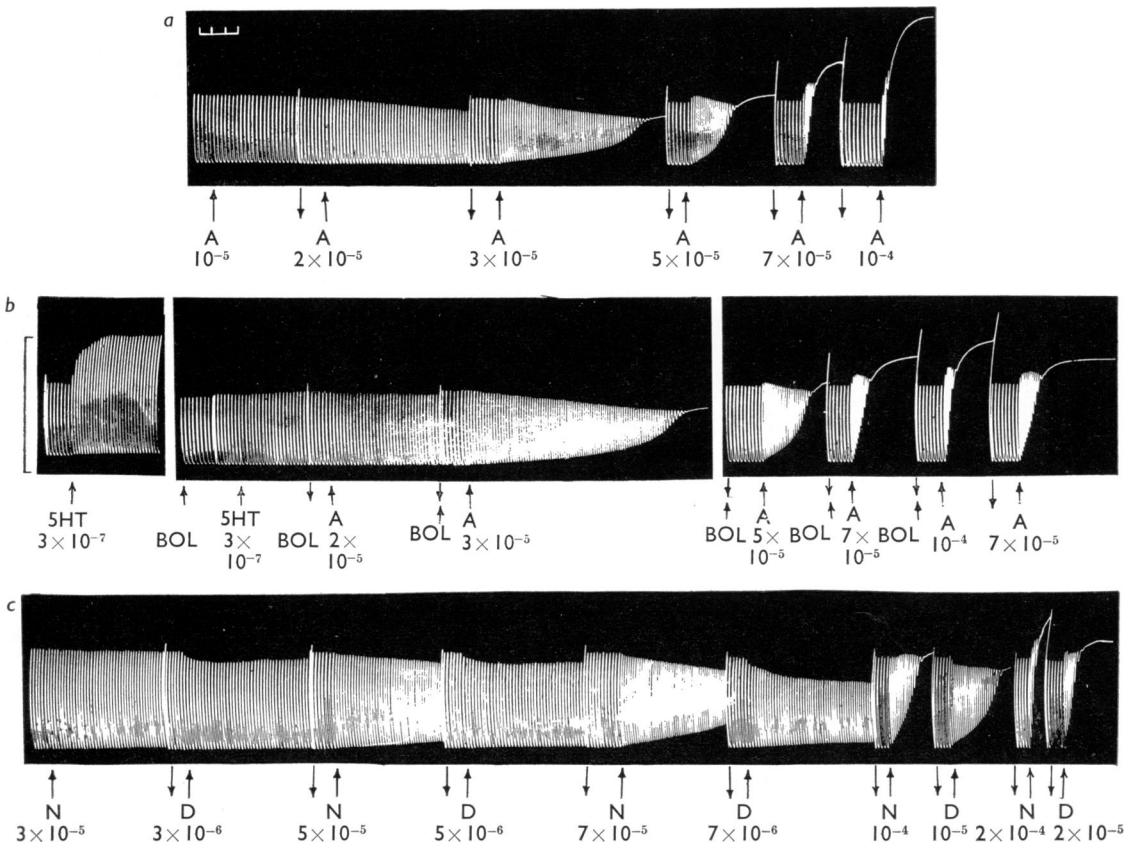

FIG. 1.—The response to catechol amines of the isolated *Venus* heart preparation. (*a*) The effect of adrenaline A. (*b*) Same preparation. The effect of adrenaline (A) in the presence of 2-bromo-(+)-lysergic acid diethylamide (BOL) (10^{-6} g./ml.). First dose of BOL added 20 min. before excitator agent (5-hydroxytryptamine (5HT)); maintenance doses immediately after washing and 5 min. before each dose of exciter. (*c*) Comparison of noradrenaline (N) and dopamine (D) on the same preparation. Benzoquinonium chloride (10^{-5} g./ml.) added to bath 5 min. before each dose. Downward-pointing arrows indicate washing. Dose: moles/litre. Tension: 1,000 mg. Temperature: 15°. Time scale: 30 sec. Amplitude scale: 5 cm.

were usually produced by a dose of 5 to 20 times greater than threshold.

The responses to catechol amines were not blocked by Brom LSD (10^{-5} g./ml.) (Fig. 1b); this distinguishes these responses from those to 5-hydroxytryptamine.

It is important to know whether the catechol amine effect is merely an unspecific response of the heart to high concentrations of drug or whether it results from an action at specific sites in the tissue. A partial answer to this question can be obtained by examining the responses of the heart to various analogues of the catechol amines.

Responses to Other Phenethylamine Analogues.—Tyramine and phenethylamine were tested at concentrations between 10^{-5} M and 10^{-4} M. The response of the heart was 5-hydroxytryptamine-like, although 500 to 4,000 times weaker. The positive inotropic effect was antagonized by Brom LSD. This surprising result is dealt with elsewhere (Greenberg, 1960).

The actions of ephedrine and mescaline were relatively feeble even at such high concentrations as 10^{-4} M to 10^{-3} M. The effects of these compounds were qualitatively similar to those of tyramine and phenethylamine. Mescaline had about one third, ephedrine one fiftieth the potency of phenethylamine. No attempt was made to block the actions of these two compounds with Brom LSD.

None of the above analogues of phenethylamine tested produced an effect resembling that of the catechol amines at any concentration.

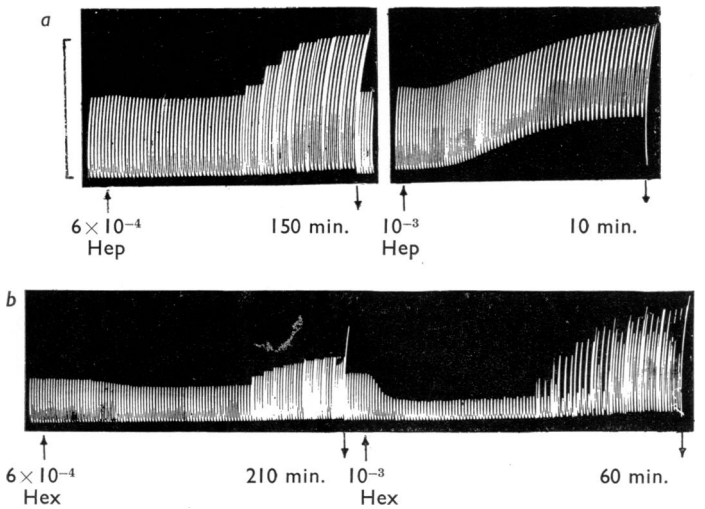

FIG. 2.—The response of the isolated *Venus* heart preparation to *n*-alkylamines. (*a*) Heptylamine (Hep). (*b*) Hexylamine (Hex). Total time for each response is indicated in min. Downward-pointing arrows indicate washing. Benzoquinonium chloride (10^{-5} g./ml.) was added to the sea water 5 min. before each dose. Dose: moles/litre [illegible] 00 mg. Temperature: 15°. Time scale: 30 sec. Amplitude scale: 5 cm.

Responses to Histamine (*4-Ethylamino Imidazole*).—Histamine is a well-known smooth muscle exciter. It acts occasionally on lamellibranch hearts in relatively high concentrations (Pilgrim, 1954; Waddum and Paasonen, 1955). Histamine has a curious effect on the *Venus* heart. Threshold concentration, when there was any action at all, was about 10^{-6} M. The response was a rapid increase in amplitude of beat which fell off again fairly rapidly. The increase in amplitude was equivalent to that produced by about 4×10^{-9} M 5-hydroxytryptamine. Increasing doses of histamine, up to 10^{-4} M, did not increase the effect; in fact, the response sometimes became smaller.

The action of histamine was not blocked by Brom LSD and hence histamine was not acting on the 5-hydroxytryptamine site. No sign of a catechol-amine-like effect has been observed in response to histamine.

Responses to n-*Alkylamines*.—The *n*-alkylamines tested excited the *Venus* heart in high concentrations. However, the mode of excitation was unique: neither like that of the catechol amines, nor histamine, nor 5-hydroxytryptamine.

Threshold, for amyl-, hexyl-, and heptylamine, was between 10^{-4} and 3×10^{-4} M. The effect was a transient decrease in amplitude lasting about 10 min. and followed by an increase in amplitude which took about an hour to develop (Fig. 2). Both the positive and negative inotropic effects increased only slightly with increasing doses up to 8×10^{-4} M. At 10^{-3} M the amines had a sudden violent effect the nature of which depended upon the compound used. With heptylamine there was a great increase of tone and a slowing of rate (Fig. 2). Hexylamine and amylamine produced an augmentation of the transient decrease and then a great irregularity of beat (Fig. 2). As might be expected the alkylamine effect was not blocked by Brom LSD (10^{-5} g./ml.).

The *n*-alkylamines with a chain length of less than five carbons had a smaller potency. Ethylamine and butylamine had about one third of the activity of hexylamine; ammonia had one sixth to one tenth of the activity of hexylamine.

The perfusion fluid, sea water, is buffered naturally. In the course of storage, prior to use, the *p*H dropped to about 7.8. The addition to the [illegible] of relatively large concentrations of these basic amines caused the *p*H to increase to as much as 8.4. Four heart preparations were tested to determine their tolerance to changes of hydrogen ion concentration. The normal functioning of the hearts was independent of *p*H. The frequency was stable at about 10 beats per min. between *p*H 5.9 and 8.6. The amplitude, while stable between *p*H 4.8 and 9.0, dropped noticeably at 4.2. Thus, between *p*H 7.8 and 8.4, the range employed in the study of the *n*-alkylamines, there was no *p*H-induced variation in the normal amplitude and frequency of the hearts.

The Effect of 5-Hydroxytryptamine.—The response of the *Venus* heart to 5-hydroxytryptamine has three components: an increase in amplitude, an increase in frequency, and an increase in the resting tone of the muscle. The relative importance of these components varies with the concentration (see Figs. 3 and 4).

Between threshold concentration (about 10^{-9} M) and moderately high doses (about 10^{-6} M) the response of the *Venus* heart to 5-hydroxytryptamine was mainly an increase in the amplitude of beat. There was also an increase in tone, which increases with concentration, and an increase in frequency which, however, was not dependable. A plot of the final amplitude of the response (measured from the original baseline) against log concentration is a

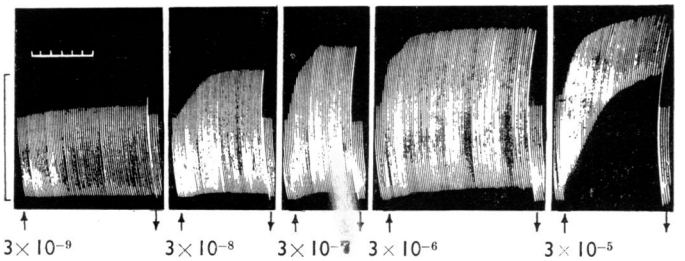

FIG. 3.—The effect of 5-hydroxytryptamine (5HT) on the isolated *Venus* heart preparation. Downward-pointing arrows indicate washing. Benzoquinonium chloride (10^{-5} g./ml.) added to sea water 5 min. before each dose of 5HT. Dose: moles/litre. Tension: 1,000 mg. Temperature: 15°. Time scale: 30 sec. Amplitude scale: 5 cm.

sigmoid curve which levels out at about 10^{-6} M and then rapidly increases with higher concentrations.

Above 10^{-6} M 5-hydroxytryptamine the relative increase of the tone became larger. At 10^{-5} M the effect was almost all increase in tone while the rhythmical excursions of the heart were very small. The ventricle was often arrested in systole. When the increase in tone is plotted as a function of log concentration a really large increase in the slope starts rather suddenly at about 3×10^{-6} M (Fig. 4). At these high concentrations the frequency of beat often doubled or trebled.

Thus, there appears to be two distinct responses of the *Venus* heart to 5-hydroxytryptamine. One, at low to moderate concentrations, is dominated by a positive inotropic effect. Tone is relatively unimportant and frequency changes are not dependable. The other response, evoked by high concentrations, is dominated by a large increase in muscle tone. The inotropic effect no longer followed the relationship implied by the sigmoid curve found at lower concentrations. The frequency always increases noticeably.

A measure of the 5-hydroxytryptamine effect at lower concentrations is the difference, in mm., between the amplitude of beat before and after the addition of a dose (b–a in the inset of Fig. 4). It is interesting that, from threshold to about 10^{-6} M 5-hydroxytryptamine, the plot of this effect against log concentration was the expected sigmoid curve. Above 10^{-6} M, when the tone also suddenly increased greatly, the points ceased to follow this relationship (Fig. 4). The action of 5-hydroxytryptamine in concentrations below 10^{-6} M is dealt with elsewhere (Greenberg, 1960). The present work concerns the effects of doses greater than 3×10^{-6} M.

Brom LSD (10^{-5} g./ml.), given 20 min. previously, antagonized the action of moderately high doses of 5-hydroxytryptamine ($3–10\times10^{-7}$ M). This blockade was usually surmountable by concentrations of

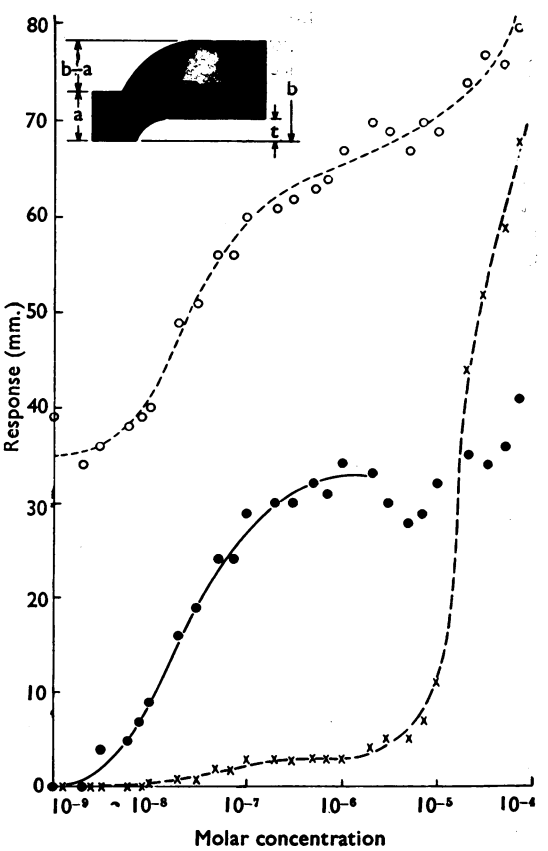

FIG. 4.—The effects of increasing concentrations of 5-hydroxytryptamine on the performance of the isolated *Venus* heart preparation (see Fig. 4). (X) indicates the amount of tone (t) in the response. (O) indicates the final amplitude (b) of the response. (●) represents the difference (b–a) between the final (b) and initial (a) amplitudes. (t), (b), (a), and (b–a) are explained in the inset, which is a diagram of a 5-hydroxytryptamine response.

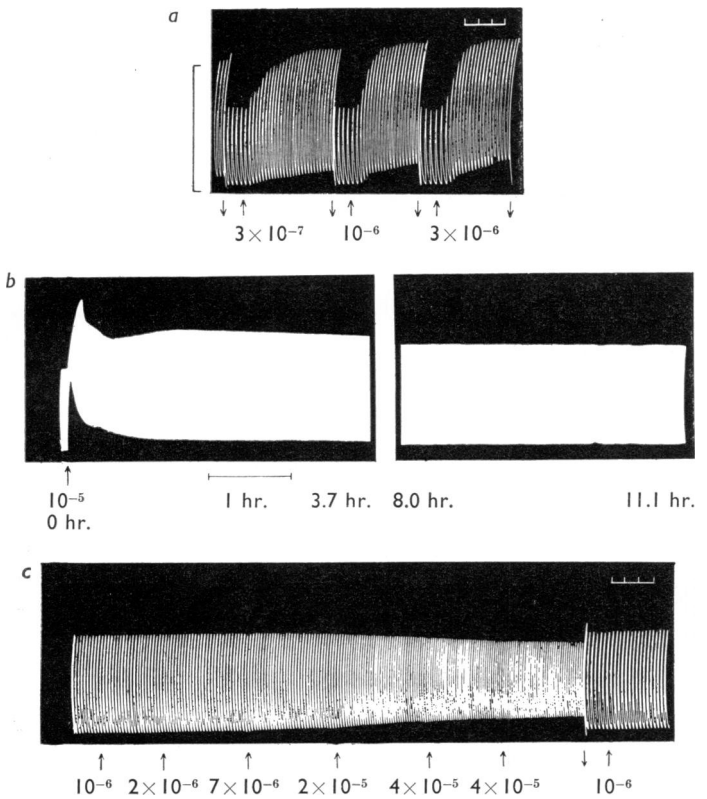

FIG. 5.—Tachyphylaxis of a *Venus* heart preparation to 5-hydroxytryptamine. (*a*) Three standard doses of 5-hydroxytryptamine (5HT) successively washed out (indicated by down-pointed arrow). (*b*) Record made at slow drum speed (Time scale : 1 hr.) showing the secondary decrease in amplitude following a large dose of 5HT. (*c*) Large doses of 5HT now added to tachyphylactic preparation. (*a*), (*b*), and (*c*) are consecutive records from the same preparation. Benzoquinonium chloride (10^{-5} g./ml.) added after every washing. Dosage: moles/litre. Tension: 500 mg. Temperature: 15°. Time scale for (*a*) and (*c*) : 30 sec. Amplitude scale: 5 cm.

5-hydroxytryptamine greater than 10^{-6} M or by smaller doses if the preparation was a sensitive one. Often, the *Venus* heart was excited by 10^{-5} g./ml. Brom LSD (see Greenberg, 1960). Increased time of application or increased dose resulted in substantial excitation. Consequently, Brom LSD is not useful as an inhibitor of high 5-hydroxytryptamine doses.

Tachyphylaxis to 5-Hydroxytryptamine.—When 10^{-5} M 5-hydroxytryptamine was present in the bath the tone of the heart increased greatly. If the dose was not washed out, this effect slowly diminished. After from 4 to 12 hr. the ventricle was beating almost normally (Fig. 5b). The tone and amplitude may be larger than before the application of the large dose of 5-hydroxytryptamine, but usually not as large as it is in response to even moderate concentrations (10^{-8} to 10^{-7} M). Such a preparation was then tachyphylactic. It did not respond to the further addition of increasing doses of 5-hydroxytryptamine up to 10^{-5} M (Fig. 5c). It also did not respond to 5-hydroxytryptamine analogues, such as tryptamine and bufotenine (Fig. 6a). Furthermore, hearts could be made tachyphylactic to tryptamine (Fig. 8a), bufotenine, or 5-hydroxy-α-methyltryptamine (Fig. 6b), and afterwards these hearts will be unresponsive to 5-hydroxytryptamine as well as to the desensitizing drugs. On the other hand, the catechol amines still excited desensitized hearts in their characteristic manner, although the magnitude of the response may be reduced (Fig. 6c).

Isolated *Venus* hearts which have been left untreated for 12 hr. responded to *all* 5-hydroxytryptamine concentrations in the same manner as fresh preparations. Thus, tachyphylaxis does not depend upon the age of the preparation. It was not possible to restore the sensitivity of the tachyphylactic heart to 5-hydroxytryptamine even after prolonged washing. Specific tachyphylaxis to 5-hydroxytryptamine has been described in guinea-pig ileum and other mammalian tissues (Gaddum, 1953; Gaddum and Hameed, 1954). Tachyphylaxis, in these tissues, occurs relatively rapidly and is reversible.

High 5-Hydroxytryptamine Concentrations after Tachyphylaxis.—Desensitized hearts were insensitive to doses of 5-hydroxytryptamine up to 10^{-5} M. However, when challenged with 2×10^{-5} M, the beat of such a heart *decreased* in amplitude. With increasing doses, up to 10^{-4} M (which caused the heart to be arrested immediately in systole), responses were produced which were almost identical with those to adrenaline and noradrenaline (Fig. 7). These catechol amine-like effects were also elicited by 5-hydroxytryptamine if the heart had been made tachyphylactic by one of its analogues (Fig. 8*a*). Furthermore, the decrease in amplitude at about

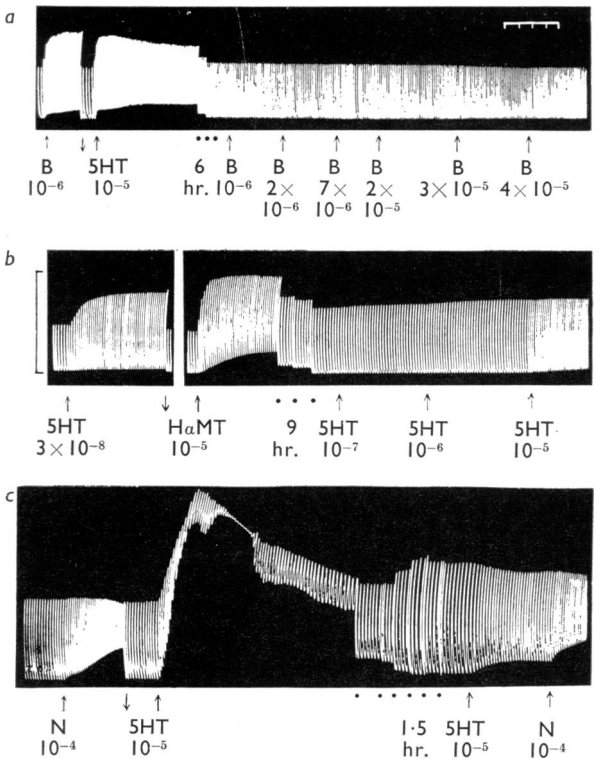

FIG. 6.—Responses of the tachyphylactic *Venus* heart preparation. (*a*) After a standard dose of bufotenine (B), the heart is made tachyphylactic to 5-hydroxytryptamine (5HT). Bufotenine, subsequently, has no effect. (*b*) After a standard dose of 5HT the heart is made tachyphylactic to 5-hydroxy-α-methyltryptamine (HαMT). Subsequent doses of 5HT are ineffective. (*c*) Tachyphylaxis produced by 5HT. The noradrenaline (N) response is relatively unaffected. (●) indicates stopping of drum. The total time required for tachyphylaxis is indicated. Washing was at the downward-pointing arrows. Benzoquinonium chloride (10^{-5} g./ml.) was added to the sea water after each washing. Doses: moles/litre. Tension: 500 mg. Temperature: 15°. Time scale: 30 sec. Amplitude scale: 4 cm.

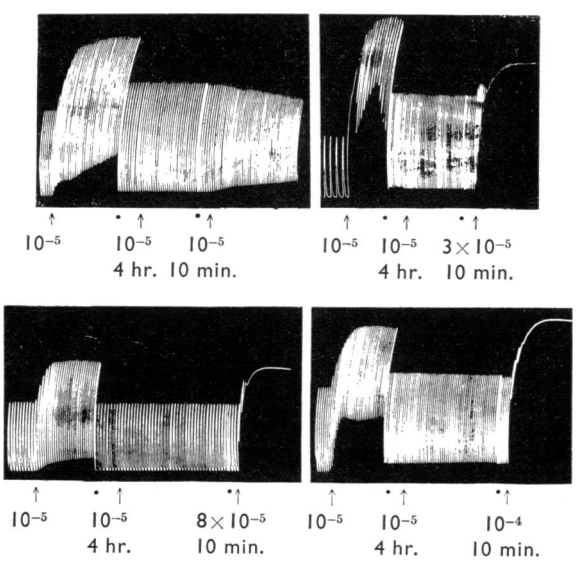

FIG. 7.—Catechol-amine-like responses (see Fig. 1) produced by 5-hydroxytryptamine (5HT) on tachyphylactic *Venus* heart preparations. (●) indicates that the drums were stopped for the time shown. Doses: moles/litre. Tension: 500 mg. Temperature: 15°.

2×10^{-5} M occurred even if the positive inotropic effect, due to a high dose of a tryptamine analogue had not disappeared (Fig. 8b). Such a decrease in amplitude after 10^{-5} M 5-hydroxytryptamine occurred when an exciting dose of Brom LSD (10^{-5} mg./ml.) was left in the bath for 7 hr. (Fig. 8c). The similarity of responses and concentrations suggests that the 5-hydroxytryptamine is acting at the same sites as the catechol amines.

It has been impossible to obtain this distinctive series of responses with either tryptamine or bufotenine. Tryptamine, at 10^{-3} M, after tachyphylaxis had, in a few experiments, produced effects resembling the *n*-alkylamine effect. Bufotenine, at least up to 4×10^{-5} M, produced only a slight decrease in amplitude with a small increase in tone on a tachyphylactic heart.

The Effect of 5-Hydroxytryptamine at High Temperatures.—High bath temperatures reduced the response of the heart to 5-hydroxytryptamine. At 30° to 35° the muscle lengthened and the addition of doses of 5-hydroxytryptamine, up to 10^{-7} M, had very little effect. When 10^{-5} M 5-hydroxytryptamine was then added there followed the decrease in amplitude which had been associated both with 5-hydroxytryptamine after tachyphylaxis and with threshold doses of catechol amines. When the bath is cooled to 15° the usual 5-hydroxytryptamine effect, at 10^{-5} M, with its high tone and small rhythmical excursions, is unmasked. Thus, it was possible to demonstrate the catechol-amine-like response to high concentrations of 5-hydroxytryptamine without desensitizing the heart to 5-hydroxytryptamine.

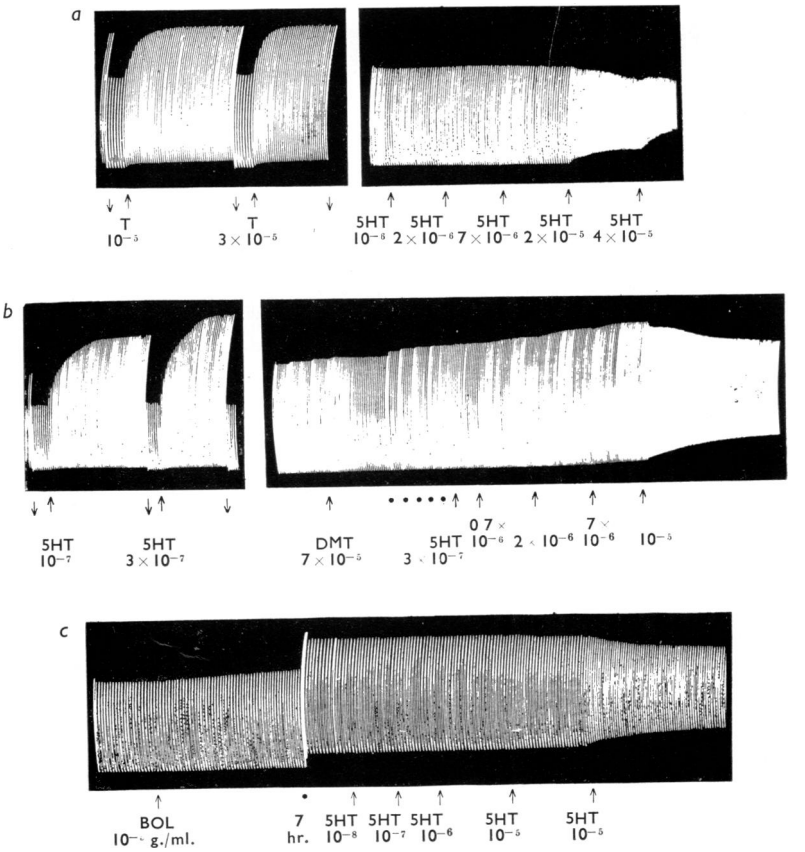

FIG. 8.—Conditions resulting in catechol-amine-like responses of 5-hydroxytryptamine. (*a*) Response of a *Venus* heart preparation to tryptamine (T) before tachyphylaxis and to 5-hydroxytryptamine (5HT) afterwards. Tachyphylaxis produced by 10^{-4} M tryptamine over a period of 11.3 hr. (*b*) Following the normal response of a heart to 5-hydroxytryptamine (5HT) are 8 hr. of application of increasing doses of *N'N'*-dimethyltryptamine (DMT), the last of which is shown. Successive doses of 5HT are then added. Note the small positive inotropic effects, up to 2×10^{-5} M 5HT, indicating the absence of tachyphylaxis. (●) indicates that drum was stopped for 7 min. (*c*) Catechol-amine-like effect of 5HT after prolonged treatment with 2-brom-(+)-lysergic acid diethylamide (BOL). Doses: moles/litre. Tension: 1,000 mg. Temperature: 15°.

DISCUSSION

A *Venus* heart preparation may be either excited or depressed by the catechol amines, depending upon the dose. Although previous workers have reported diverse effects for adrenaline and noradrenaline on lamellibranch hearts (Krijgsman and Divaris, 1955 ; Fänge, 1955 ; Gaddum and Paasonen, 1955), only for *Anodonta cygnea* is there a suggestion of more than one mode of action for

these compounds. Fänge (1955) mentions not only a negative inotropic effect, but also immediate systolic arrest at a ten-fold higher concentration. Similarly, Ten Cate (1923) and Hendrickx (1945) noted an enfeeblement of *Anodonta* heart beat prior to systolic arrest.

It seems possible that the hearts of some animals, such as *Amblema peruviana* (Motley, 1934), *Cardium edule* (Gaddum and Paasonen, 1955), and the oyster (Jullien, 1936), for which a depression by catechol amines has been reported, are especially sensitive to the negative inotropic effect of these compounds. Presumably, higher doses would evoke the excitation which has been seen in *Venus* and others. One would also expect to produce, with lower concentrations than have been used in the past, a decrease in amplitude from clam heart preparations which have been noted to be stimulated by the catechol amines.

The exciter effect of adrenaline and noradrenaline on the *Venus* heart preparation was not only of lower potency than that of 5-hydroxytryptamine below 10^{-6} M but also qualitatively different. Welsh made this distinction between the two excitatory actions in 1953.

While other lamellibranch hearts are, in general, between 1,000 and 10,000 times more sensitive to 5-hydroxytryptamine than to adrenaline, only *Cardium edule* (Gaddum and Paasonen, 1955) and *Anodonta cygnea* (Fänge, 1955) show any qualitative distinction between the two exciter effects.

The difference, on *Venus* heart, between the actions of catechol amine and 5-hydroxytryptamine suggests that these actions originate from different sites in the tissue. This contention is supported by two sorts of evidence. First, when a preparation is insensitive to low or moderate doses of 5-hydroxytryptamine, due to tachyphylaxis or high bath temperatures (30° to 35°), it will display the various effects associated with the catechol amines if challenged either by high doses of these amines or by 5-hydroxytryptamine. Second, Brom LSD (10^{-5} g./ml.), which inhibits 5-hydroxytryptamine, has little effect on the response to adrenaline. Brom LSD also produces tachyphylaxis; this has been observed only when it was exciting the heart. Therefore, it is impossible to say whether the catechol-amine-like effect would also follow an inhibition of 5-hydroxytryptamine when not preceded by excitation. However, Brom LSD does not antagonize the effect of 5-hydroxytryptamine seen in the desensitized heart; this strengthens the presumption that this effect does not occur as a result of excitation at the usual 5-hydroxytryptamine site.

It would be interesting to know whether there is in *Venus*, a physiological basis for the response to catechol amines or whether it is merely an unspecific effect of toxicological significance only. There is some evidence which suggests that a physiological role exists.

As far as has been studied, there seem to be relatively exacting structural requirements for molecules producing this effect. Thus, in contrast to 5-hydroxytryptamine, tryptamine is inactive. Phenylethylamine analogues which either lack hydroxyl groups or have methoxy groups (mescaline) in any two of the three hydroxyl positions in noradrenaline are similarly ineffective. The activity of dopamine, which has no side chain hydroxyl, suggests that only the two phenolic groups of noradrenaline are necessary for the production of the characteristic response. Whether the hydroxyl on the side chain would suffice, instead of that in the 3-position of the nucleus, must be established by testing *p*-hydroxyphenylethanolamine (octopamine). This prospect is interesting since octopamine is a natural product found abundantly in the posterior salivary glands of *Octopus vulgaris* (Bacq, Fischer and Ghiretti, 1952). The *n*-alkylamines and histamine, while excitatory, each have actions which are distinctive. The structure-activity relations of the response to the catechol amine have not been worked out. However, present information indicates that a structurally specific receptor in the cell is involved.

The responses of the tachyphylactic or overwarmed *Venus* heart to 5-hydroxytryptamine are duplicated seasonally in nature. During the summer months (late June to August) the threshold of the ventricle to 5-hydroxytryptamine may increase by ten-fold or more. In fact, normally beating hearts sometimes cannot be excited until concentrations of 10^{-6} M are reached, and then the response is small. Furthermore, in the summer, a *decrease* in amplitude of beat in response to low 5-hydroxytryptamine doses (about 10^{-8}) has occasionally been observed (B. M. Twarog ; M. K. Paasonen ; personal communications). The decrease is not acetycholine-like (see Welsh and Taub, 1948) ; the rate of beat increases and the response resembles, in fact, the effect of noradrenaline between 10^{-5} and 2×10^{-5} M. The production of the catechol amine effect by the normal excitatory neurohumour, 5-hydroxytryptamine, under naturally induced circumstances thus occurs. This suggests that the series of responses evoked experimentally by the catechol amines are physiological. No explanation for the increase of threshold of the 5-hydroxytryptamine effect in the summer is at hand. The possibility of a

relationship between summer and high temperature insensitivity and the irreversible tachyphylaxis with its slow onset is obvious and intriguing, but it has not been explored.

Finally, catechol amines have been found in the ganglia of *Venus mercenaria* (Welsh, personal communication). This strongly suggests that the catechol amines, themselves, have a role in the normal functioning of *Venus* and, more specifically, in the seasonal variation seen in the performance of the heart.

A practical result of the present work is that, as a consequence of the response of the heart to high 5-hydroxytryptamine concentrations, two tests for the specificity of action of excitatory compounds become available. The first involves the assumption that when the heart preparation is tachyphylactic to 5-hydroxytryptamine the receptors are blocked and there cannot subsequently be a response to any compound which initiates its actions by attachment to this receptor. Conversely, it is to be expected, and has been shown, that long applications of high concentrations of specifically acting compounds will also result in insensitivity to 5-hydroxytryptamine. Gaddum (1953) first used tachyphylaxis to 5-hydroxytryptamine in this way to demonstrate the existence of specific tryptamine receptors in the guinea-pig ileum.

The application of 2×10^{-5} M 5-hydroxytryptamine to the tachyphylactic *Venus* heart preparation results in a *decrease* in amplitude ; a ten-fold higher dose causes systolic arrest. This phenomenon constitutes the second means of distinguishing 5-hydroxytryptamine-like substances from other excitatory agents. It is especially useful in studies of test compounds which act irreversibly and cause tachyphylaxis, with a slow onset, after high doses.

It is a privilege to acknowledge the advice and encouragement offered by Professor J. H. Welsh during the course of this investigation.

This work was supported, in part, by a fellowship (HF-7571) from the National Heart Institute of the United States Public Health Service and by a University of Illinois Faculty Fellowship.

The author is indebted to the following for their generous gifts of chemicals: Dr. R. V. Heinzelman and Dr. M. E. Speeter, Upjohn Co. (bufotenine, 5-hydroxy-α-methyltryptamine, N'N'-dimethyltryptamine); Dr. R. Bircher, Sandoz Pharmaceuticals (2-brom-d-lysergic acid diethylamide); Dr. A. M. Lands, Sterling-Winthrop Research Institute (benzoquinonium chloride); Dr. R. K. Richards, Abbott Laboratories (tyramine).

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STRUCTURE-ACTIVITY RELATIONSHIP OF TRYPTAMINE ANALOGUES ON THE HEART OF *VENUS MERCENARIA*

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(RECEIVED JANUARY 28, 1960)

A number of tryptamine analogues and other exciter agents have been tested on the heart of *Venus mercenaria*. The method of estimation of potency, especially for irreversibly acting compounds, is discussed. Specificity of action with respect to the site of action of 5-hydroxytryptamine is defined experimentally. The specific activity of tyramine and phenethylamine and the non-specific excitatory action of indole and skatole indicate that the indole ring is neither necessary nor sufficient for 5-hydroxytryptamine-like activity. Tryptamine analogues differ in mode of action as well as potency. Congeners without a 5-hydroxyl group tend to act more slowly and irreversibly as well as less strongly than 5-hydroxytryptamine. Methyl substitution also increases the time of action and difficulty of reversal. However, the potency of such compounds may be increased or decreased depending upon the position of substitution and the presence of the 5-hydroxyl group. The relations between structure and potency and mode of action are discussed. Suggestions are made concerning the effective conformation of the 5-hydroxytryptamine molecule and the nature of its receptor.

5-Hydroxytryptamine might be a chemical transmitter in mollusca (Bacq, Fischer and Ghiretti, 1952 ; Welsh, 1953, 1954 ; Twarog, 1954 ; Hill, 1958). The evidence supporting this contention has recently been summarized by Welsh (1957).

The structure-activity relations of 5-hydroxytryptamine have been explored only rarely in preparations of mollusc (Marczyński, 1959), although isolated mammalian organs have often been used for this purpose (Page, 1958 ; Barlow and Khan, 1959a, b ; Vane, 1959). The great sensitivity to 5-hydroxytryptamine of the isolated ventricle of the clam, *Venus mercenaria,* makes it an excellent preparation for such a study.

In the course of this work a number of indole analogues and other excitor agents have been tested on the *Venus* heart preparation. Some suggestions are made regarding the binding sites and effective conformation of the 5-hydroxytryptamine molecule as well as the shape of its receptor.

METHODS

Preparation.—Hearts of *Venus mercenaria* (the quahog) were removed from the animals by the method of Welsh and Taub (1948). The hearts were set up in a 10 ml. perfusion bath at 15°. Treatment of the hearts in the bath, drug administration and recording were as previously described (Greenberg, 1960).

The Effect and its Measurement.—5-Hydroxytryptamine increases the force of contraction of the *Venus* heart and, at high concentrations, augments the tone as well. The measure of exciter effect used in this study is the difference, in mm., between the amplitude of beat before and after the response to an agent. Any increase of tone which occurs is included in the measurement of the final amplitude. A given effect of 5-hydroxytryptamine, or of any other compound tested, was considered completed, and therefore measurable, when there was no longer any change of amplitude following administration of a given dose ; that is, when the drug bound to the receptors was in equilibrium with the drug in the perfusion fluid. Consequently, time of action varies with dose, drug and preparation. However, the time of action for a given dose of any drug, relative to that of 5-hydroxytryptamine, is approximately the same in every preparation.

The log dose-response curve for 5-hydroxytryptamine is sigmoid between about 10^{-9} and 10^{-6} M (Greenberg, 1960). Between about 10^{-8} M and 3×10^{-7} M the curve is nearly linear and the increase in tone is never more than 15% of the response. In the experiments to be described, concentrations of 5-hydroxytryptamine above 3×10^{-7} M were rarely used.

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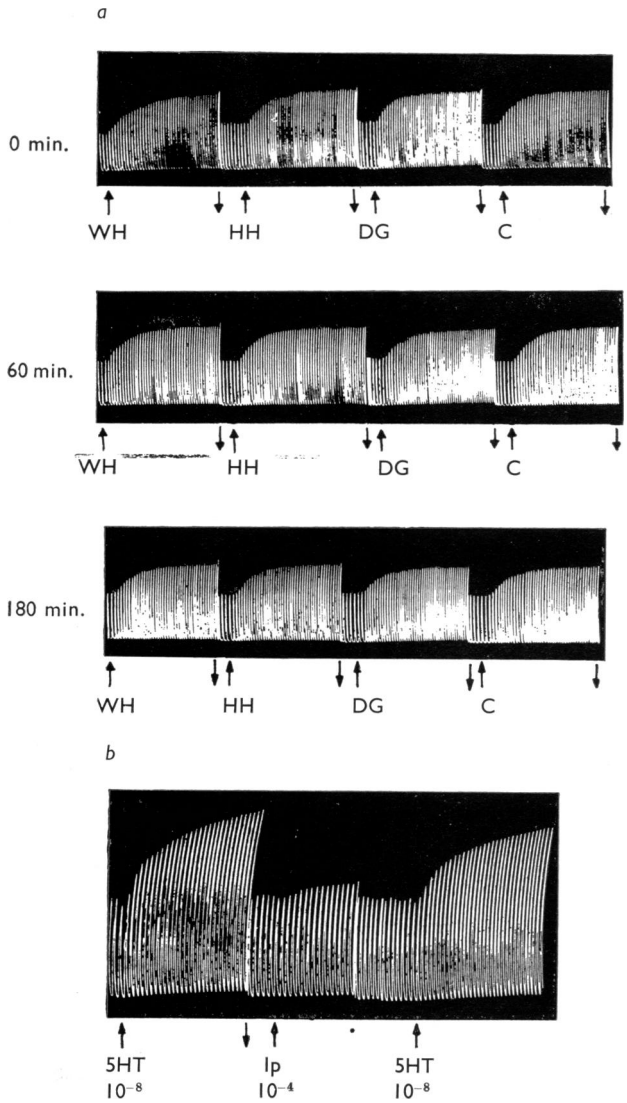


FIG. 1.—Absence of amine oxidase activity in *Venus* heart. (a) The effect, on a heart, of 3×10^{-8} M 5-hydroxytryptamine incubated with whole heart (WH), homogenized heart (HH) and homogenized digestive gland (DG) at room temperature. (C) is the untreated control dose of 5-hydroxytryptamine. Length of incubation period is indicated. (b) Ineffectiveness of iproniazid (Ip) in enhancing the effect of 5-hydroxytryptamine (5HT). (●) indicates that the drum was stopped for 1·5 hr. Washing is at downward-pointing arrows. Benzoquinonium chloride (10^{-5} g./ml.) added to bath after each washing. Dosage: moles/l. in the bath. Tension: 500 mg. Bath temperature: 15°.

The potency of the various compounds tested is expressed as the equiactive molar ratio. 5-Hydroxytryptamine is the standard and has a value of 1.0. The ratios are obtained directly from the log dose-response curves of 5-hydroxytryptamine and the test analogue from the same preparation. The points for these curves, except in the special cases mentioned below, are obtained by adding, and then washing out, successively larger doses of the agent. When the relative potency is independent of concentration, these curves are parallel. Often, however, they are not parallel. In such instances the estimation of the equiactive molar ratio is made at the inflection point of the curve of the test compound. In a few instances individual responses of the test analogue were matched by equiactive doses of 5-hydroxytryptamine to obtain the equiactive molar ratio.

Some tryptamine congeners produce irreversible effects so that relative potency is dependent upon concentration. The points for the dose-response curves of such compounds cannot be obtained by washing out a series of successively larger doses. Estimation of potency by matching is also impossible. Therefore in such cases the following method was employed to obtain the equiactive molar ratio. A low concentration of the irreversibly acting drug was added to the bath. After 20 min. with no response, or after a completed response, another dose-increment was added to the bath without washing to make the next concentration in the series, and so on. For any dose, the initial amplitude taken for computation was that prior to the first small dose.

This method of demonstrating the dose-response relationships of slow, irreversibly acting compounds is justified as follows. Any amine oxidase which is present in *Venus* heart and liver tissue is pharmacologically undemonstrable. 5-Hydroxytryptamine incubated with whole heart, homogenized heart, or homogenized digestive gland in no instance decreased in potency over 3 hr. incubation at room temperature (Fig. 1a). Also, pretreatment of hearts with an amine oxidase inhibitor, iproniazid phosphate (Hoffmann-LaRoche) (10^{-4} M), for 1 hr. has no effect on the response to 5-hydroxytryptamine (Fig. 1b). Therefore, there is no need to suppose that the drugs are slowly destroyed during the course of the experiment. Finally, 5-hydroxytryptamine dose-response curves produced on the same heart are similar, whether or not the previous dose was washed out (Fig. 4).

Specificity.—Three tests have been applied to determine whether an exciter agent is acting at the 5-hydroxytryptamine site.

(1) The only effective inhibitor of 5-hydroxytryptamine on the *Venus* heart is (+)-2-bromolysergic acid diethylamide (Brom LSD). A concentration of 10^{-5} g./ml. of this compound present in the bath for 20 min. will usually completely inhibit the action of even high doses of 5-hydroxytryptamine (Welsh and McCoy, 1957). On the other hand, adrenaline, noradrenaline, histamine and n-alkylamines excite the *Venus* heart, but are not antagonized by Brom LSD (Greenberg, 1960).

(2) Drugs which are rendered ineffective by tachyphylaxis of the preparation to 5-hydroxytryptamine are thought to act specifically (Greenberg, 1960). Such compounds in high concentrations can, furthermore, make the heart insensitive to 5-hydroxytryptamine or other specifically acting analogues.

(3) When specific tryptamine analogues in concentrations greater than 10^{-6} M have been left on the heart for over 2 hr., the addition of 10^{-5} M or 2×10^{-5} M 5-hydroxytryptamine will cause a decrease in amplitude. This is usually accompanied by a small increase in tone and a chronotropic effect characteristic of the action of the catechol amines on this preparation (Greenberg, 1960).

Drugs Used.—The compounds used in this study were 5-hydroxytryptamine creatinine sulphate, gramine, 5-hydroxytryptophan, indol-3-ylacetic acid, indol-3-ylpropionic acid (Nutritional Biochemicals); tryptamine [3-(2-aminoethyl)indole] hydrochloride, 5-hydroxyindol-3-ylacetic acid (Mann Research Laboratories); (+)-lysergic acid diethylamide, (+)-2-bromolysergic acid diethylamide (Sandoz Pharmaceuticals); indole, skatole (3-methylindole), phenethylamine (Eastman Kodak Co.); tyramine (*p*-hydroxyphenethylamine) (Abbott Laboratories); tryptophan (California Foundation for Biochemical Research); bufotenine [5-hydroxy-*N'N'*-dimethyltryptamine; 3-(2-dimethylaminoethyl)-5-hydroxyindole], α-methyltryptamine [3-(2-aminopropyl)indole], 5-hydroxy-α-methyltryptamine [3-(2-aminopropyl)-5-hydroxyindole] creatinine sulphate, *N'*-methyltryptamine [3-(2-methylaminoethyl)-indole], *N'N'*-dimethyltryptamine [3-(2-dimethylaminoethyl)indole], *N'*-ethyltryptamine [3-(2-ethylaminoethyl)indole], *N'N'*-diethyltryptamine [3-(2-diethylaminoethyl)indole], 3-(3-dimethylaminopropyl)indole (Upjohn); 5-methoxy-2-methyltryptamine [3-(2-aminoethyl)-5-methoxy-2-methylindole] hydrochloride, 5-hydroxy-2-methyltryptamine [3-(2-aminoethyl)-5-hydroxy-2-methylindole] hydrochloride (Merck Sharp and Dohme); benzoquinonium chloride (Mytolon) (Sterling-Winthrop).

Drugs were made up in M/10 to M/1,000 stock solutions and diluted with distilled or sea water. All doses are expressed as molar concentrations in the organ bath.

RESULTS

The Effects of Phenethylamine and Tyramine.—The action of phenethylamine is a positive inotropic effect, the threshold concentration being about 10^{-5} M. At 10^{-4} M the increase is large and takes about 75 min. to develop completely. Its potency compared with that of 5-hydroxytryptamine is small; the equiactive molar ratio is 2,000. Tyramine, the *p*-hydroxy derivative of phenethylamine, is about 4 times more potent and 1.5 times faster acting than the latter. The actions of both compounds are antagonized by Brom LSD (10^{-5} g./ml.), indicating that they act at the same site as 5-hydroxytryptamine (Fig. 2a, b).

The Effects of Indole and Skatole.—Indole and its 3-methyl derivative, skatole, elicit a positive inotropic response from the *Venus* heart. Threshold is about 3×10^{-6} M. The effect at 10^{-4} M is large and equal to that of 3×10^{-8} M 5-hydroxytryptamine; however, this increase is not blocked by Brom LSD (Fig. 2c, d). Consequently, the action of the indole ring is non-specific.

Close Congeners of 5-Hydroxytryptamine.—The compounds to be discussed here are all closely related in structure to 5-hydroxytryptamine. The actions have, in most cases, been shown to be specific by one or more of the tests previously mentioned. The experimental results are listed in Table I.

Group A.—Tryptamine is about 10 times less active than 5-hydroxytryptamine. Furthermore, the effect of an equiactive dose of tryptamine takes about twice as long to develop (Fig. 3a) and to wash out. The slow recovery from the response is especially evident at higher concentrations. The log dose-response curves for 5-hydroxytryptamine and tryptamine are usually parallel or only slightly divergent (Fig. 4a).

Group B.—Tryptamine analogues with no 5-hydroxyl group, and with methyl or ethyl groups substituted on either the nitrogen or carbon atoms of the side-chain, produce a response which is in certain respects different from that to 5-hydroxytryptamine.

Firstly, the effects are very slow to develop. For example, the response to 3×10^{-9} M *N'*-methyltryptamine may take 5 min. to become noticeable and between 30 and 40 min. to be completed (Fig. 3a). At higher doses this time is often longer.

Secondly, the response is irreversible. While prolonged washing decreases the effect slightly, the original amplitude is never regained. Due to the irreversibility of these compounds, points for dose-response curves were found by adding successively larger doses without interposed washing, as described under methods.

Thirdly, the relationship between concentration and effect of the *N'*-alkyl-substituted compounds is unlike that of 5-hydroxytryptamine. Threshold

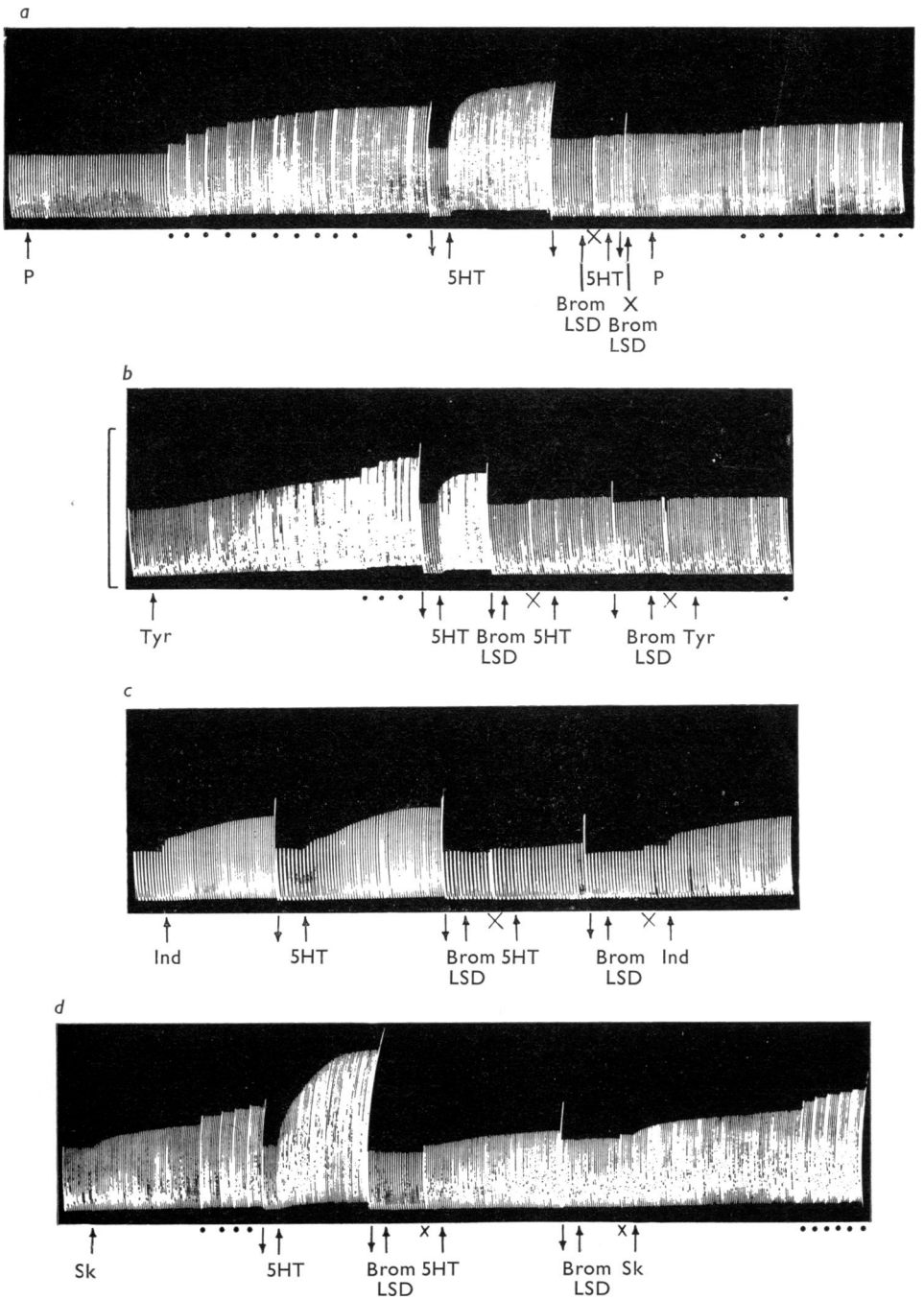
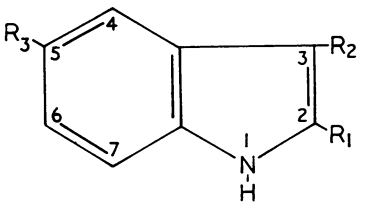


FIG. 2.—The effect of 10^{-5} g./ml. (+)-2-bromolysergic acid diethylamide (Brom LSD) on the responses of the *Venus* heart to (a) 5-hydroxytryptamine 3×10^{-7} M (5HT) and phenethylamine 10^{-4} M (P); (b) 5-hydroxytryptamine 3×10^{-7} M (5HT) and tyramine 10^{-7} M (Tyr); (c) 5-hydroxytryptamine 3×10^{-8} M (5HT) and indole 10^{-4} M (Ind); (d) 5-hydroxytryptamine 10^{-7} M (5HT) and skatole 10^{-4} M (Sk). Following each dose of Brom LSD the drum was stopped (at X) for 20 min. (●) indicates drum was stopped; total elapsed time is 60 min. in (a) and (b) and 40 min. in (d). Downward-pointing arrows indicate washing. Benzoquinonium chloride (10^{-5} g./ml.) added to bath after washing. Tension: 1,000 mg. Temperature: 15°. Heart rate: 10/min. Amplitude scale: 6 cm.

TABLE I

RELATIVE POTENCIES OF TRYPTAMINE ANALOGUES ON *VENUS* HEART

The equiactive molar ratio of 5-hydroxytryptamine is taken as unity. N.E. means that no effect could be elicited from the preparation.



Group	Compound	R_1	R_2	R_3	Equiactive Molar Ratio: Individual Values	Equiactive Molar Ratio: Mean
A	Tryptamine	H	$-CH_2-CH_2-NH_2$	H	15, 14, 13, 11, 10, 6, 6, 4	9·9
B	*N'*-Methyltryptamine	H	$-CH_2-CH_2-NH(CH_3)$	H	6, 5, 4, 4, 3·3, 2·2, 1·4	3·7
	N'-Ethyltryptamine	H	$-CH_2-CH_2-NH(C_2H_5)$	H	15, 14, 10, 10, 7, 4·5, 3	9·1
	N'N'-Dimethyltryptamine	H	$-CH_2-CH_2-N(CH_3)_2$	H	30, 15, 12·5, 10, 4, 2, 1·7	10·7
	N'N'-Diethyltryptamine	H	$-CH_2CH_2-N(C_2H_5)_2$	H	12, 12, 10, 10, 8·6, 1·7, 1	7·9
	α-Methyltryptamine	H	$-CH_2-CH(CH_3)-NH_2$	H	10, 8·7, 7	8·6
C	Bufotenine	H	$-CH_2-CH_2-N(CH_3)_2$	-OH	0·04, 0·03, 0·025, 0·017	0·028
	5-Hydroxy-α-methyltryptamine	H	$-CH_2-CH(CH_3)-NH_2$	-OH	6, 6	6
D	5-Hydroxy-2-methyltryptamine	$-CH_3$	$-CH_2-CH_2-NH_2$	-OH	60, 35, 30, 17, 15	31·4
	5-Methoxy-2-methyltryptamine	$-CH_3$	$-CH_2-CH_2-NH_2$	$-OCH_3$	65, 50, 35, 25	43·8
E	Gramine	H	$-CH_2-N(CH_3)_2$	H	N.E., N.E., N.E., 10,000, 7,000, 170	—
	N'N'-Dimethyltryptamine	H	$-CH_2-CH_2-N(CH_3)_2$	H	30, 15, 12·5, 10, 4, 2, 1·7	10·7
	3-(3-Dimethylaminopropyl)indole	H	$-CH_2-CH_2-CH_2-N(CH_3)_2$	H	3,300, 1,700, 1,000	2,000

is usually 10^{-9} M or 3×10^{-9} M. In 82% of the experiments the response to low concentrations (10^{-9} to 10^{-8} M) of the *N'*-alkyltryptamines was equal to, or greater than, that to the same dose of 5-hydroxytryptamine. The maximum effect was achieved at 10^{-5} to 10^{-4} M. However, both the inotropic effect and the increased tone resulting from this maximal response are only equivalent to those of 3×10^{-8} M to 3×10^{-7} M 5-hydroxytryptamine.

The log dose-response curves which result vary greatly in shape (Fig. 4). In general the curves are sigmoid with a lower limb which may cross the 5-hydroxytryptamine curve of the same preparation (Fig. 4b). The middle, steeply-rising, portion of the *N'*-alkyltryptamine curves starts at about 10^{-7} M and is usually divergent from that of 5-hydroxytryptamine. On the other hand, with *N'N'*-diethyl- or *N'*-ethyl-tryptamine this portion of the curves is often parallel to the standard (Fig. 4c). In about a third to a half of the experiments, once a response at low concentrations was established, further increases in dose (up to about 10^{-7} M) produced either small or negligible increases in amplitude. The log dose-response curves which resulted were biphasic with a

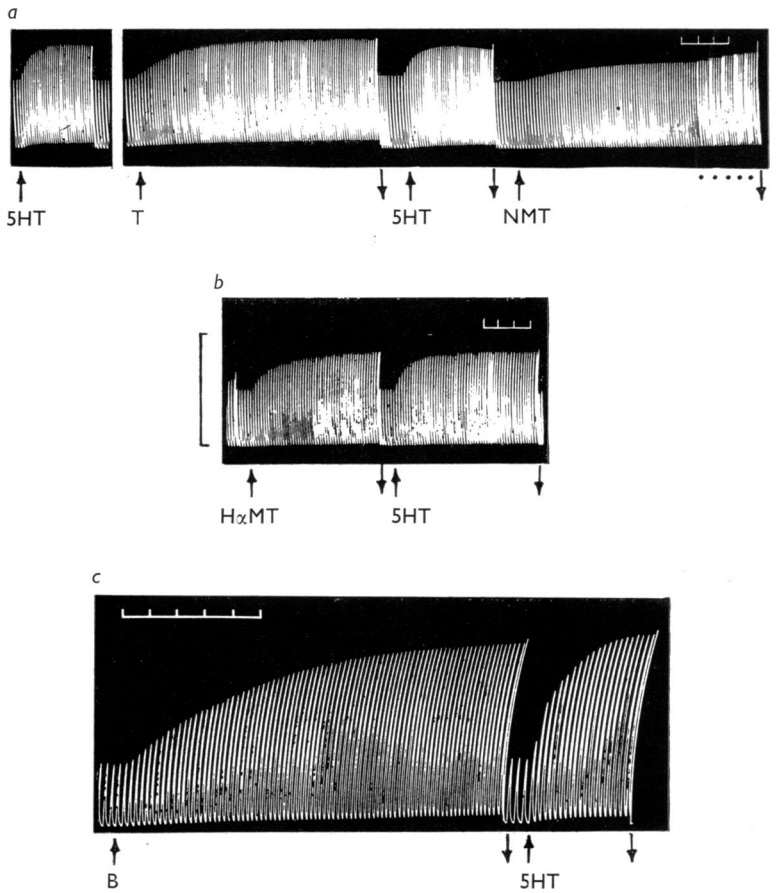


FIG. 3.—Comparison of velocities of *Venus* heart preparations in response to (a) 5-hydroxytryptamine 3×10^{-7} M (5HT), tyramine 3×10^{-6} M (T), 5-hydroxytryptamine 10^{-7} M (5HT) and *N'*-methyltryptamine 10^{-8} M (NMT); (b) 5-hydroxy-α-methyltryptamine 10^{-7} M (HαMT) and 5-hydroxytryptamine 2×10^{-8} M (5HT); (c) bufotenine 10^{-8} M (B) and 5-hydroxytryptamine 10^{-7} M (5HT). At (●) the drum was stopped for 5 min. Washing is at downward-pointing arrows. Benzoquinonium chloride (10^{-5} g./ml.) added to the bath immediately after washing and 5 min. before succeeding dose. Tension: 500 mg. Temperature: 15°. Time scale: 30 sec. Amplitude: 3 cm.

plateau between about 10^{-8} M and 10^{-7} M (Fig. 4c). Such variation in the shape of the curves, and their divergence from those of 5-hydroxytryptamine, suggest that there should be some uncertainty in potency measurements. This is confirmed by the results shown in Table I. The equiactive molar ratios of α-methyl-, *N'*-ethyl-, *N'N'*-diethyl- and *N'N'*-dimethyl-tryptamine are of the same order of magnitude as that of tryptamine itself. However, *N'*-methyltryptamine has a potency about double that of tryptamine.

Group C.—5-Hydroxy-α-methyltryptamine and bufotenine are the 5-hydroxy analogues of two methyl-substituted amines previously discussed (Group B). The presence of the hydroxyl group results in some marked changes in action. First, the responses are reversible. Second, the speed of action increases greatly.

5-Hydroxy-α-methyltryptamine is not significantly more active than α-methyltryptamine, but bufotenine is about 500 times as active as *N'N'*-dimethyltryptamine.

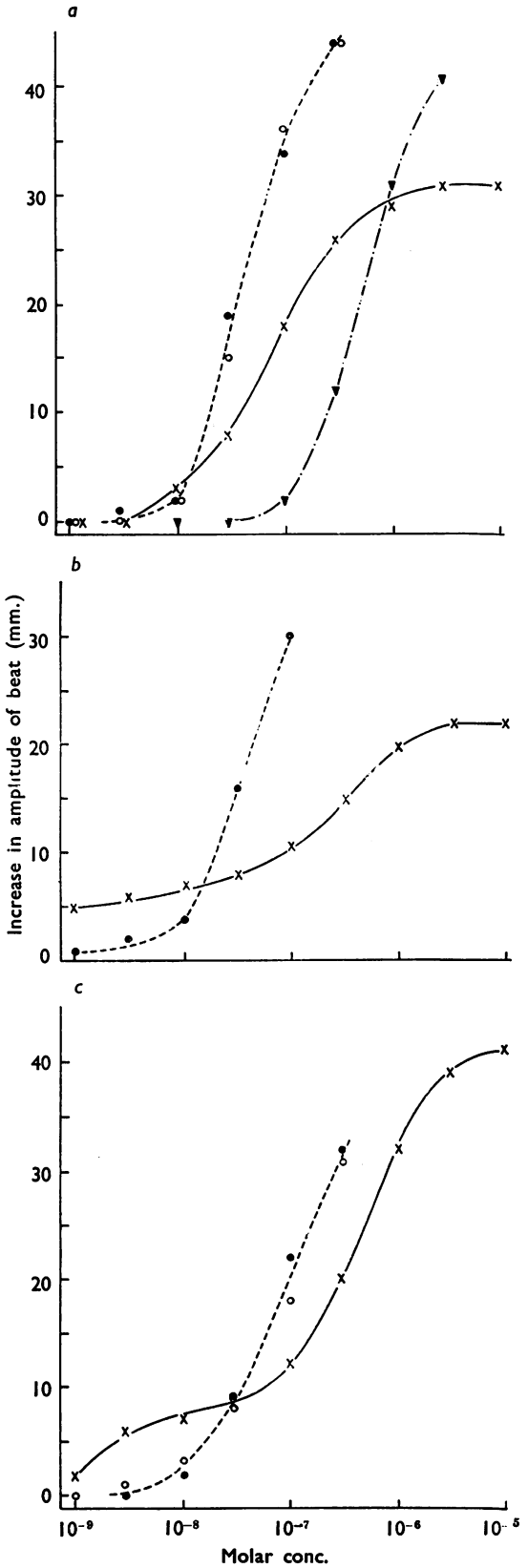


FIG. 4.—Dose-response curves illustrating the variation in the effects of non-hydroxylated tryptamine analogues on three *Venus* heart preparations. The effects of 5-hydroxytryptamine (— — —) are compared with those of: (a) *N'*-methyltryptamine (X——X) and tryptamine (▼—.—▼); (b) *N'N'*-diethyltryptamine (X——X); and (c) *N'*-ethyltryptamine (X——X). In preparations (a) and (c) one set of 5HT points were obtained by washing between successive doses (**O**) and another by making additions without washing (●).

Of all the compounds which have been tested, 5-hydroxy-α-methyltryptamine produces effects which are most similar to those of 5-hydroxytryptamine. Its time of action is only slightly longer than that of 5-hydroxytryptamine (Fig. 3b). The effects, even of relatively high doses (3×10^{-7} M), are quickly washed out. The log dose-response of the two substances are parallel (Fig. 5a); this is similar to the results with tryptamine.

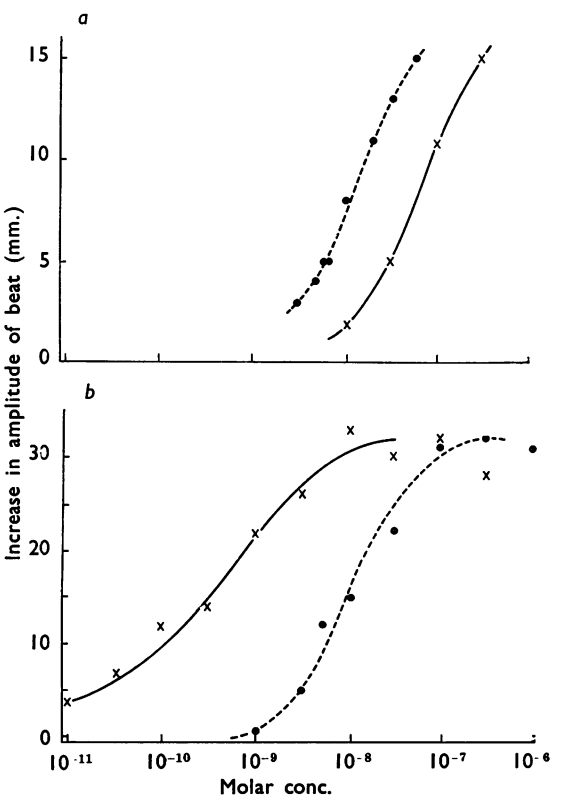


FIG. 5.—Dose-response curves contrasting the effects on *Venus* hearts of 5-hydroxytryptamine (●— — —●) and (a) 5-hydroxy-α-methyltryptamine (X——X) and (b) bufotenine (X——X).

However, compared with tryptamine, 5-hydroxy-α-methyltryptamine is more potent, faster in action, and easier to wash out.

Bufotenine is more potent than 5-hydroxytryptamine. Its structure is intermediate between that of 5-hydroxytryptamine and *N'N'*-dimethyltryptamine, and its mode of action is also intermediate. First, while its effects are not irreversible, the washing time for a similar response is almost twice that of 5-hydroxytryptamine. Second, at threshold or slightly higher concentrations (10^{-11} M to 10^{-9} M), the response to bufotenine may take between 20 and 30 min. to develop and at any concentration it is slower than the equipotent 5-hydroxytryptamine response (Fig. 3c). Third, the log dose-response curve of bufotenine is always less steep than that of 5-hydroxytryptamine (Fig. 5b). Accordingly, the equiactive molar ratio varies with concentration. At threshold it is 1/100. Near the maximum response of bufotenine, at about 3×10^{-8} M, it is approximately 1/10.

Group D.—The compounds of this group have a substituent in the 2-position of the indole ring. This substitution serves to decrease the potency greatly. Thus, 5-hydroxy-2-methyltryptamine is about 30 times less potent than 5-hydroxytryptamine. Its threshold is 3×10^{-7} M. Also, the time of action is slow and the effect is difficult to wash out, especially at high concentrations. 5-Methoxy-2-methyltryptamine is slightly less potent than its 5-hydroxy congener, although its threshold is about the same. It is also far more difficult to wash out. The effects are, in fact, not completely reversible.

Brom LSD might be included here in its capacity as an excitor agent (Greenberg, 1960) with a substituent in the 2-position. The exciter action is extremely undependable. When it occurs, the equiactive molar ratio of a 3×10^{-5} M dose varies between 300 and 30,000 depending upon the preparation. The time of action is slow and the effect is irreversible.

Group E.—The substances included here differ only in the length of the side-chain at the 3-position. In gramine it is one, in *N'N'*-dimethyltryptamine it is two, and in 3-(3-dimethylaminopropyl)indole it is three carbon atoms long. All three compounds have two methyl groups on the amino-nitrogen atom. None has a 5-hydroxyl group in the indole ring.

It is clear that increasing or decreasing the length of the side-chain results in a profound loss of activity (Table I). This loss is greatest with gramine. Gramine is well known as a 5-hydroxytryptamine antagonist in a variety of preparations. It also acts in this way, although weakly, on *Venus* heart. Applied to the heart for an hour, 10^{-4} M gramine decreases the response by more than half. 3-(3-Dimethylaminopropyl)indole is only slightly inhibitory. Its excitatory action at high concentrations usually masks any inhibition which might occur.

No attempt was made to block the action of gramine or 3-(3-dimethylaminopropyl)indole with Brom LSD. Since these two compounds antagonize 5-hydroxytryptamine, it was assumed that they act at the 5-hydroxytryptamine site. However, the specificity of the antagonism has not been demonstrated.

The Effects of Indol-3-yl Acids.—Of the indol-3-yl acids which have been examined, some are in the 5-hydroxytryptamine metabolic pathway and others are not. It might be expected that tryptophan (α-aminoindol-3-ylpropionic acid) and 5-hydroxytryptophan, which are precursors of 5-hydroxytryptamine (Udenfriend, 1958), would be converted by enzymes in the heart. Their effect should then be a slow increase in the amplitude of the heart. In fact, tryptophan is completely inactive up to 10^{-3} M. 5-Hydroxytryptophan excites feebly and occasionally at 10^{-3} M. Its potency relative to 5-hydroxytryptamine is about 100,000.

5-Hydroxyindol-3-ylacetic acid is a product of the enzymic breakdown of 5-hydroxytryptamine (Udenfriend, Wiessbach, and Bogdanski, 1957). This acid does excite feebly at 10^{-4} M. The equiactive molar ratio is 20,000.

Indol-3-ylacetic acid and indol-3-ylpropionic acid are essentially inert although indol-3-ylacetic acid excites at 10^{-3} M. The equiactive molar ratio is over 100,000.

These acids are so feebly active that no attempt was made to antagonize their action with Brom LSD. Indeed, the exciter response to the latter at 2×10^{-5} g./ml. is usually of greater magnitude.

At least two attempts were made, using each of these acids, to block the actions of 5-hydroxytryptamine. None were successful.

Lysergic Acid Diethylamide.—Characteristics encountered in the *N'*-alkyltryptamines are emphasized in lysergic acid diethylamide. The usual threshold concentrations (10^{-16} M–10^{-15} M) often result in a nearly maximal response which, however, takes 4 hr. to be completed (Welsh and McCoy, 1957). The log dose-response curves from such preparations cross, almost perpendicularly, those of 5-hydroxytryptamine (Fig. 6a). Estimation of potency in such cases is meaningless. However, not all hearts respond in this

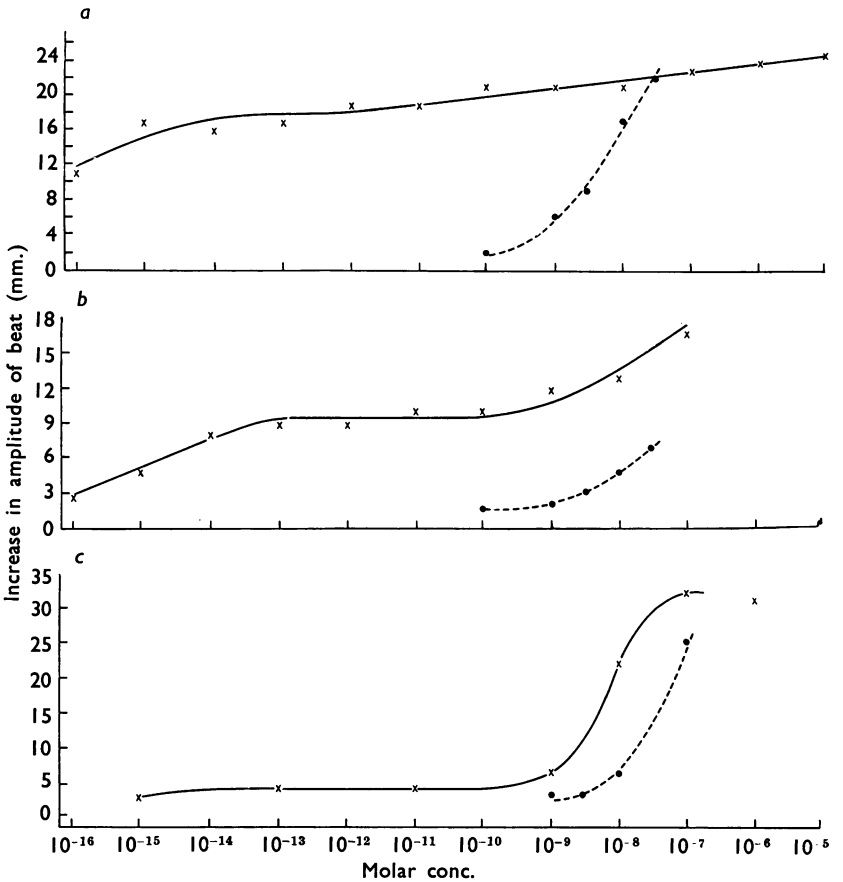


FIG. 6.—Dose-response curves showing variations in the responses of different *Venus* heart preparations to (+)-lysergic acid diethylamide (X——X) and to 5-hydroxytryptamine (● — — — ●).

manner. Some preparations are affected less strongly by low concentrations of lysergic acid diethylamide. The curve in these cases may turn upward at about 10^{-10} M (Fig. 6b). In two experiments, there was almost complete insensitivity to low concentrations. The log dose-response curve rose abruptly, at about 10^{-10} M, parallel to that of 5-hydroxytryptamine on the same heart (Fig. 6c). In both experiments, the equiactive molar ratio in this parallel portion of the curve was 0.1.

Yohimbine.—This alkaloid excites the *Venus* heart weakly. The threshold concentration is between 10^{-6} M and 10^{-5} M. The mean equipotent molar ratio is 630. The response is slow, taking about 30 min. for completion. It is also not entirely reversible. Evidence of specificity from tachyphylaxis experiments is negative but inconclusive.

DISCUSSION

The simplest structural requirement for 5-hydroxytryptamine-like activity on the *Venus* heart preparation seems to be embodied in phenethylamine and tryptamine: a flat aromatic nucleus with a 2-aminoethyl side-chain. These requirements are established largely by the ineffectiveness of some of the substances tested.

Neither the primary alkylamines, nor indole alone, have a 5-hydroxytryptamine-like action on the *Venus* heart. The n-alkylamines have been shown to be excitatory but unspecific in their action relative to 5-hydroxytryptamine (Greenberg, 1960). Evidently an alkyl side-chain cannot take the place of a proper ring substituent. Indole and its close congener skatole are likewise excitatory but unspecific in action. Indeed, these two compounds have been shown to be inactive

(Erspamer, 1952), or unspecifically depressant (Izquierdo and Stoppani, 1953), on a variety of smooth muscle preparations.

The characteristically different actions of histamine and dopamine on *Venus* heart (Greenberg, 1960) point to the ineffectiveness of imidazolyl- or dihydroxyphenyl-ethylamines in producing 5-hydroxytryptamine-like effects. However, if these compounds had such an action, it would probably be no stronger than that of tyramine. Hence, it might be completely masked by the strong generic effects of histamine and dopamine.

The importance of the amino-group itself is illustrated, firstly, by the ineffectiveness of indol-3-ylacetic acid, in which the amino-group is absent.

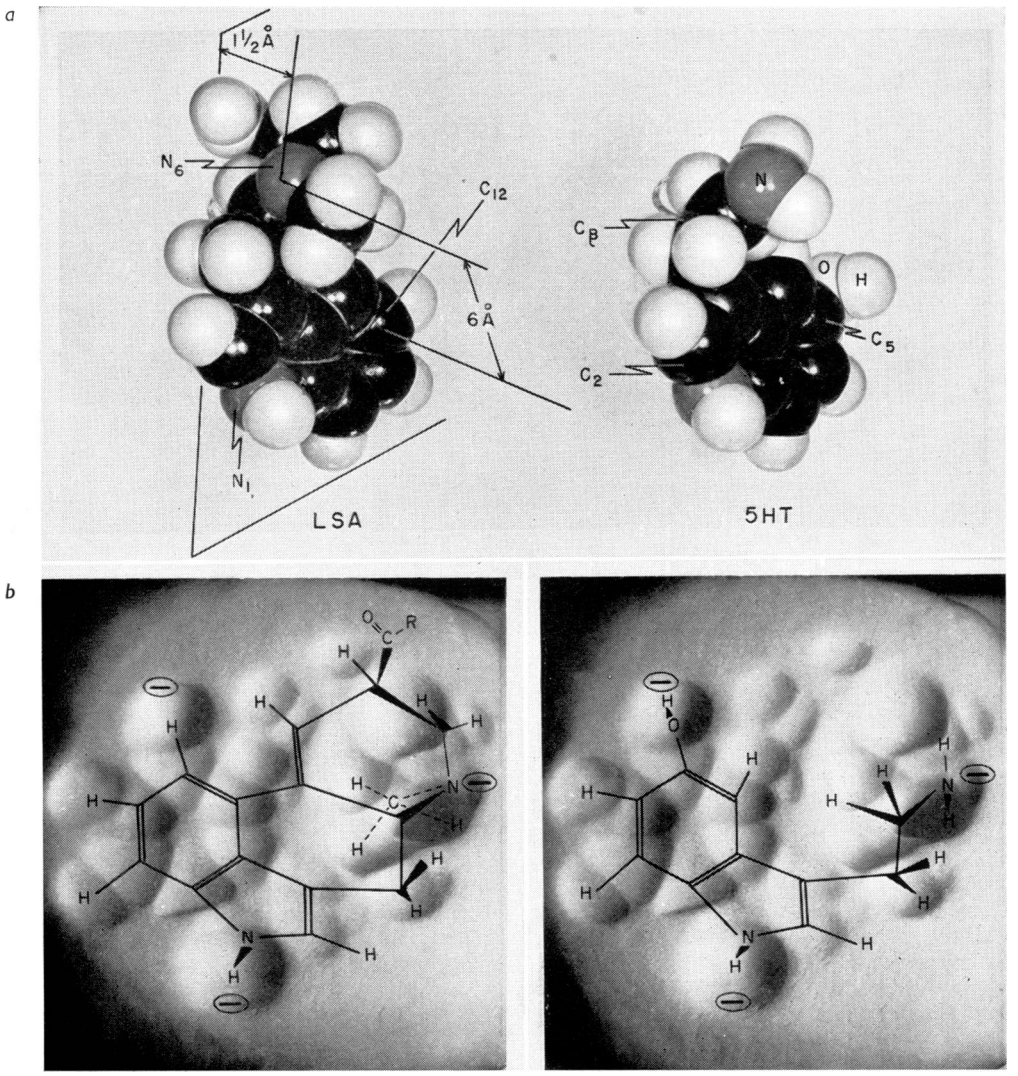


FIG. 7.—(a) Taylor-Hirschfelder models of (+)-lysergic acid (LSA) and 5-hydroxytryptamine (5HT). (b) Composite negative imprint of the molecules in (a) as a model of 5-hydroxytryptamine receptor area. Structural formulae of (+)-lysergic acid diethylamide (left) and 5-hydroxytryptamine (right) show orientation of molecules. The H at C_5 of (+)-lysergic acid diethylamide is omitted; R is $-N(C_2H_5)_2$. Three proposed negative binding sites for the 5-hydroxyl group, 1-nitrogen atom and terminal amino-group of 5-hydroxytryptamine are indicated.

Secondly, in tryptophan and 5-hydroxytryptophan solutions, the α-amino-groups are hydrogen-bonded to the carboxyl groups. These two tryptamine analogues are also feeble exciter agents on the *Venus* heart. Finally, the amino-nitrogen atom must be two carbon atoms distant from the ring. Pronounced reduction in potency accompanies either a decrease (gramine) or an increase [3-3-(dimethylaminopropyl)indole] of only about 1.5 Å. in the length of the aminoalkyl side-chain. That gramine excitation might not even be specific is suggested by the fact that it antagonizes the effect of adrenaline as well as 5-hydroxytryptamine in *Spisula solida* (Gaddum and Paasonen, 1955).

The arrangement in space of the aminoethyl side-chain of tryptamine and phenethylamine analogues is variable. Consequently, the effective conformations of these molecules and the corresponding shapes of the receptors are in question. A partial answer resides in two properties of (+)-lysergic acid diethylamide: Firstly, its extremely low threshold on the *Venus* heart suggests that the molecule must fit some cellular receptor site precisely. Secondly, the dimensions and conformation of the (+)-lysergic acid nucleus are fixed by closure of the C and D rings. Thus, lysergic acid diethylamide might well be used as a tool in establishing at least one active conformation and complementary receptor of tryptamine and its congeners.

Three salient points define the spatial arrangement of lysergic acid diethylamide (Cookson, 1953): (1) The lysergic acid nucleus is planar up to a line joining C_5 and C_8 across the upper (D) ring. (2) Along this line the D ring is folded so that N_6 (corresponding to the primary amino-nitrogen atom of 5-hydroxytryptamine) is out of the plane by 1 to 1.5 Å. (3) The distance between C_{12} (corresponding to C_5 in 5-hydroxytryptamine) and N_6 is between 5.5 and 6 Å.

Of the many possible conformations of 5-hydroxytryptamine, one exists (Fig. 7) which satisfies the spatial requirements defined by lysergic acid. (1) Only one of the three most probable positions of C_β, that in which the C_β hydrogen atoms are symmetrically disposed about the C_2 hydrogen atom, permits the carbon atoms of the aminoethyl side-chain of 5-hydroxytryptamine to lie in the same plane as the indole ring. Such a conformation is energetically favoured by the positioning of the C_2 and C_β hydrogen atoms at an energy minimum which, however, is not necessarily the lowest minimum (Dauben and Pitzer, 1956). (2) With C_β in the position described, the primary amino-nitrogen atom can be displaced 1 to 1.5 Å. out of the plane of the molecule by the rotation of C_α. (3) The distance between C_5 and the primary amino-nitrogen atom then varies between 4.5 and 6.5 Å. Thus, a receptor (for example, Fig. 7) which accommodates lysergic acid diethylamide will also accept 5-hydroxytryptamine—but only when the latter molecule is in the conformation described above. This conformation, it must be mentioned, is not the most probable one which exists for (2-aminoethyl)indole analogues. However, if the free energy barrier between this conformation and the most probable one is between 4,000 and 6,500 cal./mole, then for every 10^3 to 10^5 molecules having the probable conformation there will be one in the planar conformation hypothesized. Now, lysergic acid diethylamide requires about 10^6 to 10^7 times fewer molecules to produce a threshold response than do its tryptamine congeners. If we assume (comparing Table I and Fig. 6c) that lysergic acid diethylamide is between 10 and 100 times more potent than these analogues, then 1 out of 10^3 to 10^5 tryptamine molecules would indeed be sufficient to produce the observed effects. Thus, the improbability of the proposed conformation would seem to explain, in part, the great difference in the number of molecules required for the responses of lysergic acid diethylamide and its tryptamine analogues.

Two regions are seen to be necessary in the receptor area. Firstly, there must be a flat surface, about 11 Å. by 9 Å., which is complementary to the indole, or benzene, ring. Secondly, a contiguous ovoid depression, 6 Å. by 4 Å. and up to 3.5 Å. deep, should be present. The depression accepts the groups in the lysergic acid D ring and the terminal amino-groups of tryptamines or phenethylamines which are folded out of the plane of the indole ring.

On the basis of this model some deductions can be made regarding the binding forces between the various compounds employed and the receptor. The forces which bind the aromatic nuclei to the receptor are probably of the weak, unspecific van der Waals type which depend for strength on exact conformity between many atoms in the drug and its receptor. Also, the possibility of hydrogen bonding between the 1-nitrogen of the indole ring and the receptor exists. The relative impotency of the benzene nucleus in phenethylamine (compared to indole in tryptamine) could be attributed to a deficiency in either of these sorts of binding forces.

Since the pK_a of 5-hydroxytryptamine is 10.0, the amino-group is mostly ionized at physiological *p*H (Vane, 1959). This fact, together with the

loss of potency with lengthening of the side-chain (and presumably with distance from a binding site), suggests that the terminal amino-group of tryptamines, and probably of phenethylamines, is hydrogen bonded to some negative site in the receptor.

Structural modifications of the basic compounds, tryptamine and phenethylamine, affect both the potency and mode of action of the resulting analogues. The presence of the 5-hydroxyl group in 5-hydroxytryptamine, bufotenine and 5 - hydroxy - α - methyltryptamine renders these analogues more potent, faster acting, and more rapidly reversible than their unhydroxylated congeners. The potency relationship has been observed in other molluscan preparations. Gaddum and Paasonen (1955) found that tryptamine was about one thousand times less effective than 5-hydroxytryptamine in stimulating the heart of *Spisula solida*. The threshold of both 5-hydroxytryptamine and bufotenine excitation of *Anodonta* heart is 7×10^{-9} g./ml., while that of *N'N'*-dimethyltryptamine is found to be only 10^{-5} g./ml. (Marczyński, 1959). These data suggest the possibility of a hydrogen bridge between a negative site in the receptor (Fig. 7) and the 5-hydroxyl group which is unionized at physiological hydrogen-ion concentrations (Vane, 1959).

While tyramine is more potent and has a smaller response time than phenethylamine, it cannot be assumed that the *p*-hydroxyl group of tyramine necessarily occupies the same binding site as the 5-hydroxyl group of 5-hydroxytryptamine. A position of the molecule in which the benzene ring is flat on the planar portion of the receptor and the amino-group is in its appropriate site is compatible with binding of the *p*-hydroxyl group of tyramine either at the 5-hydroxyl site or the 1-nitrogen site of 5-hydroxytryptamine. The conformation of tyramine in either position is relatively improbable, which helps account for its low potency. Incidentally, if binding of the *p*-hydroxyl group at the 5-hydroxyl site occurs, an additional planar receptor area must be hypothesized. Both receptor positions, as well as others, are probably assumed by these small, flexible molecules.

Tryptamine analogues which have alkyl substituents on the primary amino-nitrogen atom have a lower potency than 5-hydroxytryptamine. However, in no case is potency significantly lower than that of tryptamine; in one instance (*N'*-methyltryptamine) it is higher. In this regard, an *N'*-methyl substituent, homologous with that of lysergic acid, would be expected to provide the most complementary fit to the hypothesized receptor.

Since the *N'*-alkyltryptamine effects are also irreversible, it becomes clear that increased binding alone seems to lead to little or no increase in potency if there is, at the same time, no 5-hydroxyl group. The hydrogen bridge at the latter location is essential for the precise positioning of the indole ring in the receptor area. If this is correct, then *N'*-alkyltryptamine analogues which have a 5-hydroxyl group should have a greater potency than 5-hydroxytryptamine. In fact, bufotenine is about thirty-five times as potent as 5-hydroxytryptamine, while 5-hydroxy-*N'*-methyltryptamine is about ten times as potent (Bumpus and Page, 1955). On the other hand, bufotenidine (5-hydroxy - *N'N'N'* - trimethyltryptamine) is only about as active as tryptamine on *Venus* heart (Twarog and Page, 1953). The amino-nitrogen atom in bufotenidine is quaternary. Consequently, it probably also acts on the acetylcholine receptors of the ventricle which would result in an observed effect with a negative inotropic component (Welsh and Taub, 1948).

Substitution of a methyl group in the tryptamine molecule, in positions other than on the primary amino-group, either lowers, or has little effect on, the potency of the resulting compound. Although such compounds are also irreversible indicating strong binding, the analogues with 5-hydroxyl groups, unlike the *N'*-alkyl tryptamines, are not much more potent than 5-hydroxytryptamine. Thus, 5-hydroxy-2-methyltryptamine is barely more potent than 5-methoxy-2-methyltryptamine. Again, while the presence of a 5-hydroxyl group in α-methyltryptamine changes the character of the response, the potency remains essentially unchanged. Substitution of a methyl group in positions other than on the primary amino-group probably sterically hinders the binding of the side-chain (α-methyltryptamines) or the indole nucleus (2-methyltryptamines) by lifting them off the planar portion of the receptor. For example, a methyl group or bromine atom in the 2-position would raise C_2 and the adjacent tertiary nitrogen atom almost an Ångstrom, thus, of course, decreasing the strength of the interaction. If the displacement is sufficient, the presence or absence of the 5-hydroxyl group seems to have relatively little further effect on the positioning, and hence the potency, of the analogue.

Conformations of 5-hydroxytryptamine other than that represented by the hypothetical receptor exist, of course. Some of these might also be

effective on the *Venus* heart. For example, another spatial arrangement is represented in the structure of the harmala and rauwolfia alkaloids. As is well known, both 5-hydroxytryptamine-like action and inhibition of 5-hydroxytryptamine-like action by these compounds have often been correlated with the presence of the 3-(2-aminoethyl)-indole configuration (Woolley and Shaw, 1957). However, two features of yohimbine, in particular, suggest that such a conformation of 5-hydroxytryptamine is ineffective on *Venus* heart.

First, it is obvious that when the planar indole rings of lysergic acid diethylamide and yohimbine are congruent, the important ring nitrogen atoms (N_6 and N_4 respectively) are 4 to 5 Å apart. Thus, judging from the lack of potency of gramine and 3-(3-dimethylaminopropyl)indole, yohimbine and similar alkaloids cannot be effective at the *Venus* heart receptors with which lysergic acid diethylamide interacts. It follows that 5-hydroxytryptamine in a conformation corresponding to that of yohimbine (and involving, at least, rotation of C_β through about 90°) would also be inactive at the lysergic-acid-like site. Second, not only is the structure of yohimbine theoretically incompatible with effective action at lysergic-acid-like sites but also it is, *in fact,* relatively inactive. This suggests that, in *Venus* heart, there is no receptor area complementary to yohimbine or to 5-hydroxytryptamine in this conformation.

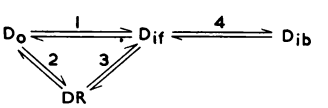
It seems possible that the well-known differences in action between the rauwolfia and ergot alkaloids (Brodie, 1958) are due to the fact that these two groups of compounds are structurally incapable of acting at the same cellular receptors. The relative smallness and flexibility of 5-hydroxytryptamine, on the other hand, would permit interaction of this molecule with the sites of both sorts of alkaloids. However, differences between such interactions would be expected and, in fact, occur in a variety of preparations (Woolley and Shaw, 1953; Gaddum and Hameed, 1954; Salmoiraghi and Page, 1957).

Tissues may have a population of cellular receptors representing the different conformations (and presumably the different effects) of small, active molecules such as 5-hydroxytryptamine. The relative effectiveness of large molecules with fixed conformations would be a measure of the distribution of such a population. The *Venus* heart receptors, in contrast to those of mammalian brain, apparently respond to a smaller range of possible conformations of 5-hydroxytryptamine.

Many analogues of tryptamine are more or less irreversible. The ease with which a drug will leave the tissue for the bath solution is determined, in part, by the oil-water partition coefficient of the compound. Vane (1959) showed that the coefficient of tryptamine is about twenty times that of 5-hydroxytryptamine due to the absence, in the former, of the polar hydroxyl group. It is well known that alkyl substitution increases the partition coefficient and it is in these terms that the irreversibility of such compounds can most readily be explained.

There is no evidence from experiments on the *Venus* heart preparation which indicates whether the *N'*-alkyltryptamines are merely strongly bound to the cell membrane or whether they actually penetrate. However, data from other studies suggest that these compounds enter the cell. Vane (1959), for example, using the enhancement of the excitor activity by amine oxidase inhibition as a test, was able to show that tryptamine analogues with high oil-water partition coefficients (for example, tryptamine, *N'*-ethyltryptamine, *N'*-methyltryptamine) entered the cells of the rat fundus while 5-hydroxytryptamine did not. Similarly, lysergic acid diethylamide and bufotenine, as well as *N'N'*-dimethyltryptamine and *N'N'*-diethyltryptamine (Szara, 1957), are hallucinogens in man and thus cross the blood-brain barrier. 5-Hydroxytryptamine, which cannot cross, is not a hallucinogen.

If the alkyl-substituted tryptamines enter the cell, then the effective drug molecules must be considered as being divided into two pools: those inside (D_i) and those outside (D_o) the cell. The following equilibria might obtain at any bath concentration:



where D_{if} and D_{ib} are, respectively, free and bound drug inside the cell. DR is drug-receptor complex at the cell surface. The following points need to be made regarding the above model:

(1) The action of lysergic acid diethylamide and the *N'*-alkyltryptamines suggests that the drug accumulates slowly in the cells from low bath concentrations. However, it is impossible, on present evidence, to distinguish between steps (1) and (2+3). It is important to note that the response is assumed to be a measure of the concentration of drug-receptor complex. Consequently, even if step (1) does occur, only internal

free drug accumulating in this manner in close proximity to the receptor can be measured.

(2) The biphasic dose-response curves which are obtained in a third of *N'*-alkyltryptamine experiments (Fig. 4c) suggest that the "tryptamine space" of the cell can be saturated. Thus, when the bath concentration is about 10^{-8} M the net movement of drug into the cells ceases. The ratio of dose-response becomes constant until the bath concentration is made large enough (about 10^{-7} M) so that $d(D_oR)/dt$ becomes larger than $d(D_iR)/dt$. Naturally, compounds such as the 2-methyl analogues, whose thresholds are greater than the maximum internal drug concentration, will have no effect even though they enter the cell and accumulate there to the same extent as do the *N'*-alkyltryptamines.

(3) If drug is bound in the cell, it is not bound to the receptors (that is, $D_{ib} \neq DR$). As drug concentration is increased above the levels which saturate the cell interior, the response again increases, indicating the availability of more free receptors. Lipid associated with the cell membrane is probably responsible for intracellular binding if the latter occurs.

The relationship between doses of lipid-soluble tryptamine analogues and their effects on the *Venus* heart is obviously a complex one. The individual variation found in the responses of different hearts to the *N'*-alkyltryptamines clearly reflects this.

The author wishes to acknowledge the advice and encouragement offered by Professor J. H. Welsh during the course of this investigation.

This work was supported in part by a fellowship (HF-7571) from the National Heart Institute of the United States Public Health Service, and by a University of Illinois Faculty Fellowship.

The author is indebted to the following for their generous gifts of chemicals: Dr. R. V. Heinzelman and Dr. M. E. Speeter, Upjohn Co.; Dr. L. H. Sarett, Merck, Sharpe & Dohme; Dr. R. Bircher, Sandoz Pharmaceuticals; Dr. A. M. Lands, Sterling-Winthrop Research Institute; Dr. R. K. Richards, Abbott Laboratories.

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THE EFFECTS OF ADRENALINE AND NORADRENALINE ON THE METABOLISM AND PERFORMANCE OF THE ISOLATED DOG HEART

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(RECEIVED MARCH 22, 1960)

In 16 dog heart-lung preparations modified to permit a more accurate measurement of coronary flow, adrenaline or noradrenaline was infused at a rate of 4 μg. base/min. After a 30-min. pause during which the increased oxygen consumption and heart rate, but not the coronary flow, returned to pre-infusion levels, the other sympathomimetic amine was infused for the same length of time. It was found that, mole per mole, noradrenaline is as effective, and probably more so, than adrenaline in raising the oxygen consumption of the heart-lung preparation. The positive chronotropic and coronary dilating action of both amines appear to be equal. It was observed that in any one experiment the second dose of the sympathomimetic amine was slightly more effective than the first dose in raising the oxygen consumption. The level of high-energy phosphorus compounds does not change after adrenaline or noradrenaline administration even at the time when the oxygen consumption rises to as much as 200%. During this period there are no signs of cardiac hypoxia, as can be judged by the good oxygen saturation of coronary venous blood. Single doses of 5 μg. adrenaline or noradrenaline have a consistent positive inotropic effect that lasts about 15 min. when tested on a failing heart. In 12 experiments on non-failing modified heart-lung preparations, a single dose of 5 μg. adrenaline fails to cause a measurable increase in oxygen consumption after 1, 3, 6, or 11 min. in spite of a mild positive chronotropic action. The significance of these findings is discussed and the suggestion made that, when noradrenaline infusions are effective in treating cardiogenic shock in man, part of this effect may be due to its positive inotropic action, thus correcting an element of heart failure that might exist.

The main actions of adrenaline on the heart are: positive inotropic and chronotropic effects, increased atrio-ventricular conduction, and increased excitability. In addition to these, there is the "calorigenic" effect. This is the increase in cardiac oxygen consumption which cannot be explained solely by the rise in heart rate and increase in work performed (for literature see Gollwitzer-Meier, Kramer and Krüger, 1936). All these effects of adrenaline need not have the same mechanism of action at the cellular or subcellular (enzymatic) level. Krayer (1949), for instance, has shown that the chronotropic action but not the inotropic action can be inhibited by veratramine. It is also conceivable that a certain dose of adrenaline would evoke one effect and not the other. The mechanism of the coronary dilating action of adrenaline is not completely understood and it is not clear to what extent the vasodilatation is due to the rise in cardiac metabolism.

In this study a comparison has been made between the "calorigenic," chronotropic, and coronary dilating actions of (−)-adrenaline and (−)-noradrenaline on the dog heart-lung preparation and the effect of these compounds on the phosphorus compounds of the heart. Furthermore, a study has been made of the effect of graded doses of adrenaline, to see if the inotropic action can be separated from the calorigenic action. This study appeared important in view of statements often encountered in the clinical literature that (*a*) adrenaline raises the blood pressure mainly by virtue of its action on the heart, increasing the cardiac output, whereas noradrenaline acts peripherally; (*b*) adrenaline is contraindicated in the treatment of cardiogenic shock as it increases myocardial oxygen consumption and produces symptoms of coronary

TABLE I

EFFECT OF ADRENALINE AND NORADRENALINE ON THE ISOLATED DOG HEART

Adrenaline added to reservoir at rate of 4 μg./min. for 15 min. followed by a 30 min. pause, then a 15 min. infusion period with noradrenaline. The next day the order was reversed, noradrenaline added first. The values at the end of the second 15 min. period are compared with those at 45 min. as a base line. The numeral (1) indicates at the start of the experiment, (2) after 15 min., (3) after 45 min., (4) after 60 min.

	Part 1								Part 2							
	Adrenaline				Noradrenaline				Adrenaline				Noradrenaline			
No. of expts. ..	16				16				8				8			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Heart rate	138	—	141	—	—	—	—	—	—	—	—	—	—	—	—	—
Mean±S.E. ..	(±4·8)		(±2·3)													
% change in heart rate	—	44·3	—	44·3	—	47·1	—	47·1	—	47·8	—	41·0	—	57·0	—	37·3
Mean±S.E. ..		(±3)		(±3)		(±4·5)		(±4·5)		(±4·6)		(±3·6)		(±5·9)		(±5·0)
Oxygen consumption (ml./100 g./min.) ..	6.67	—	—	—	6.67	—	—	—	—	—	—	—	—	—	—	—
Mean±S.E. ..	(±0·38)				(±0·38)											
% change in oxygen consumption ..	—	74·0	−3·7	74·0	—	112·0	−3·7	112·0	—	54·2	—	93·8	—	98·9	—	124·9
Mean±S.E. ..		(±11·6)	(±3·3)	(±11·6)		(±16·0)	(±3·3)	(±16·0)		(±9·9)		(±19·3)		(±27·5)		(±16·6)
Mean % change in work performed ..	—	−1·37	−8·1	−1·37	—	−0·8	−8·1	0·8	—	−2·35	—	−0·4	—	+2·2	—	−3·9
Coronary flow (ml./min.)	55	121	151	215	55	121	151	215	—	—	—	—	—	—	—	—
Mean±S.E. ..	(±6)		(±26)		(±6)		(±26)									
% change in coronary flow	—	—	—	—	—	—	—	—	—	113·6	—	78·9	—	130·7	—	49·4
Mean±S.E. ..										(±12)		(±20·8)		(±26·1)		(±20)
Phosphorus Compounds of Heart Muscle. Mean±S.E. (% Total Acid-soluble Phosphorus)																
Phosphocreatinine phosphorus		25·3 (±0·94)														
Inorganic phosphorus		17·8 (±0·87)														
Labile nucleotide phosphorus (2/3 of adenosine triphosphate)		27·7 (±0·62)														

TABLE II

EFFECT OF 5 μG. ADRENALINE ON THE ISOLATED DOG HEART

In the first five experiments adrenaline was added to the reservoir. In the next seven experiments adrenaline was injected into the venous cannula near the right heart, and in this series the first oxygen consumption measurement was made 45 sec. (4 experiments) and 1 min. after the injection; since the readings at 45 sec. and 1 min. were not different they were averaged together. Initial coronary flow was 56 (±6·6) in the experiments in which adrenaline was added to the reservoir, and 56 (±4·8) in those in which adrenaline was injected.

	Adrenaline Added to Reservoir						Adrenaline Injected					
No. of expts. . .	5						7					
Min. after injection . .	1	3	6	8	11	13	1	3	6	8	11	13
% change in heart rate	+13·5	+19	+15	+11·7	—	+11·0	27·0	+11·2	+10·4	—	+6·1	—
Mean±S.E.		(±2)				(±1·3)	(±5·9)		(±3·6)		(±2·4)	
% change in oxygen consumption . .	—	+3·8	—	−3·1	—	−2·2	+3·8	—	+3·0	—	−5·9	—
Mean±S.E.		(±1·8)		(±2·5)		(±3·1)	(±3·6)		(±2·7)		(±2·2)	
Mean % change in work performed	—	+4·4	—	+3·9	—	+2·7	+0·6	—	+0·6	—	+1·2	—
% change in coronary flow . .	—	+23·4	—	+38·6	—	+41·7	+11·9	—	+35·2	—	+45·3	—
Mean±S.E.		(±6·1)		(±12·3)		(±11)	(±2·3)		(±9·2)		(±11·2)	
Phosphorus Compounds of Heart Muscle (% of Total Acid-soluble Phosphorus)												
Phosphocreatine phosphorus . .				25·1 (±2·0)						26·0 (±0·8)		
Inorganic phosphorus . .				12·6 (±1·03)						13·9 (±0·89)		
Labile nucleotide phosphorus (2/3 of adenosine triphosphate) . .				30·3 (±0·86)						29·2 (±1·21)		

insufficiency, whereas noradrenaline does not increase cardiac oxygen consumption.

METHODS

Male and female dogs were used weighing about 12 kg. The techniques of preparing the heart-lung, of measuring coronary flow and oxygen consumption, as well as other experimental details have been described previously (Fawaz and Tutunji, 1959). Blood pressure was maintained at 100 mm. Hg, and an attempt was made to keep the left ventricular output constant. The average left ventricular output in this series was about 650 ml./min. Readings were taken at the start of the experiment, and then one of the sympathomimetic amines was added to the venous reservoir at the rate of 4 μg./min. for a period of 15 min. at the end of which measurements were again taken. After a period of 30 min. without infusion readings were taken again and an equimolar amount of the other sympathomimetic amine added over a period of 15 min.; after this the final readings were taken. The next day the order of adding the sympathomimetic amines was reversed. In all, 16 such experiments were performed over a period of a whole year. Each sympathomimetic amine was given 8 times during the first 15 min. and 8 times during the last 15 min. of the experiment. Experiments were terminated at the end of the second 15-min. infusion period and a piece of the left ventricle was excised for the determination of the phosphorus compounds.

The inotropic action of adrenaline and noradrenaline was tested on the classical Starling heart-lung preparation. Failure was produced in the course of about 15 min. by repeated additions of pentobarbitone until the systemic output fell to about half the original level with a corresponding rise in right auricular pressure. A single dose of 5 μg. of adrenaline or equimolar amount of noradrenaline was added to the reservoir or injected into the venous inflow tube near the right heart. The calorigenic action of this dose of adrenaline was tested on the modified non-failing heart-lung preparation by injection into the venous inflow tube, and the usual measurements were taken 45 sec., 1, 3, 6, 11, and 13 min. thereafter. The procedure for measuring oxygen consumption in these "acute" as well as in the infusion experiments was as follows: Coronary flow was measured during an interval of 15 sec., coronary venous and arterial blood samples were then collected simultaneously for the oxygen determination by the method of Van Slyke, a procedure that requires 5 sec., then a second coronary flow measurement was made, and the average was taken of the two coronary flow figures, which usually agreed very well.

RESULTS

Comparison of the Calorigenic, Chronotropic, and Coronary-dilator Actions of Adrenaline and Noradrenaline

It can be seen from Table I that during the 30-min. pause following the first 15-min. infusion period both the heart rate and the oxygen consumption return to pre-infusion levels. Yet we took not only the average of all 16 measurements for each of the sympathomimetic amines (part 1 of Table I), but made separate analysis of the 8 experiments during which each amine was given at the beginning or at the end (part 2 of Table I). We noticed early that the same amine, given at the end of the experiment, tends to have a greater calorigenic action and the same or slightly smaller chronotropic action than when given at the beginning of the experiment. We have no explanation for this finding, which may or may not be significant. Another reason for this separate analysis is because the coronary flow does not return to normal and may even decrease during the 30-min. pause.

"Calorigenic Action."—It is quite clear from the figures presented in Table I that one can speak of a "calorigenic" action of noradrenaline and adrenaline, for, in the absence of a change in work performed, a tachycardia with a 44 to 47% increase in rate cannot explain this rise in oxygen consumption. In a previous publication (Fawaz and Tutunji, 1959) we have shown that a tachycardia with about 65% increase in rate can result in not more than 23% increase in oxygen consumption. Our results indicate that the cardiac calorigenic action of noradrenaline is at least equal to, and probably greater than, that of adrenaline. There are marked variations in the response of different dogs to adrenaline and noradrenaline. The initial cardiac oxygen consumption, however, does not vary so much from one animal to another and in this particular series of 16 hearts it is 6.67 ± 0.38 ml./100 g./min. Due to these variations in response and the resulting high standard error it is not possible to compare the calorigenic actions of adrenaline and noradrenaline in a strictly quantitative manner. Gollwitzer-Meier, Kramer and Krüger (1936) and Gollwitzer-Meier and Witzleb (1952) have had the same experience regarding the variations in response to single doses of adrenaline and noradrenaline.

Chronotropic Action.—Our results indicate that adrenaline and noradrenaline infused at the rate of 4 μg./min. are equally effective in increasing the heart rate in the denervated heart-lung preparation. The heart rate rises rapidly and remains more or less steady during the infusion. Furthermore, we have found less variation from one animal to another in the chronotropic response to the sympathomimetic amines than in

the "calorigenic" response. Krayer (1949) and Krayer and Van Maanen (1949) have also found that the heart rate remains steady during a constant infusion of adrenaline or noradrenaline

Coronary Dilating Action.—Both adrenaline and noradrenaline given at the rate of 4 μg./min. have a definite coronary dilating action as can be seen from Table I. During the first 15 min. there was a gradual increase in coronary flow amounting, at the end of this period, to 113% in the case of adrenaline and 130% in the case of noradrenaline. It is known that coronary flow increases spontaneously in a heart-lung preparation, but the "spontaneous" rise in coronary flow during the first 15 min. is much less than that observed after the addition of sympathomimetic amines. This can be seen by comparing these values with results reported in a previous publication (Fawaz and Tutunji, 1959) and obtained under almost identical experimental conditions. These results, however, do not answer the question as to what extent the effect of the sympathomimetic amines is secondary to the marked increase in oxygen consumption.

The Inotropic Action of Adrenaline or Noradrenaline

If 5 μg. of adrenaline or noradrenaline is injected into the venous inflow tube near the right heart or into the venous reservoir in a classical Starling heart-lung preparation previously made to fail with pentobarbitone, the results are clear-cut and reproducible. There is a sudden drop of right auricular pressure almost to pre-failure level, then a gradual rise to pre-injection level in the course of about 15 min. Simultaneously there is an increase in output almost to pre-failure level followed by a gradual decrease to pre-injection level. The blood pressure rises suddenly to a high level during the first 15 sec. if the amine is injected near the heart and only slightly if injected into the reservoir. If the injection is near the heart the heart rate also increases by about 27% during the first minute and subsides to normal within 5 to 10 min. If now 5 μg. of the other sympathomimetic amine is injected the result is almost identical except that the correction of failure is not as complete. Adrenaline and noradrenaline behave identically in this respect. This experiment has been performed many times and is a student demonstration experiment. An illustration of this action can be seen in figure 12 of Krayer (1949) demonstrating the positive inotropic effect of 10 μg. of adrenaline in a failing heart-lung preparation treated with veratramine.

The Lack of Measurable Calorigenic Effect of 5 μg. of Adrenaline

The calorigenic effect of 5 μg. adrenaline was tested on the non-failing heart-lung preparation performed according to the modified procedure to allow a more accurate measurement of coronary flow (Table II). In 7 experiments, 5 μg. adrenaline produced no measurable increase in oxygen-consumption 45 sec., 1, 6 or 11 min. after injection. In another 5 experiments where 5 μg. adrenaline was added to the reservoir, a procedure known to produce definite inotropic effect, again no increase in oxygen consumption occurred after 3, 8, or 13 min.

The heart rate did increase in these experiments, by a maximum of about 27% in the first series, but this increase was not accompanied by a measurable increase in oxygen consumption.

The Effect on the High-energy Phosphorus Compounds of Heart Muscle

As can be seen from the tables, there was no change in the high-energy phosphorus compounds of heart muscle even in those experiments where the oxygen consumption rose to as much as 200%. During this period, however, the coronary flow was increased and there were no signs of cardiac hypoxia as can be judged by the high oxygen content of coronary venous blood.

Discussion

The results indicate that infusion of noradrenaline is as effective as adrenaline in raising the oxygen consumption of the isolated denervated dog heart. A quantitative evaluation of the actions of the two amines is not possible owing to the high standard errors resulting from the great individual variations in the response to the sympathomimetic amines from dog to dog. Gollwitzer-Meier and Witzleb (1952) are the only investigators who have studied the calorigenic effect of noradrenaline on the isolated denervated dog heart and they came to the opposite conclusion. They injected single doses of noradrenaline (20 to 40 μg.) and adrenaline (10 to 20 μg.). The maximum increase in oxygen consumption after noradrenaline was 21 to 154% (15 experiments) and 54 to 252% after adrenaline (6 experiments). They thus concluded that the metabolic action of noradrenaline was substantially less ("wesentlich geringer") than that of adrenaline. The experimental part of Gollwitzer-Meier and Witzleb's work was different from ours. In the first place, these authors used single injections of adrenaline and noradrenaline and noted the peak in oxygen

consumption. We, however, infused these substances at a constant rate of 4 μg./min. for 15 min. and measured the oxygen consumption at the end of this period. We have not measured the oxygen consumption during this 15-min. period. However, we have measured the heart rate during this interval and found it to be relatively constant, thus confirming the observations of Krayer (1949) and Krayer and Van Maanen (1949) with adrenaline and noradrenaline. There are also other differences in the experimental conditions of Gollwitzer-Meier and those of the present experiments. For example, in the methods for measuring coronary flow and oxygen content of the blood.

Our results also indicate that noradrenaline is at least as effective as adrenaline in increasing the heart rate and also the coronary flow. Whether or not the increase in coronary flow is secondary to the increased myocardial oxygen consumption cannot be decided from our experiments, which were not originally planned to answer this specific question. Even in experiments where single doses of 5 μg. adrenaline were given and found to be without measurable effect on oxygen consumption, the rise in coronary flow could not be distinguished from the "spontaneous" increase observed in heart-lung preparations. It has been known for some time that noradrenaline can cause coronary vasodilatation (see Lochner, Mercker and Schürmeyer, 1956), but no quantitative study has been made of its efficacy in comparison with adrenaline.

As was stated above, we found that single injections of 5 μg. adrenaline had no measurable effect on the oxygen consumption of the denervated heart-lung preparation. Here we find ourselves in complete disagreement with Gollwitzer-Meier *et al.* (1936), who state that doses of 5 to 10 μg. adrenaline increase the oxygen consumption of the denervated heart-lung preparation 100 to 250% and even 350%, the effect reaching a maximum 1 min. after injection and lasting 5 to 10 min. We have not found a measurable increase in oxygen consumption 45 sec., 1, 3, or 6 min. after injection, even when we injected the adrenaline into the venous cannula near the right heart as Gollwitzer-Meier *et al.* did. We believe that differences in experimental technique may account for this discrepancy in results. In the first place, the methods of measuring the coronary flow and thus the technique of preparing the heart-lung are different. Gollwitzer-Meier *et al.* employed a Morawitz cannula to measure coronary sinus flow and calculated from this the total coronary flow. In a previous publication (Fawaz, Hawa and Tutunji, 1957), we have given our reasons why we believe that the Morawitz cannula method cannot be relied upon to measure even total coronary sinus flow. Although both groups utilize the Fick principle for measuring oxygen consumption, we measure coronary flow and blood oxygen directly whereas Gollwitzer-Meier *et al.* use indirect methods for measuring coronary sinus flow (Weese's Stromuhr and Rein's Thermostromuhr) and blood oxygen content (Kramer's photoelectric method). We are not in a position to judge these indirect methods; however, all indirect methods have eventually to be calibrated by direct measurements with a graduated cylinder or a Van Slyke apparatus. The indirect methods have the general advantage of yielding continuous and instantaneous measurements. In this particular case there is no advantage in that, for knowledge of the oxygen content of coronary sinus blood at any one instant obviously does not tell us about the metabolic activity of the cardiac cell at that particular instant.

A dose of 5 μg. adrenaline did cause a measurable increase in heart rate, about 27%, after 45 to 60 sec., and theoretically an increased oxygen consumption is to be expected in view of the known effect of tachycardia *per se* in increasing metabolism (Laurent, Bolene-Williams, Williams and Katz, 1956; Berglund, Borst, Duff and Schreiner, 1958; and Fawaz and Tutunji, 1959). However, Laurent *et al.* found in the intact animal an 11% increase in oxygen consumption with a 27% increase in heart rate and a 5.5% increase in oxygen consumption with a 36% increase in heart rate. Badeer and Khachadourian (1958) observed no significant reduction in oxygen consumption when the heart rate was reduced from 153 to 110 beats per min. by applying a cold thermode to the sino-atrial node. Clearly, such changes in oxygen consumption are within the limits of experimental error.

The observation that small doses of adrenaline can have a positive inotropic action but no measurable calorigenic action may be of considerable interest. Noradrenaline has been used in recent years in the treatment of shock due to myocardial infarction (Binder, Ryan, Marcus, Mugler, Strange, and Agress, 1955), with the belief that noradrenaline acts peripherally to raise blood pressure whereas adrenaline acts mainly in the heart. The observation that adrenaline injections may sometimes produce symptoms of coronary insufficiency helped to support this hypothesis.

Perhaps if the clinicians had known that noradrenaline could raise the myocardial oxygen consumption to the same extent as adrenaline they would have hesitated to use it in cases of shock due to myocardial infarction. However, it is quite possible that, where noradrenaline is of use in cardiogenic shock, its efficacy may be due precisely to its cardiac (inotropic) action. The element of cardiac failure in shock—even haemorrhagic shock—has hitherto received little attention (for review of literature see Walton, Richardson, Walton and Thompson, 1959) and it may well be that the doses of noradrenaline used clinically have no effect on raising the cardiac oxygen consumption but produce a positive inotropic action, thus counteracting any existing element of cardiac failure. Noradrenaline is to be preferred to adrenaline in this treatment because the latter, by raising the basal metabolic rate—a property not shared by noradrenaline—indirectly puts an extra burden on the heart. The increase in cardiac output observed after adrenaline may thus be due to such a general metabolic action and be similar to the action of the thyroid hormones.

This work was supported by a grant from the American Heart Association. We are grateful to the Sterling-Winthrop Research Institute, Rensselaer, N.Y., for a generous supply of (−)-adrenaline and (−)-noradrenaline.

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THE ANTI-ANAPHYLACTIC AND HISTAMINE-RELEASING PROPERTIES OF THE ANTIHISTAMINES. THEIR EFFECT ON THE MAST CELLS

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(RECEIVED FEBRUARY 26, 1960)

It has been shown that, depending upon their concentration, antihistamines act in three different ways: (*a*) by competitive inhibition of histamine as already known; (*b*) by destroying mast cells and releasing histamine; and (*c*) by preventing mast cell damage and histamine release in anaphylaxis. Furthermore, antihistamines potentiated mast cell damage and histamine release by compound 48/80, when acting on guinea-pig tissues, and inhibited these same phenomena when acting on rat tissues. It is concluded that the effect of antihistamines in anaphylaxis is possibly due both to their competitive inhibition of histamine on smooth muscle receptors and to their inhibition of mast cell damage and histamine release by antigen.

It is known that the release of histamine in anaphylaxis is accompanied by damage to the mast cells (Mota, 1953; 1958; 1959; Mota and Vugman, 1956; Humphrey and Mota, 1959). It has been suggested that antihistamines may act either by blocking tissue receptors to circulating histamine or by preventing the release of histamine from the tissues (Dale, 1950). If the latter is true, antihistamines should protect mast cells from damage induced by anaphylaxis. Our experiments have shown that antihistamines can act not only as inhibitors of mast cell damage and histamine release in anaphylaxis but also as very potent histamine releasers.

METHODS

Wistar rats and guinea-pigs of either sex, and of body weight 200 to 400 g., were used in all experiments.

Sensitization.—Rats were sensitized by injecting intraperitoneally 1 ml. of horse serum plus 1 ml. *Haemophilus pertussis* vaccine containing 20,000 million organisms. The rats were used from 2 weeks after sensitization. Evidence of sensitization was provided by testing whether the mast cells presented extrusion of the granules in presence of the specific antigen; a tiny piece of mesentery was first incubated with antigen and afterwards fixed and stained for mast cell observation. Animals whose mesentery did not present mast cell disruption on contact with antigen were discarded. Guinea-pigs were passively sensitized by injecting intravenously 0.5 ml. of rabbit antiserum containing 1.2 mg. antibodies to ovalbumin and used 12 to 72 hr. after the injection of antiserum.

Antihistamines.—The following antihistamine drugs were used: antazoline hydrochloride (Antistine, Ciba), chlorpheniramine maleate (Alergon, Schering, and Polaramine, Schering), diphenhydramine hydrochloride (Benadryl, Parke-Davis), bromodiphenhydramine hydrochloride (Ambodryl, Parke-Davis), isothipendyl hydrochloride (Andantol, Homburg), mepyramine hydrogen maleate (Eulantinon, Laborterápica; Anthisan, May and Baker), methapyrilene hydrochloride (Histadyl, Lilly), phenindamine hydrogen tartrate (Thephorin, Roche), promethazine hydrochloride (Phenergan, Rhodia), and tripelennamine hydrochloride (Pyribenzamine, Ciba). The authors are grateful to the drug firms mentioned for supplies of the pure crystalline salts. Advantage was taken of the observation that bromodiphenhydramine, diphenhydramine and mepyramine lost their antihistamine activity after boiling for 10 min. with 20% hydrochloric acid, and thus the experiments concerning histamine release were performed with these three antihistamines while the effect on the mast cells was studied with all of them.

Removal and Treatment of the Tissues.—The animals were killed by a blow on the head, bled from the jugular veins and their tissues removed immediately. The skin was first shaved with an electric c ipper and cut in pieces of about 1 sq. cm. The lung was cut in slices 0.8 mm. thick with the McIlwain tissue chopper, washed for about 2 hr. in a large vol. of cold Tyrode solution, and 4 or 5 of these slices were used as a sample. The diaphragm was divided into 2 symmetrical halves each being used as a sample. The stomach and ileum were first opened and their contents washed out with cold Tyrode solution. The mesentery was dissected away from the small intestine and cut into several small pieces, which were dipped into cold Tyrode solution, 2 or 3 of which were used as a sample.

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Experiments to Study Histamine Release and Mast Cell Damage.—To study the histamine-releasing property of the antihistamines, rat or guinea-pig tissue samples were shaken gently in 50 ml. beakers closed with parafilm caps for a standard time of 15 min. at 37° in 4 ml. of Tyrode solution containing the required concentration of antihistamine, after which the tissue samples were transferred to the hydrochloric acid solution for histamine extraction or to fixative for mast cell observation. As a control in each experiment a tissue sample of similar size and thickness was incubated in Tyrode solution with no added drug.

Experiments to Study Inhibition of Histamine Release and Mast Cell Damage in Anaphylaxis.—Tissue samples were first incubated for 15 min. at 37° in Tyrode solution or Tyrode solution containing the antihistamine, washed for 5 min. in Tyrode solution and then transferred to Tyrode solution containing antigen. In the experiments with guinea-pig tissues ovalbumin was added to give a final concentration of 1 mg./ml., and in the experiments with rat tissues horse serum was added to produce a 10% solution. The tissues were then incubated for a further period of 15 min. and transferred to the hydrochloric acid solution for histamine extraction or to fixative for mast cell observation. Histamine was assayed in all the incubation fluids. By means of these experiments the histamine release by antigen in non-treated control samples, the histamine release by the antihistamine itself, and the histamine release by antigen after previous contact of the tissue with the antihistamine could be determined.

Histamine Extraction and Assay.—After incubation all the tissues were cut into small pieces and transferred to 20% hydrochloric acid solution. Acid was also added to the incubation fluids to give a final concentration of 20% and then both the extracts and incubation fluids were boiled for 10 min. to destroy antihistamines and evaporate excess acid. Later the extracts and incubation fluids were neutralized with sodium hydroxide and diluted to the required volume with Tyrode solution. The extracts were assayed against histamine acid phosphate on the atropinized guinea-pig ileum. Histamine loss in the controls was subtracted from that released by antihistamines. Histamine release was expressed as a percentage of the total tissue histamine. In order to exclude any non-histamine component, the solutions were reassayed after antagonism of histamine by mepyramine 10^{-7}.

Mast Cell Observation.—The mesentery is a very suitable preparation for the study of mast cells. The description of mast cell damage presented here is based on observations in this tissue. Guinea-pig mesentery was fixed in a 4% solution of lead subacetate in 50% ethanol plus 1% acetic acid (Mota and Vugman, 1956) and stained in a 0.1% toluidine blue solution. Rat mesentery was fixed and stained in a 10% formaldehyde solution containing 1% toluidine blue and 0.1% acetic acid. The mesentery was examined as a whole-mount preparation. Rat mast cell damage was assessed by counting the percentage of cells presenting granules extrusion, 500 cells being counted. Guinea-pig mast cell damage was assessed by counting the total number of cells containing metachromatic granules in 30 microscopical fields with a magnification of 130.

Phase Contrast Observation.—In order to observe directly the effect of antihistamines on the mast cells, rat peritoneal mast cells were isolated by the method of Padawer and Gordon (1955) but using a 30% solution of bovine serum albumin as the high density medium, and the behaviour of these cells when contacting the antihistamines was observed by phase contrast microscopy. A drop of the isolated mast cell suspension was placed on a slide and covered with a square coverslip, two sides of which were sealed with melted paraffin. A small well of paraffin was then made at each free side of the coverslip in the same way as was described by Strangeways and Canti (1927), the preparation kept in a warm stage at 37°, and a suitable group of well-preserved mast cells selected for observation. A drop of 0.1% solution of diphenhydramine, mepyramine or bromodiphenhydramine was then placed in one free side of the coverslip, sucked on to the cells with a piece of filter paper on the other side of the coverslip, and the response of the cell observed with an oil immersion objective (1,000).

RESULTS

Experiments with Guinea-pig Tissues

Histamine Release by Antihistamines from Guinea-pig Tissues.—Samples of guinea-pig skin, lung, diaphragm, stomach, ileum, and mesentery were incubated with 0.001 M bromodiphenhydramine, 0.001 M diphenhydramine, and 0.001 M mepyramine and the amount of histamine released determined. The results are shown in Table I. The antihistamines released considerable quantities of histamine

TABLE I

HISTAMINE RELEASE *IN VITRO* BY ANTIHISTAMINES FROM GUINEA-PIG TISSUES

Histamine release is expressed as a % of the total tissue histamine. Each figure represents the result of a different experiment.

Tissue	% Histamine Release by								
	Bromodiphenhydramine HCl 0·001 M			Diphenhydramine HCl 0·001 M			Mepyramine Acid Maleate 0·001 M		
Skin ..	10	12	15	9	10	13	6	9	10
Diaphragm	30	70	60	51	61	29	21	39	31
Stomach ..	0	0	2	0	0	5	2	2	0
Ileum ..	13	11	14	12	13	11	12	14	9
Mesentery	76	86	59	80	79	49	39	41	50
Lung ..	69	79	60	48	51	58	40	25	29

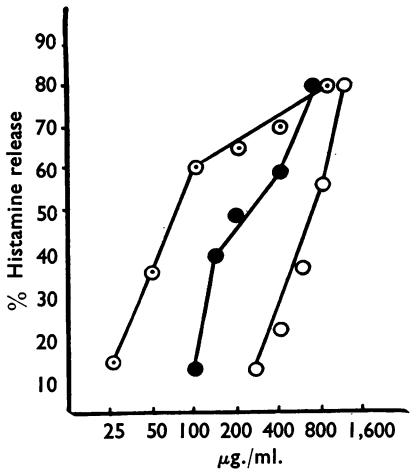

FIG. 1.—Effect of concentration of antihistamines, bromodiphenhydramine ⊙——⊙, diphenhydramine ●——●, and mepyramine O——O, on histamine release from guinea-pig lung. Ordinates: histamine release expressed as a % of the total tissue histamine. Abscissae concentration of antihistamine in μg./ml. Each point is mean of 2 experiments.

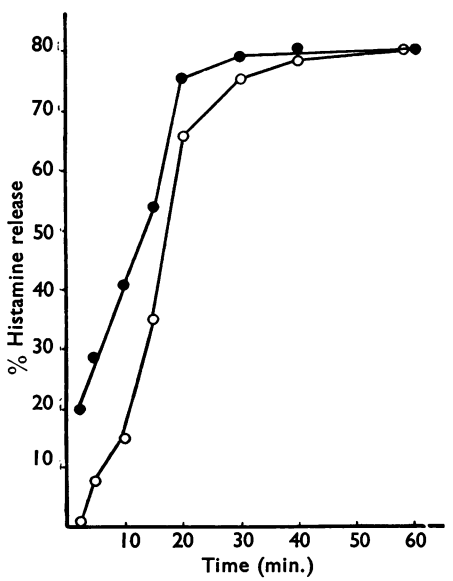

FIG. 2.—Effect of time of incubation on histamine release by antihistamines, diphenhydramine 0.001 M ●——● and mepyramine 0.001 M O——O, from guinea-pig lung. Ordinates: histamine release expressed as a % of the total tissue histamine. Abscissae: time in min. Each point is mean of 2 experiments.

from guinea-pig lung, diaphragm, mesentery, and skin, very little from the ileum and practically nothing from the stomach.

Effect of the Concentration of Antihistamines.—To study the strength of antihistamines as histamine releasers lung tissue was incubated with different concentrations of bromodiphenhydramine, diphenhydramine, and mepyramine and the amount of histamine released by each concentration determined. The results, summarized in Fig. 1, show that histamine release is related to the concentration of the antihistamine.

Effect of Time of Incubation with Antihistamines on Histamine Release.—Pieces of lung tissue were incubated with antihistamines for periods of 2 min. up to 60 min. The results are shown in Fig. 2. A significant release of histamine occurred as early as 2 min. after incubation with diphenhydramine and 5 min. with mepyramine, a maximal release being attained after 40 min. It was not possible to release more than 80% of the total histamine even when the time of incubation was increased to 2 hr.

Effect of pH on Histamine Release by Antihistamines.—To study the effect of *p*H on histamine release by antihistamines, samples of guinea-pig lung were incubated with diphenhydramine and mepyramine in Tyrode solution containing phosphate buffer to give *p*H values ranging from 6.5 to 8.5. Results are shown in Fig. 3. It was observed that the release of histamine by antihistamines was dependent upon *p*H, being greater at *p*H 8.5 and negligible at *p*H 6.5.

Effect of Temperature.—The effect of temperature was observed by incubating lung tissue with antihistamines for 15 min. at different temperatures

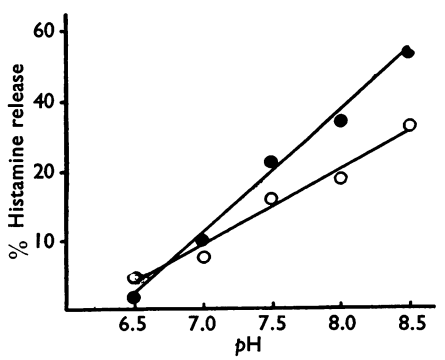

FIG. 3.—Effect of *p*H on histamine release by antihistamines, diphenhydramine 0.001 M ●——● and mepyramine 0.001 M O——O, from guinea-pig lung. Ordinates: histamine release is expressed as a % of the total tissue histamine. Abscissae: *p*H. Each point is mean of 2 experiments.

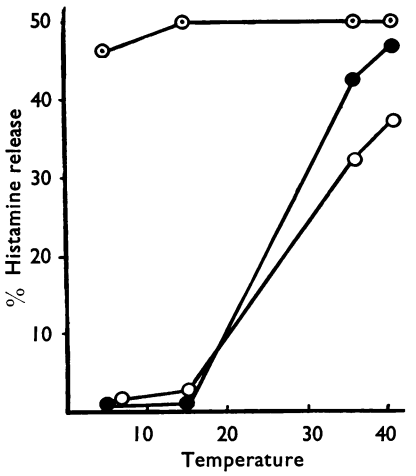

FIG. 4.—Effect of temperature on histamine release by antihistamines, bromodiphenhydramine 0.001 M ⊙——⊙, diphenhydramine 0.001 M ●——●, and mepyramine 0·001 M O——O, from guinea-pig lung. Ordinates: histamine release expressed as a % of the total tissue histamine. Abscissae: temperature. Each point is mean of 2 experiments.

ranging from 5° to 42°. Diphenhydramine and mepyramine were inactive at low temperatures, but bromodiphenhydramine was as active at 5° as at 42° (Fig. 4).

Effect of Metabolic Inhibitors, Calcium Lack and High Temperature on Histamine Release by Antihistamines.—It is known that various metabolic inhibitors, calcium lack or previous heating of the tissue at 45° inhibit histamine release in anaphylaxis, but potentiate histamine release by chemical histamine-liberators in the guinea-pig (Mongar and Schild, 1957a, 1957b, 1958). It was therefore of interest to study the effect of such treatments on histamine release by antihistamines. Lung tissue was incubated for 15 min. at 37° with either 0.001 M sodium iodoacetate, 0.001 M *N*-ethylmaleimide or calcium-free Tyrode solution containing 0.01% sodium edetate. Some samples were previously heated at 45° for 15 min. and brought back to 37°. After these treatments bromodiphenhydramine 50 μg./ml., diphenhydramine 100 μg./ml., or mepyramine 500 μg./ml. was added and the tissue was incubated for a further period of 15 min. The histamine release was then determined. Iodoacetate and *N*-ethylmaleimide potentiated histamine release by antihistamines (an average of 150% increase in 2 experiments), while calcium lack and previous heating at 45° were either without effect or only slightly potentiated histamine release by antihistamines.

Mast Cell Damage.—When pieces of guinea-pig mesentery were incubated with antihistamines in Tyrode solution at 37° damage to the mast cells occurred. Cytologically the mast cell damage induced by antihistamines was very similar to that induced by octylamine (Mota, 1959); characterized by diffusion of the metachromatic material and later dissolution of the cell. All the antihistamines tested induced the same kind of mast cell damage and caused a decrease in the number of these cells (Table II). Paralleling the histamine release this damage increased with time of incubation and concentration of the drug, and was *p*H and temperature dependent. Furthermore, iodoacetate, *N*-ethylmaleimide, calcium lack or previous heating at 45° potentiated the action of the antihistamines considerably. A concentration of antihistamine

TABLE II

MAST CELL CONTENT OF GUINEA-PIG MESENTERY INCUBATED AT 37° FOR 15 MIN. IN TYRODE SOLUTION WITH AND WITHOUT ADDED ANTIHISTAMINE

Each figure is the mean count of 30 microscopical fields (magnification 130) in each of 2 experiments.

Antihistamine	Concentration μg./ml.	Number of Mast Cells in		% Reduction
		Tyrode Alone	Tyrode with Antihistamine	
Chlorpheniramine maleate	50	12	13	0
	100	12	2	83
Bromodiphenhydramine hydrochloride	12·5	15	14	0
	25	15	3	78
Isothipendyl hydrochloride	25	19	20	0
	50	19	1	94
Antazoline hydrochloride	25	21	21	0
	50	21	4	81
Diphenhydramine hydrochloride	50	14	14	0
	100	14	1	93
Methapyrilene hydrochloride	100	17	18	0
	200	17	2	88
Mepyramine hydrogen maleate	200	24	25	0
	500	24	6	75
Promethazine hydrochloride	6·25	11	11	0
	12·50	11	3	73
Chlorpheniramine hydrochloride	100	31	31	0
	200	31	2	94
Tripelennamine hydrochloride	100	13	13	0
	200	13	3	77
Phenindamine hydrogen tartrate	25	27	29	0
	50	27	10	63

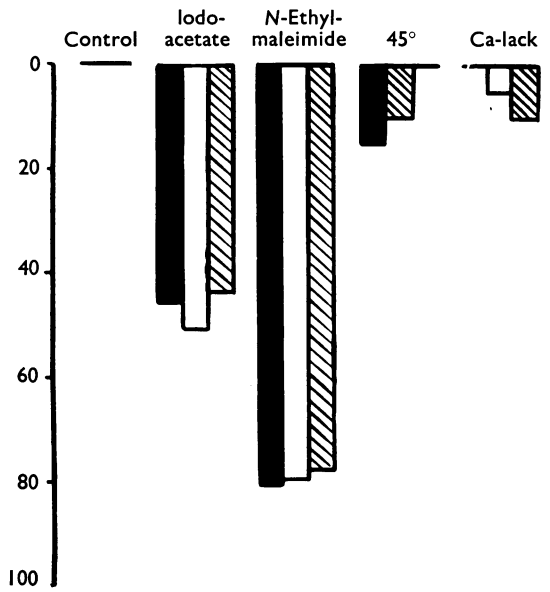

FIG. 5.—Effect of iodoacetate (0.001 M), *N*-ethyl-maleimide (0.001 M), calcium lack and previous heating at 45° on guinea-pig mast cell damage induced by bromodiphenhydramine (6.5 μg./ml.) ■, diphenhydramine (50 μg./ml.) ▩ and mepyramine (250 μg./ml.) □. Ordinates: % diminution in mast cell count. Control: the effect induced by the antihistamine alone.

incapable by itself of causing damage to the mast cells completely destroyed these cells when the tissue was previously submitted to any of these treatments (Fig. 5).

Inhibition of Histamine Release by Antigen.—When pieces of sensitized guinea-pig lung were previously incubated with antihistamines, the histamine release induced by later contact with antigen was reduced (Table III). The concentration of antihistamine necessary to produce a total inhibition of histamine release by antigen was very near to the concentration which itself released histamine. Concentrations of diphenhydramine and mepyramine able to release histamine by themselves always produced a total inhibition of histamine release by antigen. However, it was never possible to obtain a complete inhibition of histamine release in anaphylaxis with bromodiphenhydramine.

Inhibition of Mast Cell Damage by Antigen.—The minimal concentrations of antihistamines necessary to produce a total inhibition of mast cell reduction by antigen are shown in Table IV. When sensitized guinea-pig mesentery previously incubated with antihistamine was incubated with antigen, the usual mast cell diminution induced by antigen-antibody reaction did not occur.

TABLE III

EFFECT OF ANTIHISTAMINES ON HISTAMINE RELEASE BY ANTIGEN OR COMPOUND 48/80

Sensitized lung tissue was previously incubated with antihistamine, washed in Tyrode solution and transferred to antigen (1 mg./ml.) or to compound 48/80 (1 mg./ml.). Each figure is the mean of 2 experiments.

Antihistamine	Concentration μg./ml.	% Histamine Release by		% Inhibition
		Antihistamine	Antigen	
Bromodiphenhydramine hydrochloride	0	—	36	—
	5	0	34	0
	10	0	37	0
	15	0	31	14
	25	11	25	30
	30	21	23	36
Diphenhydramine hydrochloride	0	—	28	—
	25	0	30	0
	50	0	25	10
	100	11	6	78
	150	39	0	100
	200	49	0	100
Mepyramine hydrogen maleate	0	—	32	—
	25	0	31	0
	50	0	33	0
	100	0	20	38
	200	0	9·5	66
	250	0	0	100
	500	25	0	100
	800	55	0	100
			48/80	% Potentiation
Bromodiphenhydramine hydrochloride	0	—	14	—
	15	9	19	35
	30	26	25	78
Diphenhydramine hydrochloride	0	—	16	—
	100	9	20	25
	150	20	40	150
Mepyramine hydrogen maleate	0	—	16	—
	250	0	25	56
	500	20	28	75

Potentiation of Histamine Release and Mast Cell Damage by Compound 48/80.—It is known that in the guinea-pig several agents that inhibit histamine

TABLE IV

EFFECT OF ANTIHISTAMINES ON MAST CELL DAMAGE BY ANTIGEN OR COMPOUND 48/80

Sensitized guinea-pig mesentery was previously incubated with antihistamine, washed in Tyrode solution and transferred to antigen or to compound 48/80.

Antihistamine	Concentration μg./ml.	% Mast Cell Diminution by Antigen	% Inhibition
Chlorpheniramine maleate	0	50*	—
	50	0	100
Bromodiphenhydramine hydrochloride	0	37	—
	12·5	0	100
Isothiopendyl hydrochloride	0	60	—
	25	0	100
Antazoline hydrochloride	0	50	—
	25	0	100
Diphenhydramine hydrochloride	0	37	—
	50	0	100
Methapyrilene hydrochloride	0	68	—
	100	0	100
Mepyramine hydrogen maleate	0	37	—
	200	0	100
Promethazine hydrochloride	0	68	—
	6·25	0	100
Tripelennamine hydrochloride	0	50	—
	100	0	100
Phenindamine hydrogen tartrate	0	68	—
		0	100
		% Mast Cell Diminution by 48/80	% Potentiation
Bromodiphenhydramine hydrochloride	0	28	—
	12·5	64	127
Diphenhydramine hydrochloride	0	32	—
	50	76	137
Mepyramine hydrogen maleate	0	48	—
	200	92	91

* Mean of 2 experiments.

release and mast cell damage in anaphylaxis potentiate the effects of chemical histamine releasers. We decided to study the effect of antihistamines on the histamine release and mast cell damage by compound 48/80. Lung tissue or mesentery were first incubated for 15 min. at 37° with Tyrode solution or Tyrode solution containing antihistamine, washed for 5 min. with Tyrode solution and then incubated for a further period of 15 min. at 37° with Tyrode solution containing 1 mg./ml. of compound 48/80. The results in Tables III and IV show that previous incubation with antihistamines potentiated histamine release and mast cell damage by compound 48/80.

TABLE V

HISTAMINE RELEASE *IN VITRO* BY ANTIHISTAMINES FROM RAT TISSUES

Histamine release is expressed as a % of the total tissue histamine. Each figure represents a different experiment.

Tissue	% Histamine Release by		
	Bromodiphenhydramine Hydrochloride 0·001 M	Diphenhydramine Hydrochloride 0·003 M	Mepyramine Hydrogen Maleate 0·003 M
Skin ..	10 11 13 6	9 15 10	8 9 11 6
Diaphragm	60 25 69	65 60 34	38 49 30 53
Stomach ..	6 7 2	1 2 3	0 2 5 0
Ileum ..	18 13 11	13 11 6	10 14 15 12
Mesentery	28 23 6 0	30 28 21 6	20 25 20 3

Experiments with Rat Tissues

Histamine Release from Various Rat Tissues.— Incubation of rat tissue with antihistamines led to histamine liberation (Table V). The antihistamines were particularly active on the rat diaphragm but had practically no effect on the histamine content of the stomach. The concentrations of antihistamines required to release histamine from rat tissues were higher than those needed to release histamine from guinea-pig tissues.

TABLE VI

MAST CELL DAMAGE *IN VITRO* BY ANTIHISTAMINES

Rat mesentery was incubated at 37° for 15 min. with antihistamines and the % of mast cells presenting granule extrusion determined. Each column represents a different animal.

Antihistamine	Concentration μg./ml.	Mast Cell Disruption
Bromodiphenhydramine hydrochloride	1,000	100 56 0 25 100
	500	0 0 37 100
	250	0 0 0
Diphenhydramine hydrochloride	1,000	100 100 20 0 0
	500	0 100 11 0 20
	250	0 0 0 0 0
Mepyramine hydrogen maleate	1,000	0 100 25 0 0
	500	0 0 0 0 0

TABLE VII

INHIBITION BY ANTIHISTAMINES OF HISTAMINE RELEASE BY ANTIGEN OR COMPOUND 48/80

Sensitized rat tissue was previously incubated with antihistamines, washed in Tyrode solution and transferred to antigen (10% horse serum) or to compound 48/80 (0·1 mg./ml.). Each figure represents the mean of 2 experiments.

Antihistamine	Concentration μg./ml.	Tissue	% Histamine Release by Antihistamine	% Histamine Release by Antigen	% Inhibition
Bromodiphenhydramine hydrochloride	0	Skin	—	13	—
	500		10	7	46
	250		10	11	15
	100		0	14	0
Diphenhydramine hydrochloride	0	Skin	—	11	—
	1,000		0	5	55
	500		0	6	45
	250		0	8	27
	50		0	11	0
	0	Mesentery	—	40	—
	1,000		30	3	93
	500		15	4	90
	100		0	39	0
Mepyramine hydrogen maleate	0	Skin	—	13	—
	1,000		0	0	100
	500		0	4	69
	250		0	6	54
	100		0	14	0
	0	Diaphragm	—	29	—
	500		0	9	69
	0	Mesentery	—	28	—
	500		0	14	50

Antihistamine	Concentration μg./ml.	Tissue	% Histamine Release by Antihistamine	% Histamine Release by 48/80	% Inhibition
Bromodiphenhydramine hydrochloride	0	Skin	—	16	—
	500		5	10	37
	250		0	12	25
	100		0	17	0
Diphenhydramine hydrochloride	0	Skin	—	16	—
	1,000		6	6	63
	500		0	9	44
	250		0	10	37
	100		0	16	0
Mepyramine hydrogen maleate	0	Skin	—	36	—
	1,000		0	0	100
	500		0	14	61
	250		0	23	36
	100		0	37	0

TABLE VIII

EFFECT OF ANTIHISTAMINES ON MAST CELL DISRUPTION BY ANTIGEN OR COMPOUND 48/80

Sensitized rat mesentery was first incubated with antihistamine, washed in Tyrode solution and then transferred to antigen (10% horse serum) or to compound 48/80 (0.1 mg./ml.).

Antihistamine	Concentration μg./ml.	% Mast Cell Disruption by Antigen	% Inhibition
Bromodiphenhydramine hydrochloride	0	26*	—
	500	13	50
	250	21	19
	50	28	0
Diphenhydramine hydrochloride	0	60	—
	1,000	0	100
	500	0	100
	250	35	42
	100	59	0
Mepyramine hydrogen maleate	0	49	—
	1,000	0	100
	500	0	100
	250	20	59
	100	53	0

Antihistamine	Concentration μg./ml.	% Mast Cell Disruption by Compound 48/80	% Inhibition
Bromodiphenhydramine hydrochloride	0	62	—
	500	20	68
	250	60	0
	100	63	0
Diphenhydramine hydrochloride	0	76	—
	1,000	0	100
	500	0	100
	250	75	0
	100	79	0
Mepyramine hydrogen maleate	0	80	—
	1,000	0	100
	500	0	100
	250	70	13
	100	81	0

* Mean of 2 experiments.

Rat Mast Cell Damage.—All the antihistamines tested caused mast cell disruption similar to that described with compound 48/80 (Mota, Beraldo, and Junqueira, 1953). However, concentrations as high as 1 mg./ml. were frequently necessary to produce 100% mast cell disruption. Even so, this disruption was less conspicuous than that induced by 0.1 mg./ml. compound 48/80. The results with mepyramine, bromodiphenhydramine, and diphenhydramine are shown in Table VI. There was a large individual variation in the response of the mast cells to the action of the antihistamines. For example, mepyramine 1 mg./ml. in some animals produced 100% mast cell disruption and in others no disruption at all.

Using phase contrast microscopy it was observed that isolated mast cells responded to antihistamines by the sudden appearance of intracellular vacuoles and transient swelling of the cell. Simultaneously extrusion of granules was frequently seen.

Inhibition of Histamine Release by Antigen or Compound 48/80.—As with sensitized guinea-pig tissue, previous incubation of rat tissue with antihistamines caused a total or partial inhibition of histamine release in anaphylaxis (Table VII). However, higher concentrations of antihistamine were necessary to inhibit histamine release in rat anaphylaxis than in guinea-pig anaphylaxis. Antihistamines also inhibited the histamine release induced by compound 48/80 in the rat.

Inhibition of Mast Cell Damage by Antigen or Compound 48/80.—When rat mesentery was first incubated with antihistamines and then transferred to antigen (10% horse serum) or to compound 48/80 (0.1 mg./ml.) mast cell disruption was prevented (Table VIII). Concentrations of antihistamines that disrupted mast cells by themselves were avoided in these experiments since it was found difficult to distinguish the disruption induced by antihistamines from that induced by compound 48/80 when both were simultaneously present.

Discussion

The release of histamine from tissues by antihistamines was observed by Arunlakshana (1953a) and more recently by Tasaka (1957). This may explain the reported rise in human blood histamine after treatment with antihistamines (Pellerat and Murat, 1946). It has also been observed that mesenteric mast cells are destroyed after repeated intraperitoneal injections of promethazine (Smith, 1958a).

It is conceivable that antihistamines compete with histamine not only at its site of action, the histamine receptor, but also at its site of storage in the cell. If this is so it might be expected that the histamine-releasing activity and antihistamine activity of different compounds would be correlated.

In the present investigation the antihistamines behaved as typical histamine liberators; their effects increased with concentration, *p*H and temperature. Their effects were potentiated by pre-treatment with metabolic inhibitors like those of octylamine and compound 48/80 (Mongar and Schild, 1957a, 1957b; Mota, 1959). Different antihistamines differed markedly in histamine-releasing and mast cell destroying activity. We found that bromodiphenhydramine and promethazine were the most potent histamine releasers. There was also a considerable difference between different tissues. The tissues of the digestive tract released only small amounts of histamine. There was an interesting quantitative difference between the effects of the antihistamines and of the compound 48/80 in the rat and guinea-pig. In the guinea-pig the antihistamines were more active as histamine releasers than compound 48/80, and even more active than the relatively potent octylamine, judging from the data of Mongar and Schild (1953). In the rat, however, the antihistamines are less active than compound 48/80.

The observation that antihistamines act on isolated mast cells in the absence of plasma or tissue fluid suggests that they act directly on the cell. The response of the rat mast cell to antihistamines is morphologically similar to that produced by a specific antigen (Mota, Dias da Silva, and Ferreira Fernandes, 1960a).

The mechanism of the inhibitory effect of antihistamines on histamine release in anaphylaxis is not clear. Arunlakshana (1953b), who was the first to observe an inhibition of histamine release in anaphylaxis by antazoline, considered that it was due to the release, by the antihistamine, of a large proportion of the available tissue histamine, and therefore the amount releasable by antigen was diminished. Our findings suggest that this cannot be the whole explanation. With several of the antihistamines the dose which produced inhibition of histamine release in anaphylaxis was definitely less than that which produced histamine release on its own. Moreover, the histological evidence showing that mast cells fail to be disrupted by specific antigen after pre-treatment with an antihistamine demonstrates that a true protective action is being exerted. It is possible that antihistamine molecules in a critical concentration near to that required for histamine release become attached to the mast cell membrane and interfere with the antigen antibody reaction. Alternatively

they may inhibit the enzymatic system (Mongar and Schild, 1957a) required in the anaphylactic reaction.

There is no evidence that the histamine-releasing property of antihistamines and their property of preventing histamine release in anaphylaxis are correlated. For example, bromodiphenhydramine in a concentration that released histamine by itself did not completely inhibit histamine release in anaphylaxis, whereas mepyramine completely blocked an anaphylactic histamine release in concentrations which did not themselves release histamine. Furthermore, compound 48/80 in a concentration which releases a small amount of histamine does not inhibit but, on the contrary, potentiates anaphylactic histamine release (Mongar and Schild, 1952).

The concentration of antihistamines necessary to prevent a diminution in mast cell count by the specific antigen completely was frequently smaller than the concentration for complete inhibition of histamine release. For example, 100 μg./ml. mepyramine completely prevented a mast cell decrease by antigen but produced only a partial reduction of histamine release. This discrepancy may be due to qualitative alterations of mast cells which were not considered in our measurements, and also to the possible release of histamine from mast cells before any visible morphological change occurs (Smith, 1958b). The observation that antihistamines inhibited histamine release and mast cell damage by compound 48/80 in rat tissues agrees with previous results showing that the various agents that potentiate histamine release and mast cell damage by compound 48/80 in guinea-pig tissues inhibit these same phenomena when acting on rat tissues (Mota and Ishii, 1960).

The inhibition of histamine release in anaphylaxis by antihistamines may help to explain their protective action in anaphylaxis. In some cases at least the protection may be due more to inhibition of mast cell disruption than to competition at the receptor site. This, however, probably does not apply to the protective effect exerted by antihistamines in the anaphylactic shock of the intact guinea-pig, as concentrations of antihistamines necessary to inhibit histamine release *in vitro* are much higher than those likely to be produced by protective doses of antihistamines *in vivo*. On the other hand it is possible that the reduction in passive cutaneous anaphylaxis by high doses of antihistamines in rats (Brocklehurst, Humphrey, and Perry, 1955) may be due in part at least to the anti-anaphylactic effect of such doses of antihistamines. This inhibition of histamine release may possibly be attended by simultaneous inhibition of the release of other pharmacologically active substances, since Brocklehurst (1958) has shown that inhibition of histamine release in anaphylaxis is usually accompanied by inhibition of SRS-A release. The so-called unspecific effects of antihistamines in isolated tissues (Hawkins and Rosa, 1956) may also be explainable at least partially by their anti-anaphylactic actions.

As a protective agent in anaphylaxis mepyramine appears to be safer than bromodiphenhydramine or diphenhydramine as the ratio between its protective concentration and the concentration that releases histamine is greater. Mepyramine may owe its strong anti-anaphylactic action to the pyridine nucleus in its molecule, since we have observed (Mota, Dias da Silva, and Ferreira Fernandes, 1960b) that pyridine and some of its derivatives are potent inhibitors of histamine release in anaphylaxis.

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THE INHIBITION OF MAST CELL DAMAGE AND HISTAMINE RELEASE IN ANAPHYLAXIS BY PYRIDINE AND DIPHOSPHOPYRIDINE NUCLEOTIDASE INHIBITORS. COMPARISON WITH COMPOUND 48/80

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(RECEIVED MARCH 7, 1960)

In rats and guinea-pigs both mast cell damage and histamine release in anaphylaxis were inhibited by pyridine, nicotinamide, diethylnicotinamide, nicotinic acid, isonicotinic acid and isonicotinic acid hydrazide, which are known to be inhibitors of diphosphopyridine nucleotidases. On the other hand these compounds potentiated histamine release and mast cell damage by compound 48/80 in guinea-pigs although inhibiting the same phenomena in rats. The relationship of these findings with the mechanism of histamine liberation in anaphylaxis is discussed.

The isolation and synthesis of a dinucleotide of histamine was recently reported by Alivisatos (1958a). The structure of this compound is that of diphosphopyridine nucleotide (DPN) in which histamine replaced the nicotinamide moiety. Its biosynthesis involves a simple exchange reaction between free histamine and the nicotinamide of DPN. Alivisatos suggested that the exchange reaction of DPN with histamine could be involved in the mechanism of anaphylaxis. As this biosynthesis takes place in the presence of diphosphopyridine nucleotidase (DPN-ase), it was of interest to investigate the effect of compounds known to inhibit the activity of this enzyme on the mast cell damage and histamine release which occur in anaphylaxis. In addition, the effects of these compounds on the mast cell damage and histamine release by compound 48/80 were also investigated.

METHODS

Wistar rats and guinea-pigs of either sex and body weight 200 to 400 g. were used in all experiments. Guinea-pigs were sensitized with a 2% solution of egg albumen in 0.5% aqueous phenol, of which 1 ml. was injected subcutaneously and 1 ml. intraperitoneally. The animals were used from 3 weeks after sensitization. Rats were sensitized with 1 ml. of horse serum plus 1 ml. of *Haemophilus pertussis* vaccine, containing 20,000 million organisms, administered intraperitoneally. Evidence of sensitization in both species was provided by testing whether mast cells presented damage in the presence of the specific antigen (Mota, 1959 ; Humphrey and Mota, 1959; Mota and Ishii, 1960). Pyridine and the following DPN-ase inhibitors were used: nicotinamide, nicotinic acid, isonicotinic acid, isonicotinic acid hydrazide and diethylnicotinamide. These substances were dissolved in Tyrode solution and when necessary the *p*H was adjusted to 7.8 with sodium hydroxide or hydrochloric acid. Histamine release was measured in rat skin or guinea-pig lung and mast cells were observed in the mesentery. The animals were killed with a blow on the head and bled from the jugular veins. Rat skin was shaved with an electric clipper, cut in pieces of about 1 sq. cm., carefully removed from the abdominal wall and kept in cold Tyrode solution. One skin piece was used as a sample. Guinea-pig lung was cut in slices 0.8 mm. thick with a McIlwain tissue chopper. The slices were washed in cold Tyrode solution for about 1 hr. and 3 or 4 of them were used as a sample. Rat or guinea-pig mesentery was dissected out from the small intestine and cut into several small pieces, which were dipped into cold Tyrode solution. Three or four of these pieces were used as a sample. To study the effect of pyridine and DPN-ase inhibitors on mast cell damage and histamine release by antigen or compound 48/80, rat or guinea-pig tissue samples were shaken gently in 50 ml. beakers closed with parafilm caps for a standard time of 15 min. at 37° in 4 ml. of Tyrode solution containing different concentrations of the substances to be tested. After this treatment either antigen or compound 48/80 was added and the tissues incubated for a further period of 15 min. after which the mesentery was transferred to fixative and the skin or lung samples were transferred to N-hydrochloric acid, cut in very small pieces and boiled for 2 or 3 min. for histamine extraction.

The incubation fluid was acidified with one drop of concentrated hydrochloric acid, boiled and kept at 4° until assayed. As a control to each experiment a tissue

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TABLE I

EFFECT OF PYRIDINE AND DPN-ASES INHIBITORS ON GUINEA-PIG MAST CELL DIMINUTION INDUCED BY ANTIGEN OR COMPOUND 48/80

Histamine-releasing Agent	Molar Concentration of Chemicals	% Diminution by: Releasing Agent Alone	% Diminution by: Releasing Agent Plus Chemical	% Inhibition (−) or Potentiation (+)
Antigen	Diethylnicotinamide 0·040	53	0	−100
	0·040	61	0	−100
	0·020	58	0	−100
	0·010	41	20	− 51
	0·001	37	32	− 13
Antigen	Nicotinamide 0·040	80	0	−100
		59	0	−100
	0·020	59	25	− 57
	0·010	59	40	− 32
	0·005	59	40	− 32
	0·001	27	28	0
48/80	0·040	55	100	+ 81
		40	40	0
		25	60	+140
		40	56	+ 40
		70	95	+ 36
Antigen	Nicotinic acid 0·040	81	41	− 49
		88	42	− 52
		94	56	− 40
		51	31	− 39
		63	30	− 52
48/80	0·040	40	56	+ 40
		31	90	+190
		41	52	+ 26
		36	36	0
Antigen	Isonicotinic acid 0·040	28	28	0
		55	42	− 24
		76	0	−100
		55	38	− 31
48/80	0·040	41	65	+ 59
		60	100	+ 67
Antigen	Pyridine 0·040	37	26	− 30
		48	20	− 58
48/80	0·040	33	64	+ 94
		66	66	0
		54	78	+ 44
		61	62	0
Antigen	Isonicotinic acid hydrazide 0·040	75	32	− 57
		81	55	− 32
		45	45	0
48/80		55	100	+ 81
		21	25	+ 19
		38	38	0

TABLE II

EFFECT OF PYRIDINE AND DPN-ASES INHIBITORS ON HISTAMINE RELEASE BY ANTIGEN OR COMPOUND 48/80 FROM GUINEA-PIG LUNG

Histamine release is expressed as % of the total tissue histamine.

Releasing Agent	Molar Concentration of Chemicals	% Histamine Release by: Releasing Agent Alone	% Histamine Release by: Releasing Agent Plus Chemical	% Inhibition (−) or Potentiation (+)
Antigen	Diethylnicotinamide 0·040	19	0	−100
		15	0	−100
		44	0	−100
		31	1	− 97
		31	3	− 90
48/80		28	28	0
		28	32	+ 14
		21	21	0
Antigen	Nicotinamide 0·040	22	0	−100
		27	0	−100
		28	8	− 71
		39	9	− 77
48/80	0·040	10	16	+ 60
		21	32	+ 52
		26	26	0
Antigen	Nicotinic acid 0·040	33	11	− 67
		15	4	− 73
		19	12	− 36
48/80		21	33	+ 57
		23	23	0
		45	45	0
Antigen	Isonicotinic acid 0·040	34	24	− 29
		31	12	− 61
		25	8	− 68
48/80		28	37	+ 32
		16	24	+ 50
		17	17	0
Antigen	Pyridine 0·040	34	23	− 32
		30	18	− 40
		31	20	− 35
48/80		21	27	+ 28
		17	17	0
Antigen	Isonicotinic acid hydrazide 0·040	34	30	− 12
		44	32	− 27
		28	20	− 28
48/80		21	29	+ 38
		27	27	0

sample of similar size and thickness was incubated in Tyrode solution alone. At the end of the experiments the tested compound was added to the incubation fluids so that all of them contained the same concentration of the compound. Thus, any possible difference in the histamine level due to interference of the tested compound with the response of the ileum to histamine during the assay was eliminated. To assay histamine the extracts and incubation fluids were neutralized with NaOH and when necessary diluted with Tyrode solution to the required volume. Histamine was assayed on the atropinized guinea-pig ileum. In order to detect any non-histamine component the extracts and incubation fluids were reassayed after inhibition of histamine by mepyramine 10^{-8}. Histamine loss in controls was subtracted from that released by antigen or compound 48/80. Histamine release is expressed as a percentage of the total tissue histamine. Rat mesentery was fixed and stained in a 10% formaldehyde solution containing 1% toluidine blue and 0.1% acetic acid; guinea-pig mesentery was fixed in a 4% solution of lead sub-acetate in 50% ethanol, plus 1% of acetic acid (Mota and Vugman, 1956). The mesentery was observed as a whole-mount preparation. Rat mast cell damage was assessed by counting the percentage of cells presenting granules extrusion, 500 cells being counted; guinea-pig mast cell damage was assessed by counting the total number of metachromatic granules containing cells in 30 microscopical fields using a magnification of 120.

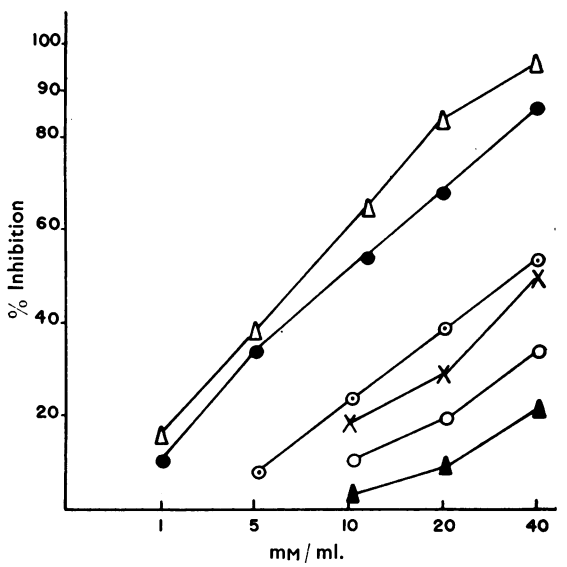

FIG. 1.—Effect of different concentrations of pyridine (O——O) and DPN-ase inhibitors (nicotinamide ●——●, nicotinic acid X——X, diethylnicotinamide Δ——Δ, isonicotinic acid hydrazide ▲—▲, isonicotinic acid ⊙——⊙) on histamine release by antigen from guinea-pig lung. Ordinates: %. Inhibition was calculated from the difference between the amount of histamine released by antigen plus inhibitor and that released by antigen alone. Abscissae: concentration of inhibitor. Each point is mean of 2 experiments.

RESULTS

Effect of Pyridine and Inhibitors of Diphosphopyridine Nucleotidase on Mast Cell Damage and Histamine Release by Antigen from Sensitized Guinea-pig Tissues.—When sensitized guinea-pig lung or mesentery was previously incubated with 0.04 M pyridine, diethylnicotinamide, nicotinamide, nicotinic acid, isonicotinic acid and isonicotinic acid hydrazide, the subsequent mast cell damage and histamine release by antigen was greatly reduced as shown in Tables I and II. Diethylnicotinamide was the most effective inhibitor of mast cell damage and histamine release, followed closely by nicotinamide. Isonicotinic acid hydrazide was the weakest inhibitor of all.

It was also observed that this inhibition was directly proportional to the concentration of these compounds present (Fig. 1). When nicotinamide was added simultaneously with antigen to the sensitized tissue, the subsequent histamine release was markedly reduced, indicating that it acts very quickly in inhibiting histamine release (Fig. 2). A maximal inhibitory effect was seen after 15 min.

Effect of Pyridine and DPN-ase Inhibitors on Mast Cell Damage and Histamine Release by Compound

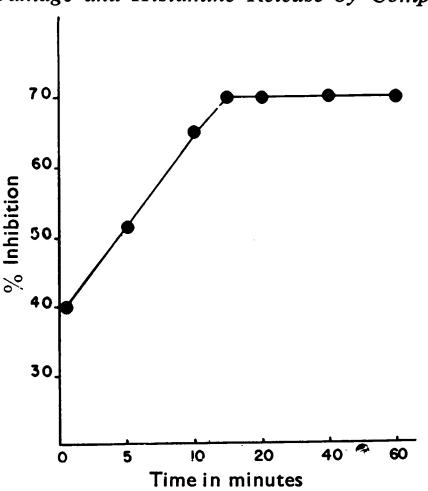

FIG. 2.—Effect of time of pre-incubation with nicotinamide (0.020 M) on the subsequent inhibition of histamine release by antigen. At 0 min. the antigen was added simultaneously with nicotinamide to the tissue. Ordinates: %. Inhibition calculated from the difference between the amount of histamine released by antigen plus inhibitor and that released by antigen alone. Abscissae: time in min. Each point is mean of 2 experiments.

TABLE III

INHIBITION BY PYRIDINE AND DPN-ASES INHIBITORS OF RAT MAST CELL DISRUPTION INDUCED BY ANTIGEN OR COMPOUND 48/80

Releasing Agent	Molar Concentration of Chemicals		% Disruption by Releasing Agent Alone	% Disruption by Releasing Agent Plus Chemical	% Inhibition
Antigen	Diethylnicotinamide	0·040	67	0	100
			31	0	100
			56	0	100
			48	15	69
48/80		0·040	36	0	100
			77	0	100
			73	60	18
			29	21	28
Antigen	Nicotinamide	0·040	36	0	100
			45	20	56
			64	0	100
			13	0	100
48/80		0·040	31	10	68
			89	0	100
			44	21	52
			35	30	14
Antigen	Isonicotinic acid	0·040	26	0	100
			33	0	100
			64	10	84
			28	0	100
48/80		0·040	68	34	50
			71	35	50
			60	0	100
Antigen	Nicotinic acid	0·040	56	0	100
			58	34	41
			20	0	100
			29	20	31
48/80		0·040	90	20	78
			87	40	54
			76	76	0
Antigen	Isonicotinic acid hydrazide	0·040	38	20	47
			49	0	100
			34	34	0
			16	10	38
48/80		0·040	81	81	0
			73	73	0
			98	98	0

TABLE IV

INHIBITION BY PYRIDINE AND DPN-ASES INHIBITORS OF HISTAMINE RELEASE BY ANTIGEN OR COMPOUND 48/80 FROM SENSITIZED RAT SKIN

Histamine release is expressed as % of the total tissue histamine.

Releasing Agent	Molar Concentration of Chemicals		% Histamine Release By Releasing Agent Alone	% Histamine Release By Releasing Agent Plus Chemical	% Inhibition
Antigen	Diethylnicotinamide	0·040	12·8	2·7	78
		0·040	13	0	100
		0·040	7·8	0	100
		0·020	7·8	2·4	69
		0·010	7·8	3	61
		0·001	7·8	7·9	0
48/80		0·040	8	8	0
			14	14	0
			31	19	39
			18	9	50
Antigen	Nicotinamide	0·040	12·8	0	100
		0·040	16	0	100
		0·040	7·8	0	100
		0·025	7·8	2	75
		0·020	7·8	0	100
		0·010	7·8	3·6	54
		0·001	7·8	7·8	0
48/80		0·040	14	14	0
			20	9	55
			31	26	16
			18	10	44
			11	11	0
Antigen	Isonicotinic acid	0·040	12·8	3·8	70
			16	0	100
		0·020	16	3	81
		0·010	16	12	25
		0·005	16	14	12
		0·001	16	16	0
48/80		0·040	18	7	61
			31	15	52
			8	5	38
			14	9	36
			20	14	30
Antigen	Isonicotinic acid hydrazide	0·040	12·8	5·8	55
			16	14	12
		0·020	16	12	25
48/80		0·040	14	14	0
			20	20	0
			31	31	0
			16	10	27

48/80.—It is known that in the guinea-pig all the substances which inhibit histamine release and mast cell damage by antigen potentiate the effects of chemical histamine releasers. It was therefore of interest to investigate the effect of pyridine and DPN-ase inhibitors on the mast cell damage and histamine release by compound 48/80. The results of these experiments are shown in Tables I and II. The mast cell damage and histamine release by compound 48/80 were potentiated by all the compounds studied.

Effect of Pyridine and DPN-ase Inhibitors on Mast Cell Damage and Histamine Release by Antigen or Compound 48/80 *from Sensitized Rat Tissues.*—The results of these experiments are summarized in Tables III and IV. When rat skin or mesentery was left for 15 min. in contact with pyridine or any of the DPN-ase inhibitors studied, the subsequent mast cell damage and histamine release by antigen or compound 48/80 was reduced. Both nicotinamide and diethylnicotinamide were very effective inhibitors of mast cell damage and histamine release in anaphylaxis but less effective inhibitors of mast cell damage and histamine release by compound 48/80. Isonicotinic acid was the most effective compound in preventing histamine release by compound 48/80.

Discussion

Inhibition of mast cell damage and histamine release in anaphylaxis has been obtained with a large number of unrelated substances (Mongar and Schild, 1957; Mota, 1959; Mota and Ishii, 1960), most of which are toxic enough to prevent their use *in vivo*. These new inhibitors of anaphylaxis are outstanding for their low toxicity. In this respect nicotinamide is the most interesting compound since it has a very high therapeutic index and so can be safely used in patients. However, it will be necessary to test nicotinamide *in vivo* under the more complex conditions of the anaphylactic reaction in the intact animal. It will also be necessary to test whether pyridine and the other compounds here studied inhibit cellular metabolism in general, since so far the chemical inhibitors of anaphylaxis have been found to depress other cellular functions, too (Mongar and Schild, 1957).

It is interesting that nicotinamide is an essential constituent of diphosphopyridine nucleotide and triphosphopyridine nucleotide and that the other compounds studied are analogues of the vitamin which might interfere with the synthesis of breakdown of the coenzymes containing nicotinamide. The ability of diphosphopyridine nucleotidase to catalyse an exchange reaction between the nicotinamide moiety of diphosphopyridine nucleotide and histamine was recently reported by Alivisatos (1958a, 1958b). This author suggested that histamine released in anaphylaxis would combine with DPN, in presence of DPN-ase, displacing nicotinamide. This exchange reaction would be inhibited by nicotinamide and similar compounds due to the well-known inhibitory effect of nicotinamide on animal DPN-ases (Zatman, Kaplan and Colowick, 1953). However, if following its release in anaphylaxis, histamine becomes bound to DPN, in presence of nicotinamide, which inhibits this exchange reaction, more free histamine should be found in the incubation fluid. This does not agree with our present findings that nicotinamide and other compounds known to inhibit DPN-ases inhibited histamine release by antigen. Nevertheless, DPN-ases may be implicated in the mechanism of histamine release in anaphylaxis. Thus, histamine may be bound to the tissues in a way that requires the activity of DPN-ases for its release. In this case, nicotinamide or any other inhibitor of DPN-ases would inhibit histamine release by antigen.

The observation that mast cell damage and histamine release by compound 48/80 in the guinea-pig are potentiated by DPN-ase inhibitors suggests that the mechanism through which histamine is released by compound 48/80 is different from that acting in anaphylaxis, as already concluded by Mongar and Schild (1957). However, it seems that if the same compound which inhibits histamine release in anaphylaxis potentiates histamine release by compound 48/80, the mechanism of histamine release in the two cases, although certainly different, must be in some way related. It seems interesting to point out here that in rats histamine release by either antigen or compound 48/80 is inhibited. This has already been observed in connexion with other inhibitors of anaphylaxis in the rat (Mota and Ishii, 1960) and suggests that in this species the mechanism of histamine release by compound 48/80 is closer to that of antigen that in the guinea-pig.

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THE ISOLATED CHICK BIVENTER CERVICIS NERVE-MUSCLE PREPARATION

BY

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(RECEIVED FEBRUARY 24, 1960)

The isolated biventer cervicis nerve-muscle preparation can be used to distinguish between neuromuscular blocking agents which cause depolarization and those which do not. Both reduce the contractions caused by nerve stimulation, but depolarizing drugs also cause a contracture of the muscle.

Child and Zaimis (1954), Child (1955) and Tyler (1960) have described the use of certain muscles of the chick, the semispinalis cervicis and the biventer cervicis, as isolated preparations. Like the rectus abdominis of the frog, these respond to the application of depolarizing substances by giving a contracture.

We have found it possible to obtain a nerve-muscle preparation by using the innervated lower belly of the biventer cervicis whose nerve supply is enclosed by the tendon between the two bellies. A stimulus applied via electrodes in contact with this tendon results in a contraction of the muscle. The preparation may therefore be used to test simultaneously both for neuromuscular blocking activity, as indicated by a reduction in the contraction produced by nerve stimulation, and for depolarizing activity, as indicated by contracture. This preparation was demonstrated to the Physiological Society in July, 1959.

METHODS

Chickens (50 to 250 g.) were anaesthetized with sodium phenobarbitone (9% solution in water; 0.2 ml./100 g.) injected into a wing vein. The dissection was similar to that described by Child (1955) (compare Tyler, 1960). The back of the neck was plucked and the skin incised along the midline from the skull to below the base of the neck, exposing the two biventer cervicis muscles on either side of the midline and immediately below the skin. A thread was tied round the upper belly of one muscle which was then cut free from its attachment to the skull. When the thread was gently pulled the tendon joining the two bellies of the muscle and the lower (caudal) belly of the muscle could be identified and separated from the underlying semispinalis cervicis

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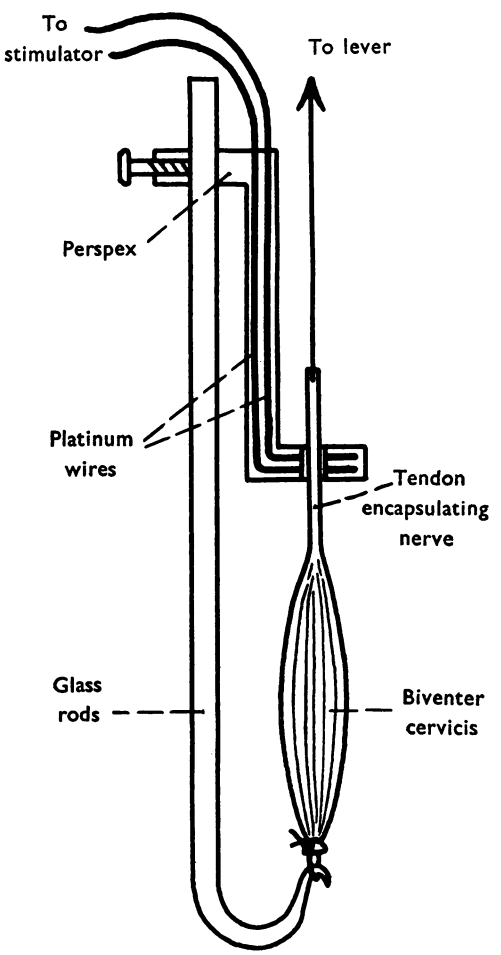

FIG. 1.—Electrode assembly.

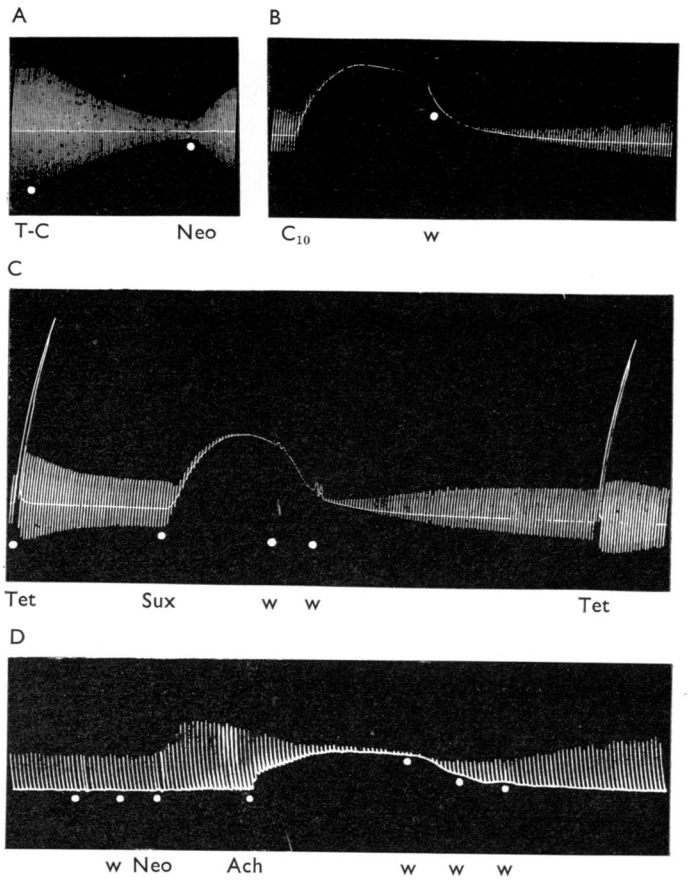

FIG. 2.—Contractions of isolated biventer cervicis muscle evoked by supramaximal nerve stimulation at 12/min. The tetani (Tet) in (C) were evoked by stimulation at 125/min., the maximum tetanic tension being 12 g. The contractions in (A), (B), and (C) were recorded with an "isometric" lever, and those in (D) were recorded using the mechano-electric transducer valve RCA 5734. (A) At T-C tubocurarine chloride was added to give a bath concentration of 1.4×10^{-5} M. At Neo, neostigmine bromide (3.3×10^{-6} M) was added. (B) At C_{10}, decamethonium iodide (3.4×10^{-8} M). (C) At Sux, succinylcholine dichloride (2.8×10^{-6} M) was added. (D) At Ach, acetylcholine chloride (1.3×10^{-6} M) was added before and after the addition of neostigmine bromide (6.6×10^{-6} M). At W, the bath was washed out.

muscle. The tendon and muscle were carefully removed together with the lower tendon which attaches the muscle to the supraspinous ligament. The anatomy of the chick has been described by Chamberlain (1943).

A loop which served to attach the preparation to the hook on the electrode assembly (Fig. 1) was tied around the lower tendon and the thread on the upper end of the muscle was passed through the electrode and attached either to a light semi-isometric lever writing on a smoked drum or to the lever of an RCA 5734 transducer valve suitably connected to a pen recorder. The electrode was lowered until it was in contact with the tendon surrounding the nerve. The organ bath (30 to 50 ml. capacity) contained Krebs-Henseleit (1932) solution which was maintained at a constant temperature between 37° and 40° and which was well stirred with a mixture containing 95% oxygen and 5% carbon dioxide. The preparation remained in good condition for several hours when stimulated supramaximally at a frequency of 12/min.

RESULTS

Fig. 2 (A) shows the effect of tubocurarine chloride (1.4×10^{-5} M in the bath) and the reversal of the neuromuscular block by neostigmine bromide (3.3×10^{-6} M). Fig. 2 (B) and (C) illustrates the contractures caused by decamethonium iodide (3.4×10^{-8} M) and succinylcholine dichloride (2.8×10^{-6} M). Fig. 2 (D) shows the effect of acetylcholine chloride (1.3×10^{-6} M) before and after the addition of neostigmine bromide (6.6×10^{-6} M).

DISCUSSION

The simplicity of the preparation should make it suitable for students' use.

We are grateful to Professor E. J. Zaimis and Dr. K. J. Child for showing us their techniques, and to Dr. R. B. Barlow for his help.

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Brit. J. Pharmacol. (1960), **15**, 412.

A NEW BIOLOGICAL METHOD FOR THE ASSAY OF DEPOLARIZING SUBSTANCES USING THE ISOLATED SEMISPINALIS MUSCLE OF THE CHICK

BY

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(RECEIVED MARCH 18, 1960)

The isolated semispinalis cervicis muscle of a 3 to 10 days old chick has been found to provide a very useful assay method for depolarizing substances. There are three main advantages of this preparation over that of the rectus abdominis of the frog: (*a*) quick relaxation without any artificial stretching of the muscle, (*b*) greater sensitivity to depolarizing drugs, and (*c*) no falling off in sensitivity for at least 2 hr.

A particular type of avian muscle is very sensitive to substances possessing an acetylcholine-like action at the neuro-muscular junction. For example, in adult fowls and chicks an intravenous injection of a minute dose of decamethonium or suxamethonium causes a rigid extension of the limbs and retraction of the head (Buttle and Zaimis, 1949). This is a peripheral effect, the muscles reacting to the depolarizing drugs with a true contracture (Zaimis, 1954, 1959). Because of this and in the hope of obtaining a sensitive and specific method for the quantitative determination of depolarizing drugs, the isolated leg and neck muscles of the chick were investigated. Of all the muscles, the semispinalis cervicis and the biventer cervicis muscles have been found to provide the best preparations as they are thin, very sensitive to depolarizing drugs, and easy to dissect.

The method was demonstrated for the first time to the Pharmacological Society in July, 1954, and a preliminary communication was given to the Biological Methods Group of the Society for Analytical Chemistry in June, 1954.

METHOD

Chicks usually obtained soon after hatching were housed at a temperature of 35 to 38° and kept on a diet of chick pellets and water.

After the chick had been anaesthetized with ether a longitudinal incision was made in the skin at the back of the neck from the base of the skull to the region of the thoracic vertebrae; the neck was then slightly flexed by means of a small pad of cotton wool. In the middle of the neck there are two pairs of thin muscles which arise from the second thoracic vertebra, run upwards side by side, and are inserted into the medial part of the occipital bone round the area of the nuchal crest. The superficial pair is formed by the two biventer cervicis, the deep one by the two semispinalis cervicis muscles. Either the biventer cervicis or the semispinalis can be used for this assay. Our preference, however, was given to the semispinalis because it could be dissected with the least possible damage. In order to expose the semispinalis, the biventer muscle of one side was lifted gently after cutting its proximal tendon (Fig. 1). The semispinalis muscle was then freed from the oblique muscles of the neck and a thread was tied round each end. Finally, the muscle was mounted as quickly as possible in a bath containing oxygenated Tyrode solution. In the present study a 5 ml. bath was used; the magnification was about 4 and the tension was 1 g.

The sensitivity of the muscle was at its best in chicks between 3 and 10 days old. In chicks less than 3 days old the muscle was very delicate and difficult to handle, whilst in chicks older than 10 days the sensitivity diminished and the differentiation between doses was less good. Differences in sex and breed did not appear to influence the response of the muscle. It was found that careful and gentle dissection was essential and that a muscle dissected from an anaesthetized chick was more sensitive than one from a recently killed animal.

Tyrode, Locke, de Jalon and Krebs solutions were tested. The sensitivity of the muscle was greatest in Tyrode solution at a temperature of 40 to 40.5°. While a temperature higher than 41° decreased the magnitude of the response without altering the time required for complete relaxation, a temperature below 39° affected both these factors; the response became less regular and the time required for complete relaxation increased markedly.

Pure oxygen and air were equally satisfactory for the oxygenation of the Tyrode solution provided that the air was finely dispersed.

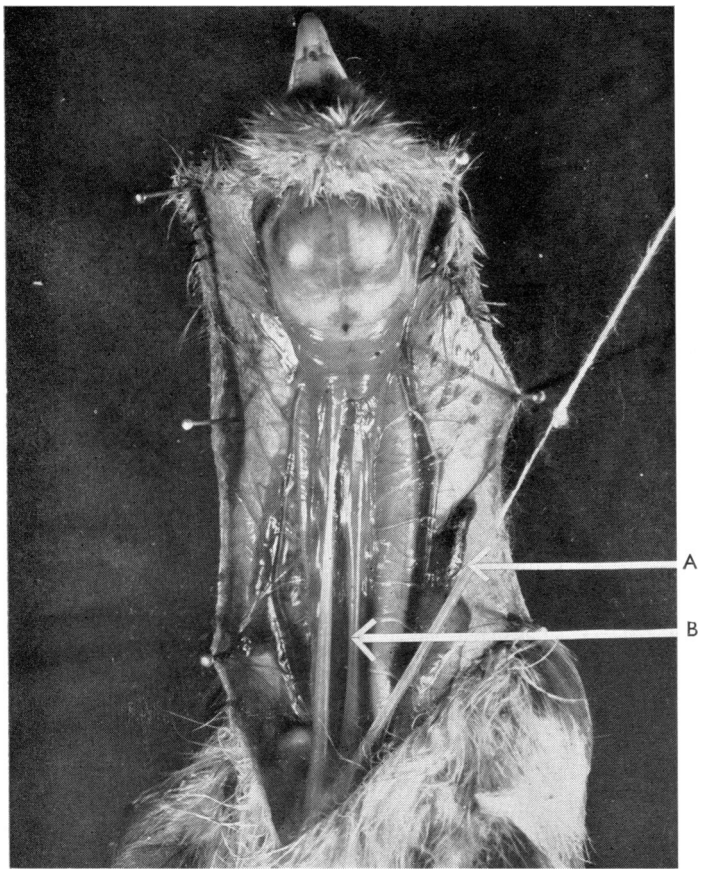

FIG. 1.—Chick; dissection of the back of neck to demonstrate (A) the biventer cervicis muscle retracted to show (B) the semispinalis cervicis muscle.

RESULTS

Substances Mimicking Acetylcholine

Decamethonium.—The semispinalis cervicis muscle responded with a sustained contracture to decamethonium and the magnitude of the response increased linearly with the logarithm of the dose (Fig. 2). The onset of the contracture was almost immediate with all but very small doses of the drug, when there was a latent period of 5 to 15 sec. The rate of development of the contracture was rapid at first but slowed down as the peak tension was approached; usually a plateau was reached in less than 1 min. The muscle remained in contracture until the bath was filled with fresh Tyrode. Relaxation was rapid and complete in less than 2 min. without any artificial stretching of the muscle. An interval of 4 min. between doses was almost always adequate.

Fig. 3 shows the result of an assay of a solution of decamethonium from which Tables I and II have been constructed. Two doses of "Standard" solution and two doses of "Test" solution were used in each assay, the doses being administered in the form of a Latin square. For the calculation of the ratio of potency and the analysis of variance of the results the method described by Schild (1942) was used with the difference that owing to the Latin square arrangement a further set of three degrees of freedom was set aside for "dose order." The value of M (log. ratio potency of unknown to standard) was estimated to be -0.0497 ± 0.003. The ratio of potency was therefore 0.892 and the potency of the test solution of decamethonium=0.892 μg./ml. The limits of error of the estimate=0.877–0.907 (P=0.05). The index of precision, $\lambda = s/b = 0.005$. In order to test the accuracy of the method the same solution of decamethonium of known strength was assayed on six fresh muscles, and the results and limits of error of the estimate of these assays are given in Table III. In each case there was a highly significant difference in activity between the two solutions and a highly significant regression between the smaller and larger doses making a quantitative estimate of activity possible. The low F value obtained for "groups" in every assay indicates that the sensitivity of the preparation did not change during the experiment.

Other Depolarizing Drugs.—Table IV shows the results obtained with suxamethonium and other substances possessing an acetylcholine-like action at the neuromuscular junction. The activity quoted for suxamethonium is the mean result of three estimations using decamethonium as the standard;

TABLE I

	Decamethonium μg./ml.	Dose ml.	Dose μg.	Responses mm.	Mean
A	1·0	0·60	0·600	32 32 32 32	32
B	1·0	0·45	0·450	14 15 16 15	15
C	0·9	0·60	0·540	25 26 24 25	25
D	0·9	0·45	0·405	9 9 8 9	8·75

TABLE II

THE ANALYSIS OF VARIANCE OF THE ASSAY RESULTS SHOWN IN TABLE I

The log. ratio of potency, M, = −0.0497 with limits of error ± 0.0074. The asterisked values of F are reciprocals.

Source of Variance	Sum of Squares	Degrees of Freedom	Variance	$F=V_1/V_2$	P
1. Groups	0·69	3	0·23	2·40*	Not significant
2. Standard and unknown ..	175·56	1	176	318	<0·001
3. Regression	1,105·62	1	1,106	2,000	<0·001
4. Dev. from parallelism ..	0·56	1	0·56	1·01	Not significant
5. Dose order	0·69	3	0·23	2·40*	,, ,,
6. Residual error	3·32	6	0·553		
Total	1,286·44	15			

the regression lines for these two substances did not differ significantly from parallel (in each case $P>0.1$).

The semispinalis cervicis muscle is very sensitive to depolarizing drugs; for example, concentrations of decamethonium and suxamethonium as low as 0.1 μg./ml. in the bathing fluid may be readily estimated. Furthermore, even less specific substances, such as carbachol and nicotine produced responses which could be easily measured.

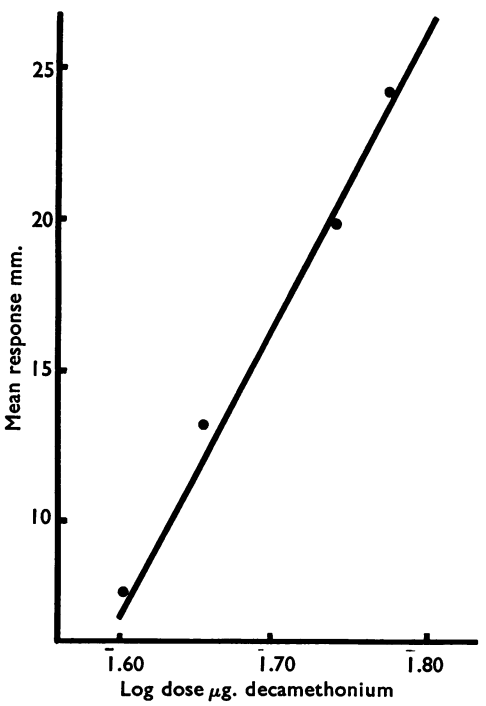

FIG. 2.—The log dose-response curve of decamethonium assayed on the isolated semispinalis cervicis muscle of the chick. S=0.59, b=93.89, λ=0.006.

TABLE III

ESTIMATION OF THE POTENCY OF A TEST SOLUTION OF DECAMETHONIUM CONTAINING 0.9 μG./ML. ON EACH OF SIX SEPARATE CHICK MUSCLE PREPARATIONS

Expt. No.	Estimated Potency μg./ml.	Limits of Error (P=0·05)	Dose Ratio	λ
1	0·908	0·878–0·939	5:4	0·009
2	0·892	0·877–0·907	4:3	0·005
3	0·889	0·864–0·915	4:3	0·009
4	0·896	0·868–0·924	4:3	0·011
5	0·889	0·865–0·913	5:4	0·009
6	0·885	0·859–0·910	6:5	0·009

TABLE IV

THE ACTIVITY OF VARIOUS DEPOLARIZING SUBSTANCES COMPARED WITH DECAMETHONIUM (=100)

All values refer to the bases.

Substance	Method of Assessment	Activity
Suxamethonium	Latin square assay	56–62 (mean=60)
Adipylcholine	Direct comparison	42
Acetylcholine	,, ,,	<1
Carbachol ..	,, ,,	<2
Nicotine ..	,, ,,	<2

Acetylcholine.—The response of the muscle to acetylcholine was unpredictable. In some preparations contracture was obtained with 5 μg. while in others 50 μg. produced no effect at all. However, in the presence of anticholinesterase drugs the sensitivity of the muscle increased and the responses became more predictable (Tyler, 1960).

Competitive Antagonists of Acetylcholine.—Competitive antagonists of acetylcholine antagonize the action of depolarizing drugs. Fig. 4 shows the effect of tubocurarine and hexamethonium; the response of the muscle to 0.5 μg. decamethonium may be reduced by approximately 50% by the previous administration of 2 μg. tubocurarine or of 100 μg. of hexamethonium. Fig. 5 shows the regression line relating log dose of tubocurarine and the percentage reduction of the response of the muscle to decamethonium.

In every experiment a potentiation of the response of the muscle to decamethonium was observed after tubocurarine had been washed out. Such an effect was not apparent following the administration of hexamethonium.

The Administration of Biological Fluids.—It was found that biological fluids containing depolarizing drugs could also be assayed provided they were approximately neutral. In acid ($pH < 6$) or alkaline ($pH > 8$) biological fluids the sensitivity of the muscle decreased very rapidly. In the alkaline fluids the decrease in sensitivity was sometimes preceded by a brief period of potentiation. Biological fluids tested included human blood and plasma, cat plasma, horse serum, and human urine. The administration of whole blood tended to reduce the rate of development of the contracture.

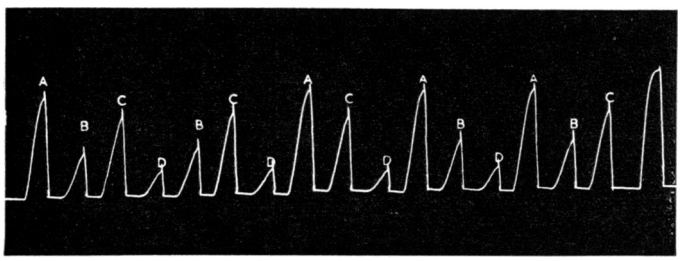

FIG. 3.—The estimation of potency of a decamethonium solution of unknown concentration on the isolated semispinalis cervicis preparation. A=0.60 ml. and B=0.45 ml. of a standard solution containing 1 μg./ml. of decamethonium. C=0.60 ml. and D=0.45 ml. of the test solution. Dose interval=3 min. 45 sec.

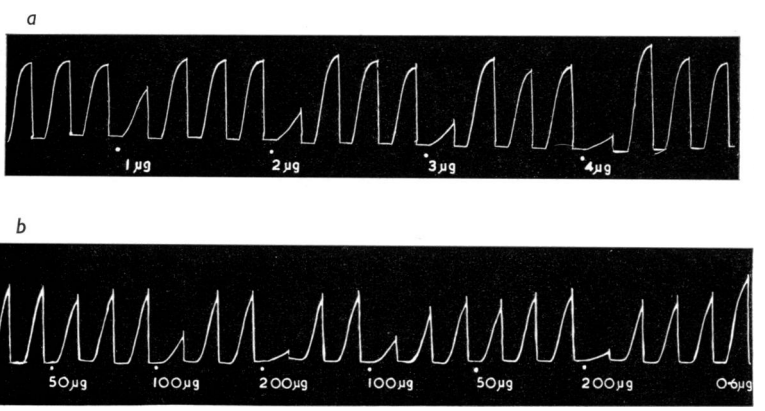

FIG. 4.—Contractions of the isolated semispinalis cervicis muscle produced by decamethonium 0·5 μg. Tubocurarine (1 to 4 μg.) (a), and hexamethonium (50 to 200 μg.) (b), were added to the bath 1 min. before the decamethonium at the points indicated. The last contraction was produced by 0·6 μg. decamethonium.

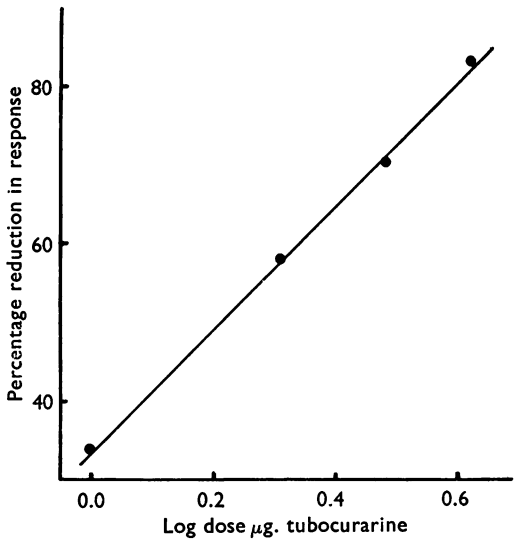

FIG. 5.—Log dose-response curve of tubocurarine assayed by its inhibitory effect on the decamethonium induced contractions of the isolated semispinalis cervicis muscle.

DISCUSSION

The semispinalis cervicis muscle of the chick, like the rectus abdominis muscle of the frog, responds by contracture to acetylcholine and to substances possessing an acetylcholine-like action at the neuromuscular junction. This muscle offers advantages over the rectus abdominis because (*a*) relaxation following contracture is much more rapid and occurs without any artificial stretching of the muscle; (*b*) the muscle is more sensitive to substances mimicking acetylcholine; and (*c*) there is no falling off in sensitivity for at least 2 hr.

The major part of this work was carried out at the Pharmacology Department of the School of Pharmacy, University of London. Grateful acknowledgement is made of a grant by the Medical Research Council (to E.Z.) for technical assistance.

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Brit. J. Pharmacol. (1960), **15**, 417.

THE RELEASE OF ACETYLCHOLINE FROM MAMMALIAN MOTOR NERVE ENDINGS

BY

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(RECEIVED MARCH 16, 1960)

The release of acetylcholine from rat and guinea-pig isolated diaphragm preparations stimulated through the phrenic nerve was optimal at 37° in Krebs solution with 5×10^{-6} neostigmine methylsulphate. The amount of acetylcholine released by a 20 min. tetanus was reduced by cooling. At frequencies of stimulation above 6/sec. the release was less than that predicted. This " failure " was unaffected by the addition of 1×10^{-6} choline. The acetylcholine release declined with continued stimulation at 25/sec. In the absence of nerve stimulation, there was a small continuous resting release of acetylcholine which seemed to originate in the muscle fibres. These results are discussed in the light of current electrophysiological knowledge of the quantal release of acetylcholine.

Nearly a quarter of a century has passed since Dale and his colleagues first showed that acetylcholine was released at the neuromuscular junction by motor nerve stimulation, and could be measured directly in the extracellular fluid if steps were taken to prevent its destruction by cholinesterase (Dale and Feldberg, 1934; Dale, Feldberg, and Vogt, 1936). Since that time it has been generally accepted that acetylcholine is the chemical mediator of nerve impulses at all neuromuscular junctions. Recently, Dale's observation has been confirmed and extended by others, in a perfused muscle preparation (Emmelin and MacIntosh, 1956) and in isolated diaphragm preparations (Burgen, Dickens and Zatman, 1949; Barnes and Duff, 1954; Brownlee, 1957; Brooks, 1954).

The present experiments are concerned with the factors which modify the amounts of the transmitter released from motor nerve endings; and in particular with the effect of changes in the frequency and duration of stimulation, changes in temperature and the presence of choline on acetylcholine release.

METHOD

The Collection of Acetylcholine

The method was based on that of Burgen, Dickens, and Zatman (1949), and has been described in detail by Straughan (1959).

Female albino rats between 200 and 300 g. and female albino guinea-pigs of 200 g. were used. After decapitation, a half diaphragm with its attached phrenic nerve was put in a flat glass diaphragm bath containing between 2.5 and 3.0 ml. Krebs solution. The small volume has the advantage of increasing the acetylcholine concentration in the bath fluid. The upper intercostal margin of the diaphragm was connected by fine stainless wire to a semi-isometric spring lever.

To ensure complete inhibition of cholinesterase, the preparation was allowed to stand for 30 min. in 5×10^{-6} neostigmine methylsulphate before an experiment was begun. The temperature of the muscle was kept constant at $37° \pm 0.25°$ and the phrenic nerve was stimulated for 20 min. periods at a rate of 25/sec. with supramaximal rectangular pulses 0.03 millisecs. in duration, except where indicated.

Less acetylcholine was found to be released by the first than by subsequent periods of stimulation; to offset this a " conditioning " 3 min. period of stimulation was applied to the preparation before each experiment. An intervening rest period of 10 min. was allowed between successive 20 min. periods of stimulation. Under these conditions the preparation could sustain up to eight successive 20 min. periods of stimulation before there was any appreciable fall in the amount of acetylcholine released.

In experiments to determine the recovery of acetylcholine at different concentrations of anticholinesterase, the initial equilibration was made at a low concentration of neostigmine, and the samples from two successive periods of stimulation collected. Subsequent samples were collected at gradually increasing concentrations of neostigmine.

When the effect of changing the frequency of stimulation on release was studied, the frequency of

stimulation in successive 20 min. periods was varied in a random order, so that there were two samples at each frequency. When the effect of duration of tetanus on release was studied, 20 min. periods of stimulation were alternated with 10 min. periods at the same frequency, so that there were three samples for each duration.

In experiments on the effect of temperature on acetylcholine release, the initial equilibration was always at 37° ; samples were either collected at this temperature and at successively lower temperatures ; or the muscle was cooled and samples collected at stages in the course of subsequent rewarming. Samples were withdrawn from the bath within a minute of the end of stimulation, acidified to about *p*H 4 with one drop of 5 N hydrochloric acid and frozen to −20° until required for assay.

The Estimation of Acetylcholine

The activity of small volumes of the bath fluid was matched against known concentrations of acetylcholine in Krebs solution containing neostigmine on the rat blood pressure preparation, as described by Straughan (1958 ; 1959). A male albino rat of 250 g. was anaesthetized with urethane 40 mg./100 g. and sodium pentobarbitone 3 mg./100 g. and allowed to cool to about 28° when a stable long-surviving preparation with a good blood pressure was obtained. Depressor artifacts which are normally a hazard of this preparation were almost completely avoided by carefully immobilizing the venous cannula after cutting the femoral nerves, and by ensuring that not more than 0.3 ml. of fluid was injected at one time. Sensitivity was highest in the winter months, when as little as 0.25 ng. acetylcholine base gave a measurable depressor response in the rat blood pressure.

The preparation also usefully discriminated between biologically active depressor substances ; equidepressor responses were seen with acetylcholine base 1 ng., choline base 10 μg., histamine 0.2 μg., adenosine triphosphate 1 mg., and potassium chloride 1 mg.

The identification of the depressor substance in the bath fluid as acetylcholine was based on the following observations.

1. The relative insensitivity of the assay preparation to other interfering biological substances.

2. The stability of the depressor substance in acid solution, and its destruction by boiling in an alkaline medium.

3. The absence of depressor activity in the bath fluid in the absence of adequate amounts of neostigmine (Table I).

4. The depressor activity of the bath fluid when present was abolished after atropine, and was enhanced by the previous administration of an anticholinesterase to the assay animal.

Krebs solution of the following composition was used (g./litre): NaCl 6.92, KCl 0.354, $CaCl_2$ 0.282, $NaHCO_3$ 2.1, KH_2PO_4 0.162, $MgSO_4,7H_2O$ 0.294, glucose 2.0.

TABLE I

ACETYLCHOLINE RECOVERY AND NEOSTIGMINE CONCENTRATION

Acetylcholine recovery in ng. base after 20 min. stimulation at 25/sec. in different concentrations of neostigmine methylsulphate. Each figure is the mean of two successive periods of stimulation.

Expt. No.	Neostigmine Concentration					
	Absent	1×10^{-7}	5×10^{-7}	1×10^{-6}	5×10^{-6}	1×10^{-5}
1	0	4	—	—	58	—
2	—	1	15	—	33	—
3	—	—	19	37	71	—
4	—	—	—	22	51	51

A mixture of 95% oxygen and 5% carbon dioxide was bubbled through the solution. A correction was applied to the results for the small volume of fluid which remains in the diaphragm bath after draining and dilutes the subsequent addition of known volumes of fluid.

The resting release was measured in most experiments, but the results were not corrected for it, because of the larger errors involved.

RESULTS

The Effect of Anticholinesterase Concentration on the Recovery of Acetylcholine

The results from a number of experiments with the rat phrenic nerve diaphragm preparation showed that when neostigmine was omitted from the bath fluid no acetylcholine could be recovered

TABLE II

RESULTS FROM DIFFERENT DIAPHRAGMS SHOWING HOW THE RECOVERY OF ACETYLCHOLINE VARIES WITH THE CONCENTRATION OF NEOSTIGMINE

Means±S.E. are given. The numbers in parentheses indicate the number of diaphragms from which the means were derived. With each diaphragm two successive 20 min. periods of stimulation at 25/sec. were applied with the stated concentration of neostigmine.

Acetylcholine Recovery	Concentration of Neostigmine Methylsulphate
0 (4)	Absent
2±0·9 (3)	1×10^{-7}
14±1·9 (4)	5×10^{-7}
28±3·4 (6)	1×10^{-6}
52±3·6 (11)	5×10^{-6}
53±0·9 (3)	1×10^{-5}

after a 20 min. period of nerve stimulation at 25/sec. Little acetylcholine was recovered at 1×10^{-7} neostigmine methylsulphate, but at higher concentrations of neostigmine the recovery increased to become maximal at a 5×10^{-6} concentration of neostigmine salt. This is illustrated in Table I.

The collected results from these and other experiments (Table II) show that there was a statistically significant increase in acetylcholine recovery at each of the following concentrations of neostigmine methyl sulphate, 1×10^{-7}, 5×10^{-7}, 1×10^{-6}, and 5×10^{-6}. Thereafter further increase in the neostigmine concentration brought about no statistically significant increase in the recovery of acetylcholine after a period of stimulation. The possibility that neostigmine might increase the amount of transmitter released by each nerve impulse cannot be ignored. But Liley (1956a) has shown that 10^{-6} neostigmine did not alter the rate of the spontaneous quantal discharge of acetylcholine in the rat phrenic nerve diaphragm preparation.

The Effect of Changes in the Frequency of Stimulation on Acetylcholine Release in the Rat and Guinea-pig Diaphragms

The amount of acetylcholine released during a 20 min. collection from the rat diaphragm increased with the frequency of stimulation from 6/sec. to 25/sec. (Table III). But the amount of acetylcholine released did not increase in direct proportion to the number of stimuli applied, so that at 25/sec. it was less than half that expected (Fig. 1). This "failure" to release acetylcholine becomes even more marked at higher frequencies, and though the response of individual diaphragms was variable, in general the amount of acetylcholine released in each 20 min. period was steady within the range 25 to 100/sec. (Fig. 1).

TABLE III

ACETYLCHOLINE RELEASE AT DIFFERENT FREQUENCIES OF STIMULATION IN THE RAT AND GUINEA-PIG DIAPHRAGM PREPARATIONS

The acetylcholine release is given in ng. base per 20 min. stimulation. Each figure is the mean of two periods of stimulation applied at random to the particular diaphragm.

Expt.	Resting	Frequency of Stimulation				
		6/sec.	12/sec.	25/sec.	50/sec.	100/sec.
Rat						
1	5	33	41	59	—	—
2	10	34	43	61	—	—
3	7	—	44	56	38	50
4	10	—	51	69	69	55
5	—	—	25	29	48	55
Guinea-pig						
1	7	28	52	71	—	—
2	9	42	63	86	—	—
3	7	35	39	68	—	—
4	13	—	—	59	58	50
5	7	—	—	77	106	64
6	8	—	—	80	110	72

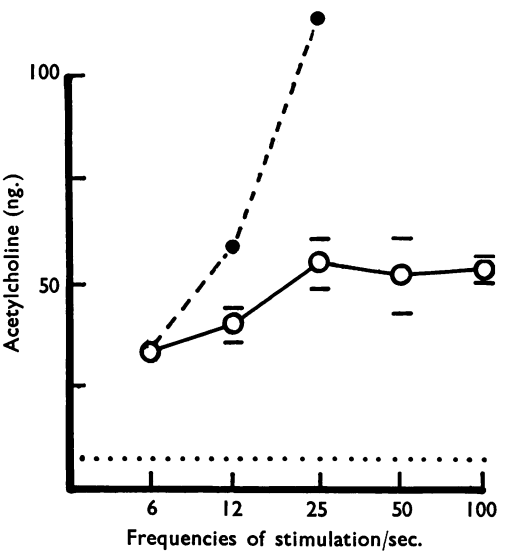

FIG. 1.—Variation in the amount of acetylcholine released from the isolated rat phrenic nerve diaphragm preparation at different rates of stimulation. Ordinate: ng. acetylcholine released per 20 min. period of stimulation in Krebs solution containing 5×10^{-6} neostigmine methylsulphate at 37°. Abscissa: rate of stimulation/sec. O——O : actual acetylcholine release. Each point is the mean ±S.E. from 2 to 6 diaphragms. ●– – –● : predicted acetylcholine release, calculated from the release at a stimulation rate of 6/sec. · · · · · : resting release of acetylcholine/20 min. collection period in the absence of stimulation.

In the guinea-pig diaphragm the amount of acetylcholine released per 20 min. period increased over the range 6 to 50 stimuli/sec. and thereafter declined (Table III and Fig. 2). "Failure" to release acetylcholine was not evident at 12/sec. and there was less "failure" at a frequency of 25/sec. than in the corresponding rat diaphragm, an observation which probably accounts for the relatively greater acetylcholine release in the guinea-pig diaphragm at this frequency.

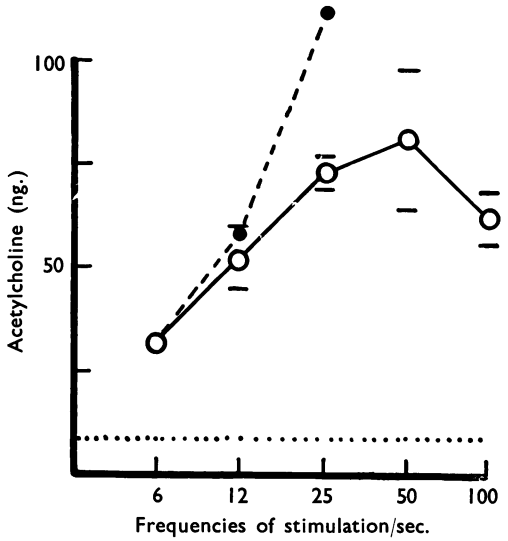

FIG. 2.—Variation in the amount of acetylcholine released from the guinea-pig phrenic nerve diaphragm preparation at different rates of stimulation. Ordinate: ng. acetylcholine base released per 20 min. period of stimulation in Krebs solution containing 5×10^{-6} neostigmine methylsulphate at 37°. Abscissa: rate of stimulation/sec. O——O : actual acetylcholine release. Each point is the mean ±S.E. from 2 to 6 diaphragms. ●- - -● : predicted acetylcholine release, calculated from the release at a stimulation rate of 6/sec. · · · · ·: resting release of acetylcholine 20 min. collection period in the absence of stimulation.

The Influence of the Duration of Stimulation on Acetylcholine Release in the Rat Diaphragm

The observed "failure" in the release of acetylcholine at some frequencies from the rat diaphragm might occur at the beginning of the period of stimulation, or might develop in intensity with continued stimulation. It is not practicable to measure the amount released each minute during a period of stimulation, so the acetylcholine released during 20 min. of stimulation at 25/sec. was compared with the acetylcholine released by 10 min. of continuous stimulation at the same frequency in each diaphragm.

In five experiments the release declined with continued stimulation during 20 min. more than 50% acetylcholine being released by 10 min. of stimulation (Table IV). In only one experiment was the acetylcholine release constant throughout 20 min. of stimulation, which suggested that uniform "failure" was present from the beginning of stimulation.

TABLE IV

DURATION OF STIMULATION AND ACETYLCHOLINE RELEASE IN THE RAT DIAPHRAGM

Acetylcholine release in ng. base. Each figure is the mean of three alternate periods of stimulation at 25/sec. in that experiment.

Expt. No.	Duration of Stimulation		
	(a) 10 min.	(b) 20 min.	$\frac{2a}{b}$%
1	24	40	120
2	39	50	158
3	25	39	128
4	45	54	166
5	23	39	120
6	25	51	98

Perry (1953) has shown that with the cat's superior cervical ganglion perfused with saline there is a sharp decline in acetylcholine release after the first few minutes during prolonged stimulation, even at low frequencies. In this respect acetylcholine release from preganglionic and motor nerve endings is comparable, though the actual mechanism of failure is probably not identical.

The Influence of Temperature Changes on Acetylcholine Release in the Rat Diaphragm

Acetylcholine release from mammalian motor nerve endings has not been directly measured at different temperatures, though there have been indirect observations from electrophysiological studies by Boyd and Martin (1956), Liley (1956a) and Li (1958). The results (Fig. 3 and Table V)

TABLE V

THE EFFECT OF TEMPERATURE ON ACETYLCHOLINE RELEASE IN THE RAT DIAPHRAGM

Release in ng. base per 20 min. at 25/sec. Each figure is the mean of two successive periods of stimulation.

Expt. No.	Temperature			
	20°	30°	37°	40°
1	6	22	31	—
2	9	17	20	—
3	6	26	37	—
4	5	17	22	—
5	—	—	63	69
6	—	—	37	38
7	—	—	63	46
8	—	—	39	29

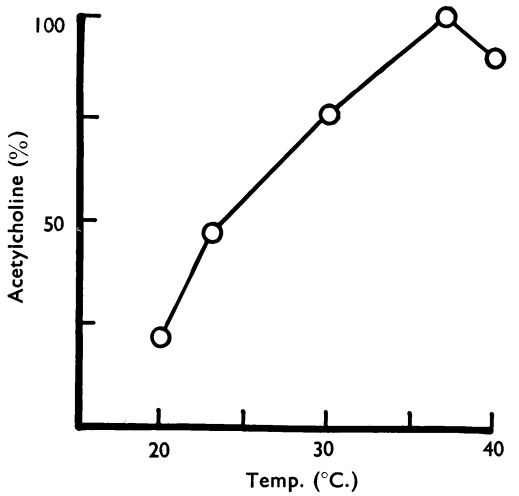

FIG. 3.—Variation in the amount of acetylcholine released from the rat phrenic nerve diaphragm preparation at different temperatures. 20 min. stimulation at 25/sec. in Krebs solution with 5×10^{-6} neostigmine methylsulphate. Ordinate: acetylcholine release as a percentage of the release at 37°. Abscissa: muscle temperature. O——O Each point is the mean release from 2 to 6 diaphragms.

showed that acetylcholine release varies directly with the temperature within the range of 20° to 37° whether acetylcholine release was studied after cooling or warming. The amount released at the two temperatures was significantly different at the 5% level of probability. The mean release of acetylcholine at 40° was less than at 37° (Table V), but this difference was not statistically significant at the 5% level of probability.

To provide a better basis for comparison between different diaphragms, the mean acetylcholine release in each experiment was plotted as a percentage of the release at 37°. The mean results from these different experiments are shown in Fig. 3.

The Effects of Choline on Acetylcholine Release in the Rat Diaphragm

The observation that the rat diaphragm preparation maintains a steady release of acetylcholine in response to repeated periods of stimulation suggests that, apart from the glucose in the Krebs solution, all the elements necessary for acetylcholine synthesis must be available in the preparation itself. Whether the absence of choline from the extracellular fluid imposed any restraint on the synthesis or release of acetylcholine was tested.

The results from four experiments (Table VI) showed that the presence of physiological amounts of choline (Bligh, 1952) brought about no significant increase in the amount of acetylcholine released by two 20 min. periods of stimulation at 25/sec.

TABLE VI

THE EFFECT OF CHOLINE ON ACETYLCHOLINE RELEASE IN THE RAT DIAPHRAGM

Acetylcholine release in ng. base/20 min./25/sec. Each figure is the mean of two successive periods of stimulation.

Expt. No.	Normal Krebs Solution	Krebs Solution + 1×10^{-6} Choline
1	46	60
2	51	41
3	37	32
4	38	42

The Resting Release of Acetylcholine from the Rat Diaphragm in the Absence of Nerve Stimulation

Brooks (1954) showed that there was a resting release of acetylcholine from the guinea-pig diaphragm preparation in the absence of nerve stimulation. A resting release has not yet been described in the isolated rat diaphragm preparation, though it was evident from the results of Burgen and others (1949) that a measurable release of acetylcholine still persisted in the isolated rat diaphragm preparation after paralysis with botulinum toxin; and Brooks (1956) has shown that any release of acetylcholine from motor nerve endings is abolished by this toxin.

The small amounts of acetylcholine involved in this resting release were measured in both rat and guinea-pig diaphragm by collecting samples over 20 min. collection periods at intervals of 1.5 hr. The mean resting release from 19 rat diaphragms was 7 ± 0.6 ng. (S.E.) acetylcholine base per 20 min.; and the mean resting release from 6 guinea-pig diaphragms (Table III) was 8.5 ± 0.6 ng. (S.E.) acetylcholine base. In both preparations the resting release appeared to be continuous and steady over many hours.

DISCUSSION

Acetylcholine Release and Frequency of Nerve Stimulation

If precautions are taken to prevent the destruction of acetylcholine, then variations in the amounts recovered after a period of nerve

stimulation under similar experimental conditions may be thought to have their origin in changes in the amounts set free at the nerve endings. This may occur in five principal ways.

1. By depletion of the stores of acetylcholine in the motor nerve terminals.

2. By altering the probability of quantal discharge in the terminal membrane. Liley (1956b) has shown that prolonged stimulation at high frequencies in the rat diaphragm has this effect, which probably accounts for the observation that less acetylcholine is released by the first period than by subsequent periods of tetanus, under identical conditions in the rat phrenic nerve diaphragm preparation.

3. By acting at some intermediate stage through which the nerve impulse co-ordinates the normal spontaneous discharge of quanta. Calcium and magnesium ions, particularly, appear to affect acetylcholine release by acting at this stage (Liley, 1956b ; Straughan, 1959).

4. By altering the level of depolarization produced by the nerve impulse in the terminals (Liley, 1956c). This is thought to be the mechanism by which small concentrations of procaine selectively reduce acetylcholine release (Straughan, 1959).

5. By altering the number of impulses invading the nerve terminals at particular synapses. Brooks (1954) suggested that high-frequency stimulation of motor nerves could cause a "blocking" reaction in the nerve terminals.

The most likely explanation for the observed "failure" to achieve the predicted release at frequencies above 6/sec. comes from the work of Krnjević and Miledi (1958b). They showed in the rat diaphragm preparation that, with stimulation of sufficient frequency and duration, there was an intermittent all-or-none failure of nerve impulse conduction, probably located in the narrow unmyelinated preterminal part of the nerve fibre. The observed decline in the amount of acetylcholine released during a 20 min. period of nerve stimulation is probably due to the fact that intermittent presynaptic failure is absent initially, and only appears and develops in degree with continued stimulation. The marked variation between different diaphragms in the amounts of acetylcholine which are released by nerve stimulation at rates above 25/sec. is perhaps due to the variation between different diaphragms in the time of onset, and degree of intermittent presynaptic failure.

It seems unlikely that there is any depletion of acetylcholine stores in the terminals during a tetanic period of stimulation even when there is complete neuromuscular block (Krnjević and Miledi, 1958b). The serial decline in the size of the end-plate potential in the curarized rat diaphragm with continued stimulation (Liley, 1956b) is due most likely to a gradual reduction in the acetylcholine sensitivity of the end plate (Krnjević and Miledi, 1958b) and an enhancement of the blocking activity of curare (Chou, 1947).

During a period of tetanus, therefore, there is a tendency for certain nerve impulses to be blocked intermittently, though when a nerve impulse does invade the terminals it will cause the normal amount of acetylcholine to be released from the terminals. This intermittent failure of conduction probably forestalls exhaustion of the terminal stores of acetylcholine (Castillo and Katz, 1956).

The Effects of Temperature on Acetylcholine Release

The most likely explanation for the observed decline in release with cooling over the range 37° to 28° is that cooling acts directly on the terminal membrane and reduces the probability of quantal discharge ; for the results are in fairly close agreement with the figures of Liley (1956a) and Li (1958), who measured the frequency of the spontaneous discharge in the rat diaphragm at these temperatures. These workers showed that with continued cooling to 20° there was a decrease and later an increase in the spontaneous discharge frequency, but Fig. 3 shows that the amount of acetylcholine released by a period of tetanus in fact decreased with continued cooling to 20°. This anomaly would be explained if, at and around room temperature, some further additive process were taking place, such as an increase in intermittent presynaptic failure at room temperature compared with 37° (as proposed by Krnjević, 1958), or possibly a reduced rate of acetylcholine resynthesis with cooling as suggested by Kostial and Vouk (1956) for the cat perfused superior cervical ganglion.

A decline in the amount of acetylcholine released with cooling over this range of temperature occurs in other nerve-muscle preparations. Boyd and Martin (1956) showed, in the curarized tenuissimus of the cat, that the amplitude of the end plate potential declined with cooling, while Bigland, Goetzee, Maclagan and Zaimis (1958) have stated that the blocking activity of curare was reduced by cooling. From their observation that the amplitude of the end plate potential in the cat tenuissimus blocked with magnesium increased on cooling, Boyd and Martin suggested that acetylcholine release was increased by cooling. But this last observation could be attributed more

easily to decreased curare-like and decreased depressant actions on acetylcholine release of magnesium ions at low temperatures.

Estimation of Acetylcholine Release per Synapse in the Rat Diaphragm

The figures in Table III show that about 2.5×10^{-8} g. of acetylcholine base is released from the rat diaphragm preparation by a 20 min. period of stimulation at 6/sec. after corrections are made for the resting release. Intermittent presynaptic failure which is thought to be precipitated by anoxia in the intramuscular portions of the motor nerve is minimal at this rate of stimulation (Krnjević and Miledi, 1958b, 1959); yet the post-synaptic changes observed in this preparation during stimulation at lower rates suggested that the deepest muscle fibres were anoxic (Creese, Hashish and Scholes, 1958). The use of neostigmine in the present experiments caused the rapid development of neuromuscular blockade, so by the end of the first minute of stimulation there was very little tension developed in the muscle. The absence of muscle activity during the greater part of stimulation thus makes it seem unlikely that severe anoxia occurred in the present experiments. If it is accepted that there is little or no "failure" in release at this frequency, then a single motor nerve volley released about 3.5×10^{-12} g. acetylcholine base. Accepting the calculations of Krnjević and Miledi (1958a) that there are 10,000 synapses in the rat diaphragm, it may be calculated that 3.5×10^{-16} g. of acetylcholine base will be released at a single synapse by a single maximal motor nerve volley in the rat diaphragm at 37° in Krebs solution. This is about three times as much as Acheson (1948) calculated was released at a nerve ending in the cat tongue. The difference may be a genuine species difference, or may arise from experimental errors.

Recent work by Liley (1956b) suggests that an individual quantum of acetylcholine in the rat diaphragm is between 1 and 2% of the amount normally released by a single nerve volley at the neuromuscular junction. From the figures presented above it would follow that each quantum would contain about 3.5×10^{-18} g. base or about 15,000 molecules of acetylcholine. This is higher than the revised estimate of 900 molecules per quantum for cat motor nerves given by MacIntosh (1959a).

It seems reasonable to suppose that most of the acetylcholine released at the nerve terminals is recovered under the conditions of the experiments, since the amounts needed to evoke substantial end plate potentials in the rat diaphragm (calculated from Krnjević and Miledi, 1958c) at 37° are only a hundred times greater than the release per synapse calculated from this present work. This discrepancy would probably disappear if it were possible to bring acetylcholine in as close proximity to the end plate as it is when naturally released.

Resting Release of Acetylcholine

The origin of the resting release was not clear; only a very small proportion of it could be accounted for by acetylcholine leakage from the cut end of the phrenic nerve, 0.6 ng. base/20 min. in one experiment; or by the spontaneous quantal discharge of acetylcholine estimated as 0.5 to 0.1 ng. base/20 min. by Straughan (1959). No great difference was apparent either between the resting release from the 7-day chronically denervated side of a diaphragm and the control acutely denervated side, in two preliminary experiments.

It would seem therefore that the resting release of acetylcholine originates in some non-nervous structure, perhaps the muscle fibres themselves. For MacIntosh (1959b) has pointed out that only half the preformed acetylcholine is lost from cat leg muscles after careful denervation.

It seems that the actual mechanisms of acetylcholine release from preganglionic and motor nerve terminals are not identical, and we must be cautious about extending results and knowledge from the one to the other. For instance, the release from motor nerve endings varies with the rate of stimulation in an almost identical manner to that observed in the cat superior cervical ganglion perfused with plasma (Birks and MacIntosh, 1957). But, in the ganglion perfused with saline the release declines sharply after the onset of stimulation so that there is little change in the amounts of acetylcholine released at different frequencies (Perry, 1953). It seems therefore that motor nerve endings maintain a high enough intracellular concentration of acetylcholine precursors to be independent of the organic composition of the extracellular fluid (apart from glucose), while the preganglionic nerve endings need choline and labile plasma factor for the optimal synthesis and release of acetylcholine (Birks and MacIntosh, 1957; MacIntosh, 1959a). It seems uncertain whether the mechanism of failure of acetylcholine release at high frequencies of stimulation in the ganglion perfused with plasma can be explained by intermittent presynaptic failure.

In contrast to motor nerve endings the acetylcholine release from the preganglionic nerve

endings of the cat superior cervical ganglion perfused with saline is not decreased by cooling (Kostial and Vouk, 1956). Thus it would appear that the acetylcholine release mechanism in preganglionic sympathetic nerve endings is not temperature-sensitive like the acetylcholine release mechanism in motor nerve endings. This implies that there is a fundamental difference between the terminal membranes of these two kinds of nerve endings. It will be of interest to see if acetylcholine release from preganglionic nerve endings is temperature-sensitive when the optimal conditions for synthesis and release are provided.

This work was included in a thesis submitted for the degree of Doctor of Philosophy in the University of London, in February, 1959, and part of it was communicated to the British Pharmacological Society in January and July, 1958. I wish to thank Professor G. Brownlee for his help and guidance throughout this work.

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THE INHIBITION BY MORPHINE OF THE ACTION OF SMOOTH MUSCLE STIMULANTS ON THE GUINEA-PIG INTESTINE

BY

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(RECEIVED MARCH 24, 1960)

Morphine and related analgesics depress the responses of the isolated guinea-pig ileum to nervous stimulation and to drugs which act by stimulating the nervous structures of the intestinal wall. The present experiments show that these analgesics also depress the responses to smooth muscle stimulants which act directly on the smooth muscle fibres.

Morphine has a depressant action on the isolated intestine which is generally explained by an action on the nervous structures of the intestinal wall (Schaumann, Giovannini, and Jochum, 1952; Schaumann, 1955, 1956a; Kosterlitz and Robinson, 1955). Morphine also inhibits the action of drugs which cause contraction of the intestine by stimulation of these nervous structures (Schaumann, 1955; Kosterlitz and Robinson, 1955, 1958; Gaddum and Picarelli, 1957; Kosterlitz, Robinson, and Taylor, 1957). Part of the inhibition which morphine exerts through its action on the nervous structures may result from inhibition of the release of acetylcholine from post-ganglionic cholinergic fibres (Schaumann, 1956b, 1957; Paton, 1956, 1957). Kosterlitz and Robinson (1958) found that morphine strongly inhibits the contractions of the isolated guinea-pig ileum to nicotine, barium and 5-hydroxytryptamine, but has only a slight effect on the response to acetylcholine, carbachol and histamine. They concluded that the strong inhibition of the responses to nicotine, barium and 5-hydroxytryptamine was due to an action on the nervous elements innervating the muscle fibres, whereas the responses to acetylcholine and histamine were mainly on the muscle fibres and therefore resistant to the depressant action of morphine.

The present experiments show that morphine apparently has a non-specific depressant action on the smooth muscle fibres as well, since it depresses the action of drugs which stimulate these fibres directly.

METHODS

The guinea-pig ileum preparation was suspended in 15 ml. of Tyrode solution at 34°. Acetylcholine chloride, histamine acid phosphate (calculated as base) and carbaminoyl choline chloride (carbachol) were added at intervals of either 2 or 3 min. and left in contact with the tissue for 15 sec. Nicotine acid tartrate, barium chloride, potassium chloride, 5-hydroxytryptamine creatinine phosphate, bradykinin (Elliott, Horton and Lewis, 1960) and substance P (11 U./mg.) were added at intervals of either 4 or 5 min. and left in contact for 30 sec.

When the action of morphine, of related drugs and of hexamethonium was examined, their concentration was maintained by additions to the bath after each washing. The following drugs were used for this purpose: morphine sulphate, diamorphine hydrochloride (heroin), methadone hydrochloride, dihydromorphinone hydrochloride, codeine sulphate, hexamethonium bromide (vegolysen).

RESULTS

Morphine reduced the contractions of the guinea-pig ileum to smooth muscle contracting substances. The experiment of Fig. 1 shows the depression of acetylcholine responses by morphine 10^{-7} at *a*, 10^{-6} at *b*, and 10^{-8} at *c*. The threshold concentration of morphine for this effect is between 5×10^{-9} and 10^{-8}, but the effect depends on the concentration of morphine only within narrow limits. When a concentration of 10^{-7} is reached no further depression occurs even when the concentration of morphine is raised to 10^{-5}.

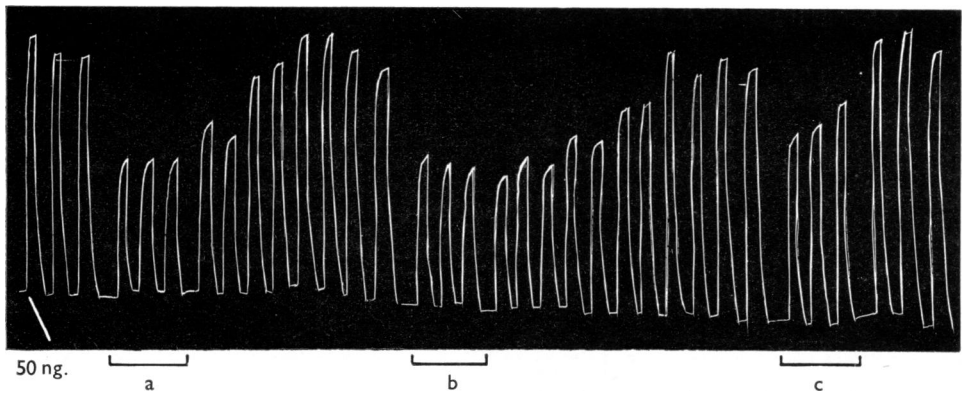

FIG. 1.—Responses of the guinea-pig ileum to acetylcholine 50 ng. alone and in the presence of morphine 10^{-7} g./ml. (*a*), 10^{-6} g./ml. (*b*), 10^{-8} g./ml. (*c*). Contractions every 3 min.; contact time 30 sec.

Usually the morphine has to be in contact with the tissue for 5 to 10 min. before it exerts its maximum effect, but some reduction in the responses is observed after morphine has been in contact for less than 1 min.

The duration of action of morphine after it is washed out will depend partly on the time it has been in contact and partly on the concentration used. With short contact times (of less than 2 min.) the time taken for the contractions to return to normal is proportional to the time of contact. Where the contact time is more than a few min. the recovery period depends on the concentration of morphine; at 10^{-8} recovery is almost immediate whereas at 10^{-5} the contractions do not return to normal for about 90 min.

Depression of the Action of Various Smooth Muscle Contracting Substances

The extent of the depressant action of morphine varies according to the drug used to contract the ileum, but in every experiment some depression was observed. The responses to acetylcholine and histamine were reduced about 20 to 40%. In the experiment of Fig. 2 morphine 2×10^{-7} reduced the response to acetylcholine 0.03 μg. to that previously given by 0.02 μg., and in the experiment of Fig. 3 it produced the same reduction with the responses to histamine. The experiment of Fig. 2 also illustrates that the depressant action of morphine still occurred when the ganglia were blocked by hexamethonium.

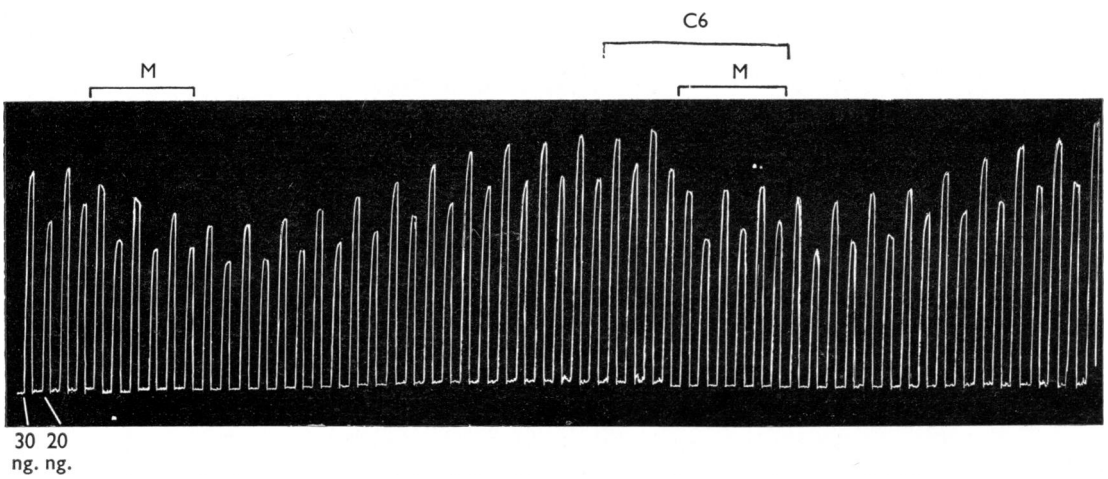

FIG. 2.—Responses of the guinea-pig ileum to acetylcholine 30 ng. and 20 ng. given alternately every 2 min. Contact time 15 sec. The bars at the top indicate the presence of morphine 2×10^{-7} g./ml. (M) or hexamethonium bromide 10^{-7} g./ml. (C6).

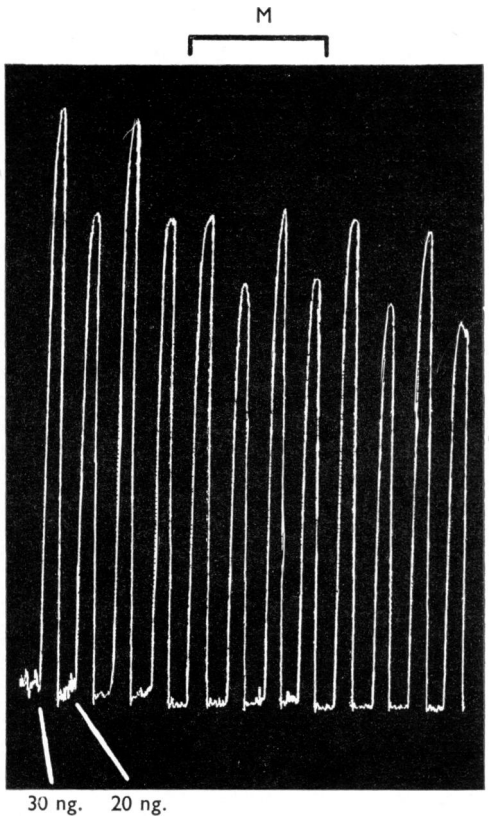

FIG. 3.—Responses of the guinea-pig ileum to histamine 30 ng. and 20 ng. given alternately every 2 min. Contact time 15 sec. The bar at the top indicates the presence of morphine 2×10^{-7} g./ml.

The experiment of Fig. 4 shows that the responses to both 5-hydroxytryptamine and nicotine were reduced to a much greater extent than those to histamine and acetylcholine, and the experiment of Fig. 5 shows that the responses to barium chloride were reduced much more than those to potassium chloride.

Morphine depressed the action of the various drugs to the same extent throughout their whole dose-response curves. In Fig. 6 the responses to acetylcholine are plotted on a graph to give the dose-response curve of acetylcholine alone and in the presence of morphine. The distance between the two curves gives the ratio of the doses of a substance causing equal contractions in the absence and in the presence of the inhibitor. This figure, which is a convenient measure of inhibition, has been called the dose ratio (Gaddum and Picarelli, 1957). In the experiment of Fig. 6 this figure was 1.7. The dose ratios for the other substances tested are shown in Table I, together with the standard errors. With the drugs in column 1, the dose ratios varied only between 1.42 and 1.82 and the variability of the morphine depression was relatively small, as shown by the standard errors. However, with nicotine, barium chloride and 5-hydroxytryptamine, the drugs in column 2, the dose ratios were not only greater but also more variable as shown by the large standard errors.

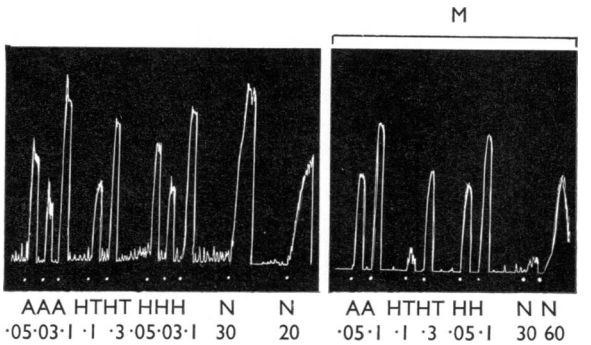

FIG. 4.—Response of the guinea-pig ileum to acetylcholine (A), 5-hydroxytryptamine (HT), histamine (H), and nicotine (N) alone and in the presence of morphine 2×10^{-7} g./ml. (M). Doses are given in μg. added to the organ bath.

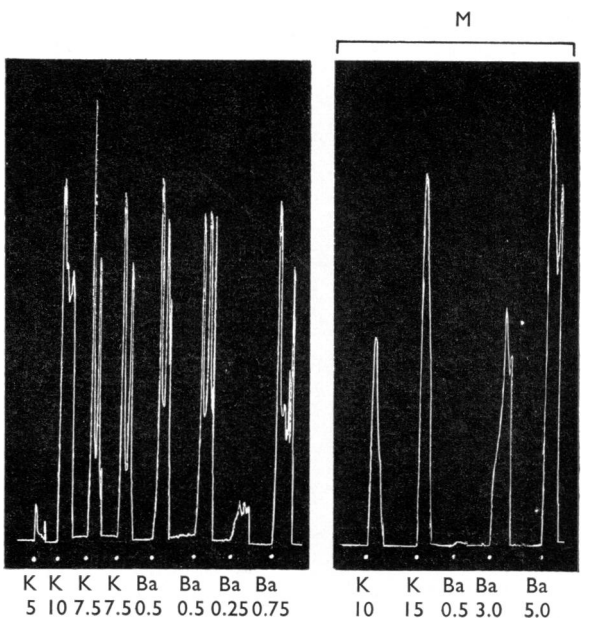

FIG. 5.—Responses of the guinea-pig ileum to potassium chloride (K) and barium chloride (Ba) alone and in the presence of morphine 2×10^{-7} g./ml. (M). Doses are given in mg. added to the organ bath.

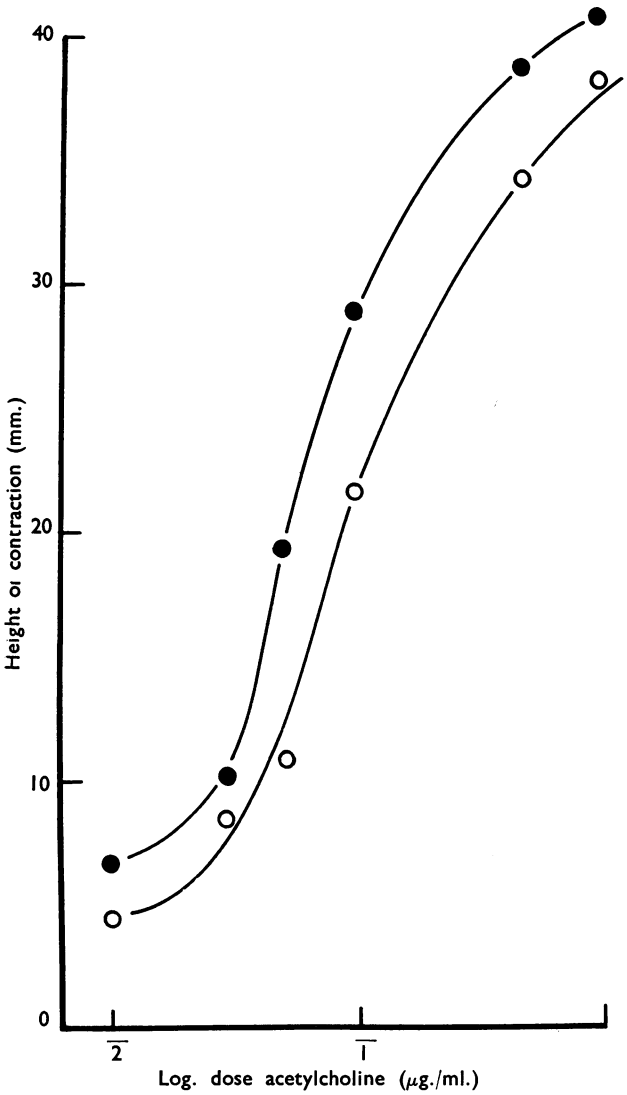

FIG. 6.—Dose-response curve of acetylcholine on the guinea-pig ileum, alone (closed circles) and in the presence of morphine 2 × 10^{-7} g./ml. (open circles). The points are plotted from the results of the experiment of Fig. 6.

The results of Table I thus show that drugs which contract the guinea-pig ileum primarily by a direct action on the muscle fibres have lower dose ratios than those which act on the nervous structures in the intestinal wall like nicotine, or on the nervous and muscle structures like barium chloride.

Drugs Related to Morphine

The inhibition of the action of smooth muscle stimulants on the guinea-pig ileum is also shown by analgesic drugs related to morphine, such as methadone, heroin, codeine, and dihydromorphinone. Usually methadone and heroin caused depressions at a slightly lower threshold concentration than morphine, whereas with codeine the threshold was higher than that for morphine. The experiment of Fig. 7 shows the depression of responses to carbachol and substance P by morphine 10^{-7} and by codeine 5×10^{-6}.

Adaptation to Morphine

In some experiments the guinea-pig intestine became tolerant to the action of morphine ; after several doses, morphine no longer depressed the action of smooth muscle stimulants. This development of tolerance was more frequently obtained with large doses of morphine, but even under this condition it did not occur in all preparations.

When an intestine had become tolerant to morphine and larger doses were then applied, they did not decrease the responses to smooth muscle stimulants but increased them. Usually in these instances another peculiar effect was seen ; when the morphine was withdrawn from the organ bath the responses were depressed, but could be restored on renewed addition of morphine to the bath, so that the intestine seemed to be dependent on the presence of morphine for its normal responsiveness. Such an experiment is illustrated in Fig. 8. Responses to barium chloride were at first inhibited by morphine 10^{-6}. Later when morphine was continuously in contact with the tissue the responses gradually recovered, and when the dose of morphine was raised to 5×10^{-6} and then to 10^{-5} the responses increased. On withdrawal of morphine the responses were depressed but were restored again when morphine was added during one contraction only. When the morphine was withdrawn again the responses were again depressed. In several intestinal strips tolerance with dependence was obtained and in some neither dependence nor tolerance could be produced.

Spontaneous Activity

When spontaneous activity of the intestine was present it was depressed by the concentrations of morphine which reduced the action of smooth muscle stimulants. This effect is seen in Figs. 4, 5 and 7. However, when the intestine became tolerant, morphine sometimes increased spontaneous movement, and on withdrawal of

TABLE I

DOSE RATIOS

The ratio of the doses of various smooth muscle stimulating drugs giving equal response before and after morphine 2×10^{-7} g./ml.

	Mean	No. of Expts.	Standard Error of Mean		Mean	No. of Expts.	Standard Error of Mean
Acetylcholine ..	1·61	10	0·086	Nicotine	3·00	6	0·465
Histamine	1·77	6	0·093	Barium chloride ..	4·17	6	0·857
Plasma kinin ..	1·75	4	0·171	5-Hydroxytryptamine	3·20	7	0·485
Substance P ..	1·42	5	0·120				
Carbachol	1·60	4	0·131				
Potassium chloride	1·82	4	0·075				

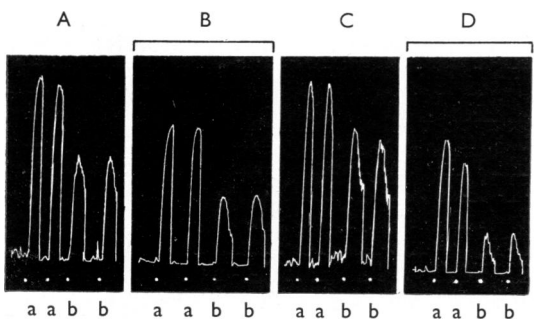

FIG. 7.—Responses of the guinea-pig ileum to carbachol 0·1 μg. (*a*) and substance P (*b*), alone (A), in the presence of morphine 10^{-7} (B), alone after 90 min. recovery (C) and in the presence of codeine 5×10^{-6} (D). Time interval 3 min.; contact time 15 sec. for carbachol, 30 sec. for substance P.

morphine during the phase of dependence, the contractions produced by barium chloride or acetylcholine were characterized by rhythmic activity as shown in Fig. 8.

DISCUSSION

It is evident from the present experiments that when we consider the action of morphine on the guinea-pig intestine we have to assume an action not only on the nervous structures of the intestinal wall, but also on the smooth muscle fibres themselves.

The finding that the contractions caused by substances which primarily stimulate smooth muscle directly are depressed to a smaller extent than those caused by substances which act on nervous structures is understandable because

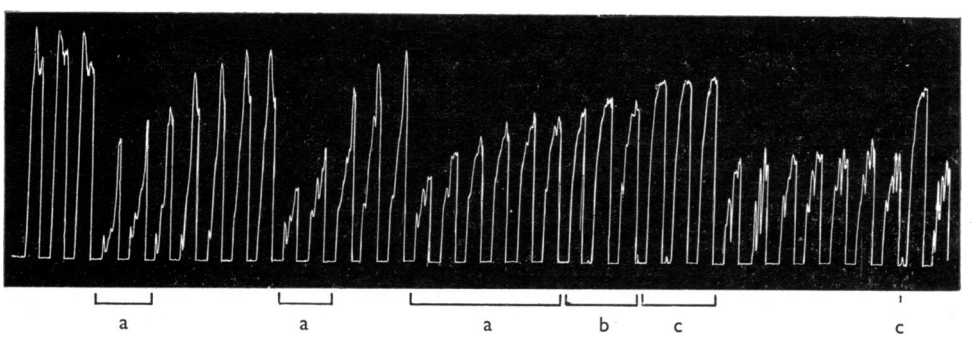

FIG. 8.—Responses of the guinea-pig ileum to barium chloride 3 mg. given every 5 min. Contact time 45 sec. The period marked by bar (*a*) indicates the presence of morphine 10^{-6}, (*b*) morphine 5×10^{-6}, (*c*) morphine 10^{-5}.

morphine has a two-fold depressing effect on the responses to substances which act on the nervous structures. In the case of histamine, for instance, morphine has only one site of action—depression of the muscle fibre—whereas with nicotine it has two sites of action—reduction of acetylcholine released from postganglionic cholinergic fibres and depression of the action of the released acetylcholine on the muscle fibre. In the case of barium chloride morphine probably has a three-fold depressing action; it reduces the release of acetylcholine, and it inhibits the action of the released acetylcholine, and it depresses the direct smooth muscle stimulating effect of barium chloride. The wide range of dose ratios (as shown by standard error) for drugs which act on nervous structure or nervous and muscle structures is consistent with the view that morphine is acting at more than one site and therefore subject to greater variability.

It is established that acetylcholine, histamine, carbachol and potassium chloride contract the guinea-pig ileum mainly by direct stimulation of the muscle and that nicotine acts wholly in the ganglia whilst barium chloride acts both on the nervous structures as well as on smooth muscle (Feldberg, 1951; Ambache and Lessin, 1955). There is also evidence that 5-hydroxytryptamine causes contraction of the guinea-pig ileum by acting on a nervous structure, although this action is different from that of nicotine and barium chloride in being insensitive to the action of hexamethonium or large doses of nicotine (Rocha e Silva, Valle and Picarelli, 1953; Robertson, 1953). Gaddum and Picarelli (1957) have postulated the existence of two kinds of tryptamine receptors, one of which they call morphine, or M receptors which can be blocked with morphine. However, the results of the present experiments do not support the idea of such a specific action of morphine. Therefore the name morphine or M receptor would appear to be misleading, although the concept of tryptamine derivatives having two sites of action would not be invalidated.

The possibility must be considered that the cholinergic nerves in the intestine like cholinergic nerves elsewhere are continually releasing quanta of acetylcholine while "at rest." Such releases, themselves producing responses below threshold for contraction, could nevertheless sum with added acetylcholine or other smooth muscle contracting substances. Morphine, by abolishing the release of such subthreshold quanta, would increase by a slight amount the concentration of added smooth muscle stimulant required to produce a given contraction. An explanation of this kind would support the view that morphine has an entirely neuronal action. However, the dose-response curve of a smooth muscle stimulating substance such as acetylcholine is shifted to a parallel position on the right by morphine. But if morphine depressed only the quanta of acetylcholine released "at rest," then the shape of the dose-response curve would be altered to indicate a greater percentage depression with small doses than with large doses.

When discussing the action of morphine in reducing the release of acetylcholine from cholinergic nerve fibres, Schaumann (1957) concluded that morphine inhibits the excitatory processes which release acetylcholine from nerve endings. Recently Schaumann (1958) has suggested that analgesics like morphine might depress the excitability of the intestine by liberating noradrenaline within the intestinal wall. According to his results, noradrenaline inhibits the release as well as the action of adrenaline. The inhibition observed in the present experiments was similar in degree to the reduction in acetylcholine release found by Schaumann. There is also a remarkable similarity to the morphine depression described by Paton in his experiments with co-axial stimulation. The threshold concentration is the same, and above a concentration of about 2×10^{-7} no further depression can be effected for the action on smooth muscle or for the release of acetylcholine. Onset and duration of both activities are similar, and both are effected not only by morphine but also by analgesic drugs related to morphine. In both cases it was sometimes possible to produce morphine tolerance and occasionally morphine dependence where, when morphine was withdrawn, the responses of the intestine were reduced and could be restored by fresh additions of morphine.

Thus morphine appears to act on the nervous structures of the intestine in much the same way as on smooth muscle and the underlying mechanism may well be an inhibition of a metabolic process common to both nervous and muscle structures.

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MAXIMAL INHIBITION OF CHOLINESTERASE IN THE CENTRAL NERVOUS SYSTEM

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(RECEIVED MARCH 24, 1960)

In experiments on mice treated with pralidoxime iodide (pyridine-2-aldoxime methiodide; PAM) and atropine, the cholinesterase activity in the brain was assayed after poisoning with very high doses of organophosphorous anticholinesterases. Acetylcholine was added to the buffer solution in which the brains were homogenized. This precaution reduced the combination between free inhibitor present in the tissue and active enzyme, and the cholinesterase activity found was below 0·5% of controls. When the experimental data were corrected for spontaneous reactivation *in vitro* during incubation, the calculated activities *in vivo* were even less. It is concluded that mice can survive complete inactivation of the cholinesterase in the central nervous system, if enough atropine is given to protect the animals against the toxic effects of the accumulating acetylcholine.

Death after poisoning with anticholinesterases which penetrate into the central nervous system is due to both central and peripheral inhibition of cholinesterase. Pralidoxime iodide (pyridine-2-aldoxime methiodide; PAM) effectively protects the peripheral cholinesterase or reactivates it after inhibition by organophosphates (Kewitz and Nachmansohn, 1957), whereas atropine protects against the actions of the accumulating acetylcholine in the central nervous system. In combination with pralidoxime iodide, atropine increases the LD50 about 10 times (Askew, 1957; Hobbiger, 1957; Kewitz, Wilson, and Nachmansohn, 1956; Wills, Kunkel, Brown, and Groblewski, 1957). The question arises whether the brains of mice surviving excessive doses of organophosphates under these conditions contain any active cholinesterase.

Several experimental difficulties must be overcome in order to answer this question:

1. After high doses of organophosphates, the brain contains large amounts of free inhibitor which must be prevented from blocking the remains of active enzyme during and after homogenization. This was done by adding acetylcholine to the buffer before homogenization (Schaumann, 1960a).

2. After poisoning with diethyl phosphates, there is spontaneous reactivation *in vitro* and the experimental values must be corrected accordingly (Schaumann, 1960a).

3. Labile organophosphates like paraoxon are destroyed enzymically in the body so quickly that reactivation of inhibited cholinesterase *in vivo* very soon prevails over inactivation by free inhibitor (Schaumann and Schiller, 1960a). Therefore, only anticholinesterases with a long half-life were used in the present experiments.

METHODS

Male white mice received 50 mg./kg. pralidoxime iodide + 10 mg./kg. atropine intraperitoneally and at the same time one of the following anticholinesterases subcutaneously: diethyl-dimethylaminothioethyl-phosphate acid oxalate (217-AO), parathion (*OO* - diethyl - *O* - *p* - nitrophenyl - phosphorothionate; E 605), or dyflos. The animals were decapitated 1 or 2 hr. after injection. The brains were dissected at once, weighed and homogenized with 1.9 ml. buffer solution per 100 mg. of brain. The buffer contained 7.5 to 10 mM. acetylcholine, 0.03 M magnesium chloride, and 0.03 M sodium bicarbonate. Two ml. of the homogenate was pipetted into Warburg vessels and at once incubated at 37°. The manometers were gassed with 95% nitogen + 5% carbon dioxide, and the production of carbon dioxide was measured from 30 to 90 and from 90 to 150 min. after inserting the manometers. The concentration of acetylcholine in the homogenates was determined colorimetrically according to the method of Hestrin (1949) at the beginning of the incubation and after 3 hr. The carbon dioxide production from brain homogenates to which an excessive amount of an anticholinesterase had been added was subtracted as a blank from the manometric values. The colorimetric readings were corrected for the spontaneous hydrolysis of acetylcholine during incubation. Cholinesterase activity

was calculated from the production of carbon dioxide (manometric determination) or the consumption of acetylcholine (colorimetric determination). The homogenate of a normal mouse brain diluted 1:20 served as a control. All determinations were done in duplicate.

RESULTS

Determination of Cholinesterase Activity in vitro

The manometric determination of cholinesterase activity during two successive hours shows a measurable increase in activity after poisoning with 217-AO and parathion (Table Ia). The rate of reactivation is practically linear during this time, and the readings taken between 30 and 90 min. after the beginning of the incubation therefore represent the activity after 60 min. (a_{60}); those of the second period the activity after 120 min. (a_{120}).

The reactivation follows the equation:

$$\ln \frac{100-a_o}{100-a_t} = K_r \cdot t \ldots\ldots (1)$$

a_o, a_t = % activity after 0 and t min. of incubation; t = duration of incubation in min., K_r = first order rate constant for the reactivation.

When the average activities for a_{60} and a_{120} from Table I are inserted together with t = 60 in equation (1), K_r can be calculated. K_r was lower after 60 mg./kg. of parathion—i.e., the rate of reactivation was slower than after 217-AO. Apparently, the high concentrations of free inhibitor found after parathion (Schaumann and Schiller, 1960b) reduced the speed of reactivation in spite of the addition of acetylcholine.

The cholinesterase inhibited by dyflos is not reactivated (Hobbiger, 1957; Davison, 1953). It was, therefore, sufficient to determine the enzyme activity once. The activities found are valid for the whole incubation period (Table II).

The colorimetric determinations give an average of the cholinesterase activity during the whole incubation period. The rate of reactivation being practically linear, the figures can be compared with the activities measured manometrically at the middle of the incubation period, i.e., after 90 min. (a_{90}). It follows from equation (1) that a_{90} is the geometric mean between $100-a_{60}$ and $100-a_{120}$. As shown in Tables I and II, there is a good agreement between the results obtained with both methods. In spite of low activities, the manometric determination gave just as dependable results as the colorimetric determination under the present experimental conditions.

Calculation of Cholinesterase Activity in vivo

No more than 3 min. elapsed between killing the animals and inserting the manometers. Therefore, the activity at the beginning of the incubation period, a_o, represents the cholinesterase activity *in vivo*. For 217-AO and parathion, a_o can be calculated by inserting into equation (1) t = 90, a_{90} from Table I for a_t and the respective values for K_r. No correction is necessary with dyflos. The results are summarized in Table III. When the mice were killed 1 hr. after the injection of 10 mg./kg. of 217-AO, a cholinesterase activity of 3.5–3.9% was found after 90 min. of incubation. From these values an activity *in vivo* of 0.04 to 0.3% of normal can be

TABLE I

MEASURED CHOLINESTERASE ACTIVITY OF BRAIN

The mice were killed (a) 1 hr. after 10 mg./kg. 217-AO subcutaneously, and (b) 2 hr. after 60 mg./kg. parathion subcutaneously; in both substances 50 mg./kg. pralidoxine iodide + 10 mg./kg. atropine was also given intraperitoneally. All values are % of control activity.

(a)

Exp. No.	Cholinesterase Activity			
	Manometrically			Colorimetrically
	a_{60}	a_{120}	a_{90}	a_{90}
3	0·9	2·6	1·75	2·10
	2·6	5·3	3·95	3·60
	2·6	5·4	4·00	3·60
	2·2	4·5	3·35	3·30
4	3·1	5·4	4·25	3·70
	4·2	6·1	5·15	4·20
	3·2	5·8	4·50	4·00
	2·8	5·0	3·90	3·50
Mean:	2·7	5·0	3·86 ±0·35	3·50 ±0·22
	$K_r = 0{\cdot}0004$			

(b)

5	1·6	3·4	2·50	2·6
	1·7	3·3	2·50	2·8
	2·1	4·0	3·05	3·1
	2·2	4·4	3·30	3·4
6	1·5	3·1	2·30	2·1
	2·5	4·2	3·35	2·9
	2·0	3·2	2·60	2·3
Mean:	1·94	3·67	2·80 ±0·16	2·74 ±0·22
	$K_r = 0{\cdot}000296$			

TABLE II

MEASURED CHOLINESTERASE ACTIVITY OF BRAIN

The mice were killed 1 hr. after injection of 80 mg./kg. dyflos subcutaneously and 50 mg./kg. pralidoxime iodide + 10 mg./kg. atropine intraperitoneally. All values are % of control activity.

Exp. No.	Cholinesterase Activity	
	Manometrically	Colorimetrically
1	0·45	0·45
	0·40	0·50
	0·20	0·45
	0·45	0·55
2	0·75	0·85
	0·70	0·95
	0·53	0·55
	0·55	0·50
Mean:	0·50 ±0·06	0·60 ±0·07

TABLE III

CALCULATED CHOLINESTERASE ACTIVITY OF BRAIN *IN VIVO*

mmt.=manometric; col.=colorimetric determination. All values are % of control activity.

	Cholinesterase Activity after Subcutaneous Injection of:		
	217-AO 10 mg./kg.	Parathion 60 mg./kg.	Dyflos 80 mg./kg.
In vitro (a_{90})			
mmt. ..	3·86	2·80	0·50
col. ..	3·50	2·74	0·60
In vivo (a_0)			
mmt. ..	0·33	0·17	0·50
col. ..	0·04	0·11	0·60

calculated. Two hr. after 60 mg./kg. of parathion the figures were even lower and from the activities found after 90 min. incubation and the rate constant of reactivation given in Table Ib, a cholinesterase activity *in vivo* of 0.1 to 0.2% of normal can be calculated. After injection of 40 mg./kg. of dyflos, Kewitz and Nachmansohn (1957) found 1.4% cholinesterase activity in the brain. In the present experiments, 80 mg./kg. was tolerated, and the activity was reduced to 0.5 to 0.6% of normal.

DISCUSSION

Using a histochemical method, McIsaac and Koelle (1959) found that physiologically liberated acetylcholine is hydrolysed only by the so-called functional cholinesterase on the cell surface and not by the reserve cholinesterase within the cells. Pharmacologically equipotent doses of two organophosphates inhibited the functional enzyme to the same degree, but only one of them penetrated into the cells; the other one left the reserve cholinesterase practically intact. In consequence, the tissue homogenates showed a considerably higher cholinesterase activity after the latter compound.

The cholinesterase activity in the brains of mice poisoned with equitoxic doses of different organophosphates decreases inversely with the stability of the respective compound in the body. In other words, the longer the half-life of a substance in the brain, the lower are the cholinesterase values found after a lethal dose (Schaumann, 1960b). This is confirmed by the present results (Table III). The free inhibitor found in mouse brain after injection of dyflos has a half-life of about 15 min. after 217-AO of about 40 min., whereas after parathion there is no measurable decrease in free inhibitor during 10 hr. (Schaumann and Schiller, 1960b). The mice were still alive 2 hr. after the injection of 60 mg./kg. of parathion, but they died 1 to 3 hr. later, when the therapeutic effects of pralidoxime iodide and atropine had worn off. A stable inhibitor will have more time to penetrate into the cell than one which is quickly metabolized. It is therefore most likely that the activities remaining after dyflos and 217-AO are due to inner or reserve cholinesterase.

Even if one assumes that the minute enzyme activities *in vitro* were due to external, functional cholinesterase, they do not constitute the absolute minimum necessary for the maintenance of life. In spite of pralidoxime iodide treatment, the animals showed severe muscular fasciculations and fibrillations, symptoms characteristic for peripheral inactivation of cholinesterase. It has previously been shown that in mice pretreated with pralidoxime iodide and atropine, the injection of a lethal dose of 217-AO reduced the cholinesterase activity in the diaphragm to the critical value for a lethal neuromuscular block in spite of the reactivator. When artificial respiration was given in addition to pralidoxime iodide and atropine, practically unlimited amounts of paraoxon and 217-AO could be given to rats without affecting the respiratory centre (Schaumann, 1959; 1960b).

When the lethal dose of an organophosphate was injected after pretreatment with pralidoxime iodide, death was due to the central inhibition of cholinesterase alone. Under these conditions, the enzyme activity found in the brains of mice was 1 to 5.5% of the control level, depending on the compound used (Schaumann, 1960b). This is the minimum amount of cholinesterase necessary to prevent the accumulation of toxic concentrations of acetylcholine in the central nervous system. However, if enough atropine is given to prevent the toxic effects of acetylcholine, the cholinesterase in the brains of rats and mice can apparently be inactivated completely without causing death.

It should perhaps be pointed out that this statement is not inconsistent with the assumption of cholinergic transmission within the central nervous system. Only the acetylcholine receptor, and not the cholinesterase, is primarily concerned in the process of transmission. The liberated acetylcholine which accumulates after the inhibition of the cholinesterase can be removed by diffusion (Ogston, 1955) and can be hydrolysed in the periphery which, due to the protective action of pralidoxime iodide, contains active enzyme.

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Brit. J. Pharmacol. (1960), **15**, 436.

SCREENING OF CESTICIDAL COMPOUNDS ON A TAPEWORM *HYMENOLEPIS NANA IN VITRO*

BY

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(RECEIVED MARCH 30, 1960)

A simple and convenient *in vitro* technique is described for the screening of compounds for action against *Hymenolepis nana* and probably many other intestinal worms. The results obtained from this test are in broad agreement with the findings of clinical experience and of a small series of *in vivo* tests. Among the substances tested, the most active ones were oil of chenopodium, dichlorophen, extract of cashew nut (*Anacardium occidentale*), antimony potassium tartrate, and BIQ 20 [eicosamethylenebis(isoquinolinium iodide)].

The testing of possible chemotherapeutic compounds upon helmintic infections in general is usually a cumbersome process which consumes great amounts of the chemical specimen, of animals and of time. In the present paper an *in vitro* technique is described for testing compounds on intestinal worms such as *Hymenolepis nana*; this technique is simple, and economical of time, animals and the specimen under test. It could be applied to many other intestinal worms. A preliminary notice of this work has been given elsewhere (Sen and Hawking, 1959).

METHODS

The worm selected for this work was *Hymenolepis nana,* which is a common tapeworm of mice, and which also occurs in man, especially in the Mediterranean area, Near East, and S. America; it can be transmitted directly by ova from mouse to mouse, since both stages of the worm (cysticercoid and tapeworm) occur in the same host. The worm was maintained in mice according to the technique described by Steward (1955). Ova were obtained from ripe segments of worms taken from infected mice. It was necessary to check under the microscope that the ova were mature since immature eggs will not infect mice. The eggs were freed from the segments of worms by light crushing under a cover slip, and they were then washed into boiled and cooled tap water. They were allowed to stand in water for 20 (or 44) hr. at room temperature (about 30°). The ova were then counted in a haemocytometer chamber, and an appropriate number (500 to 2,000) were given to mice by syringe and stomach tube.

Twenty to forty days after inoculation the mice were killed, having been starved for 24 hr. beforehand. The intestine was washed out, by inserting the needle of a syringe into the upper end and perfusing it with saline. If an isotonic solution buffered to be slightly acid (*p*H 6.8) was employed, the worms came out more readily. Alternatively, the small intestine freed from the mesentery was placed between two thick glass plates which were clamped together and examined by the naked eye or a binocular dissecting microscope. Worms *in situ* could be seen well by this technique, but they were difficult to recover from the intestine subsequently by perfusion. Newly weaned mice seem more susceptible than other mice. Some of the mice bred at this Institute contain natural infections of *H. nana* so that some older mice may possess an immunity from previous infections and some of the young mice may possess a partial immunity transmitted by immune mothers. Under good conditions the percentage of mice which later contained worms was about 80% and the number of worms was from 1 to 56 in each mouse. If only one worm was present 21 days after infection, it was usually a large one about 5 cm. long. If many worms were present they were small, from 0.5 to 1 cm. In this Institute at one season of the year, laboratory animals are often given cucumber as green food. There is some evidence that this had an anthelmintic action. Thus, of 7 control mice fed on stock diet, 6 (86%) were infected and they contained 3 to 13 worms each; while out of 5 mice fed with cucumber, only 2 were infected and they contained 3 and 5 small worms respectively. Accordingly, it is better to avoid a cucumber diet. A diet containing much potato seems to be favourable.

To expose the worms to compounds *in vitro,* the worms recovered from the intestine by perfusion were picked up free from mucus and other debris and transferred to two washes of Ringer solution. They were then placed in nutrient broth containing penicillin 500 units per ml. and streptomycin 0.1 mg. per ml.

The formula of the nutrient broth was as follows :

Peptone	10 g.
Yeast extract	2.5 g.
Sodium chloride	5 g.
Distilled water	1,000 ml.

The *p*H was adjusted to 8.5 by adding 2 ml. of 40% sodium hydroxide solution. After 0.5 to 1 hr. in nutrient broth at room temperature (30 to 34°) they were transferred to 3 ml. of the same medium in a suitable container. For this purpose small Kjeldahl's flasks of 10 ml. volume were used. Concentrations of penicillin as high as 2,000 units per ml. and of streptomycin as high as 1.0 mg./ml. were harmless to the worms. One or two worms were placed in each flask, which was then bunged. Suitable concentrations of the various compounds had been previously added to the nutrient broth in each flask. Since it was desired to make a broad survey of a large number of compounds, concentrations were chosen in multiples of 10. If the compound was insoluble in water, it was ground up, and a fine suspension was made by shaking in broth; sometimes the compound was dissolved in alcohol before adding to broth. In these cases the maximum concentration of the compound depended on its solubility in the broth, and the concentrations recorded in the table are nominal ones, the actual concentration being lower. Preliminary experiments showed that concentrations of alcohol greater than 1% are harmful to the worms and such concentrations were avoided. The flasks were incubated at 37°. For examination they were placed in a horizontal position under a binocular dissecting microscope with low magnification where the movements of the worms could easily be observed. The worms of the control flasks with drug-free medium remained actively mobile for 5 days. The action of the drug, however, was read after 24 hr. because it is unlikely that a drug given by mouth would stay in the intestine even as long as 24 hr. Bacterial growth in the flask did not occur to any appreciable extent.

Some tests were made, for comparison, of the action of the drugs *in vivo*, using the technique of Steward (1955). Groups of mice were taken on the 14th day after infection. The drug was given in a single dose by oral catheter. The mice were starved on the second day and killed on the third day after treatment. The worms were collected from the intestine and scored by the number of worms present multiplied by a factor for the size of the worm (worms more than 3 cm., 20; about 2.5 cm., 10; about 1.2 cm., 5; about 0.6 cm., 0.5; less than 0.3 cm., 0.1). The results thus obtained were compared with those from a control group of untreated mice.

Results

The results obtained during the *in vitro* experiments are shown in Table I; each figure is based on five separate experiments. The readings with carbon tetrachloride and tetrachlorethylene were very variable, ranging from 1:1,000 to

Table I

THE MINIMUM CONCENTRATIONS OF VARIOUS COMPOUNDS REQUIRED TO KILL *HYMENOLEPIS NANA* AT 37° IN 24 HR.

The extracts dichlorophen and oil of chenopodium were dissolved in alcohol and then diluted with water and Ringer solution. The solubility was greater than the minimum lethal concentration except for sulphadiazine (soluble 1:1,300), santonin (very slightly soluble), tetrachlorethylene, phenothiazine, pamaquin (insoluble), carbon tetrachloride (soluble 1:2,000).

Compound	Minimum Lethal Concentration
Male fern extract	1:100,000
Extract of cashew nut (ether extract of *Anacardium occidentale*)	1:1,000,000
Oil of chenopodium	1:10,000,000
Thymol	1:10,000
β-Naphthol	1:10,000
Arecoline	1:100,000
Santonin	1:1,000
Hexylresorcinol	1:100,000
Dichlorophen (Dicestal)	1:2,000,000
Carbon tetrachloride	1:10,000
Tetrachlorethylene	1:10,000
Phenothiazine	1:1,000
Mepacrine hydrochloride	1:100,000
Acriflavine	1:100,000
Chloroquine phosphate	1:100,000
Pentaquine phosphate	1:100,000
Pamaquin	1:100,000
Stilbamidine isethionate	1:1,000
Propamidine isethionate	1:1,000
Piperazine	1:1,000
Diethylcarbamazine	No action
Arsenamide [(*p*-carbamoylphenylarsinidenedithio)diacetic acid]	1:10,000
Melarsoprol (Mel B)	1:1,000
Antimony potassium tartrate (tartar emetic)	1:1,000,000
Copper sulphate	1:1,000
Sulphadiazine	No action
Iodochlorhydroxyquin (Vioform)	1:100,000
Bialamicol hydrochloride (camoform)	1:1,000
Lucanthone hydrochloride	1:10,000
Quinapyramine (Antrycide)	1:1,000
Prothidium (7-amino-2-(2-amino-6-methylpyrimidin-4-ylamino)-9-*p*-aminophenylphenanthridine 10,1'-dimethobromide	1:1,000
BIQ 20 [eicosamethylenebis(isoquinolinium iodide)]	1:1,000,000
Penicillin (1,000 units per ml.)	No action
Streptomycin (1 mg. per ml.)	,, ,,
Chloramphenicol ,,	,, ,,
Tetracycline ,,	,, ,,
Ethyl alcohol	1:50

1 : 100,000 in 11 experiments ; these results may be related to the insolubility of these compounds in watery solutions, although preliminary solution in alcohol or acetone did not make the results any more consistent.

The compounds tested can be divided into groups according to their ability to kill the worms *in vitro* in 24 hr. The most active compounds, which kill at a concentration of 1/million or less include oil of chenopodium (1 : 10 million), extract of cashew nut (*Anacardium occidentale*), dichlorophen, antimony potassium tartrate (tartar emetic) and BIQ 20 [eicosamethylenebis(isoquinolinium)]. The moderately active compounds (killing at 1/100,000 concentration) include male fern extract, arecoline, hexylresorcinol, mepacrine, acriflavine, chloroquine, pentaquine, pamaquine, and iodochlorhydroxyquin (Vioform). The other compounds tested are relatively or absolutely inactive against *Hymenolepis*.

A few experiments were carried out in mice to compare the *in vivo* action of several well-known anthelmintics with the findings obtained *in vitro*. The results are shown in Table II. The compounds which were active *in vivo* had already shown activity *in vitro* with the exception of tetrachlorethylene (which had given very irregular results *in vitro*).

TABLE II

THE ACTION OF CERTAIN DRUGS UPON *HYMENOLEPIS NANA IN VIVO*

Compound	Dose mg./kg.	No. of Mice Free of Worms / No. of Mice Treated	Average Score	Evaluation of Treatment
Male fern extract	750	4/5	1·1	Active
Arecoline	20	3/5	3·2	,,
Hexylresorcinol	500	4/5	1·7	,,
Dichlorophen	100	5/5	0	Very active
Tetrachlorethylene	1·5	3/5	4·2	Active
Mepacrine	300	4/5	0·4	Very active
Untreated controls	—	0/5	32·2	

DISCUSSION

The chemotherapy of helminthiasis has been surveyed in excellent reviews by Watkins (1958) and by Whitten (1956). Accordingly the present discussion will be limited to brief comments on certain aspects.

Technique of Testing.—Many techniques for testing anthelmintics have been described, but methods involving the survival or death of tapeworms *in vitro* have not been used. The method described above is simple and economical ; the worms from a few mice are sufficient to test many different drug-concentration combinations ; only a minute amount of chemical compound is required ; no previous toxicity tests are required. Theoretically, an *in vitro* method seems justified for screening compounds upon intestinal worms (although not upon parasites in the blood or tissues) since intestinal worms live in the lumen of the gut and anthelmintic drugs which have been given by mouth reach the parasites in the intestine without much opportunity for chemical modification. Of course, no single chemotherapeutic test can be guaranteed to detect 100% of active compounds, but as a compromise between expense and efficiency the present test seems good. The method is applicable to other parasites of the intestine and it is planned to apply it to some of the nematode worms. Preliminary experiments have shown that *Nippostrongylus muris* lives well for many days in simple media like Ringer-glucose plus antibiotics, and that the helminthicidal action of drugs upon it can readily be tested. When compounds have been selected by this test as having a high degree of activity compared with their probable toxicity, further investigation is of course needed by *in vivo* tests in mice infected with *Hymenolepis* and by detailed studies of oral toxicity.

Results of Testing.—The most active compounds, which kill in our test at a concentration of 1/million, include oil of chenopodium (1 : 10 million), extract of cashew nut, dichlorophen, antimony potassium tartrate and BIQ 20. Of these, oil of chenopodium is well known clinically for its action against ancylostomes and dichlorophen is used successfully for the treatment of tapeworms in dogs. Extracts of cashew nut have been tested clinically for anthelmintic action by Bhaduri, Chakravarti, Bandyopadhyay, Roy, and Arora (1958) with encouraging results ; they have also given further references. Antimony potassium tartrate is too toxic for this kind of therapy, and BIQ 20 is a new compound, synthesized by the workers of Allen & Hanburys and found to possess activity against the filarial worm *Litomosoides carinii* (Hawking and Terry, 1959) ; it will receive further investigation. The

moderately active compounds which kill at a concentration of 1/100,000 include:

male fern extract, hexylresorcinol and mepacrine	which are well-known remedies for human tapeworms.
arecoline	which has been used extensively for tapeworms in dogs, and which is an active depressant of the musculature of cestodes (Batham, 1946; Duguid and Heathcote, 1950a and b).
chloroquine	this is worth further investigation as a drug against tapeworms. It has been reported by Camero (1951) to be effective in removing *Taenia saginata* (7 cases described).
pentaquine and pamaquin	these are also worth further investigation, although their toxicity is greater than that of mepacrine and chloroquine, which would be a disadvantage.
iodochlorhydroxyquin	this would be worth investigating whether it acts on tapeworms as well as on amoebae.
acriflavine	this would presumably be too irritating and too toxic.

Tetrachlorethylene and carbon tetrachloride are difficult to test *in vitro* because of their insolubility and they gave irregular results, the minimum lethal concentrations varying from 1:1,000 to 1:100,000 in different experiments; but when tested *in vivo* tetrachlorethylene given in low dosage seemed moderately active. This is clearly one of the cases in which the *in vitro* test yields equivocal results and further investigation by *in vivo* tests is necessary.

The remaining compounds tested are inactive. In the case of phenothiazine, the inactivity may be due to the insolubility of the compound in our medium. Santonin, although clinically effective against *Ascaris*, is not effective in expelling tapeworms. The other compounds would not be expected to be active upon cestodes. On the whole, the results from our *in vitro* test are in agreement with those obtained by clinical experience.

Grateful acknowledgments are due to Dr. B. Mukerji for encouragement and for facilities for this work; to Dr. O. D. Standen and Mr. J. E. D. Keeling, Wellcome Laboratories of Tropical Medicine, London, N.W.1, for the strain of *Hymenolepis*; to Allen & Hanburys for compound BIQ 20; to Mr. Ram Govind for the extract of *Anacardium*; and to Mr. A. Dutta for technical assistance.

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Brit. J. Pharmacol. (1960), **15**, 440.

PHARMACOLOGICAL ACTIVITY OF AN AQUEOUS EXTRACT OF THE LEAVES OF THE MALAYAN RENGAS TREE *GLUTA RENGHAS*

BY

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(RECEIVED APRIL 6, 1960)

A pharmacological analysis of an aqueous extract of the leaves of *Gluta renghas* has been carried out. It has been shown that the extract contained a heat-stable, anticholinesterase agent. A heart-stimulating substance was also shown to be present in the extract. Intravenous injection of the extract into the anaesthetized cat produced changes of arterial blood pressure, venous pressure and respiration which were reduced or abolished by vagotomy. The leaves contained approximately 0·1 μg./g. of 5-hydroxytryptamine-like activity.

"Rengas" is the name given by Malays to forest trees which contain a highly vesicant sap. The trees have been classified into five genera, all belonging to the family Anacardiaceae (Corner, 1940). The commonest Malayan variety is *Gluta renghas* and the vesicant property of the latex contained in its seeds has been attributed to the presence of a monoethenoid alkyl catechol, glutarenghol (Backer and Haack, 1941). In addition, the fruit of this tree has been claimed to cause severe gastro-intestinal irritation if eaten by man (Burkill, 1935). Aqueous extracts of the bark are reported to be lethal if swallowed and the exudate from the leaves and stems has been used as a poison in Malaya and Indonesia, but it is not known if any of these effects are also brought about by glutarenghol. The present experiments show that aqueous extracts of the leaves of *Gluta renghas* possess a number of pharmacological actions although, unlike the undiluted sap, they are not vesicant.

METHODS

Preparation of the Leaf Extract

Gluta renghas is a tall, evergreen forest tree with glabrous leaves and a white sap which darkens rapidly on exposure to air and light. The extract was prepared from freshly collected leaves at various stages of maturity (Fig. 1); these were decimated in a Kenmix and then weighed. The finely divided leaves were macerated with acid-washed sand and distilled water in a mortar. The resulting suspension was squeezed through several layers of cotton gauze to remove coarse particles and centrifuged at 3,000 r.p.m. for 30 min. The supernatant fluid was acid (*p*H 5.0 to 5.5) and before use the *p*H was adjusted to 7.4 with N/3 sodium hydroxide. The colour of the extract, which was stored at 4°, changed gradually from pale yellow to dark red-brown, and for this reason only extracts which were 48 hr. old, or less, were used in all experiments, unless otherwise stated.

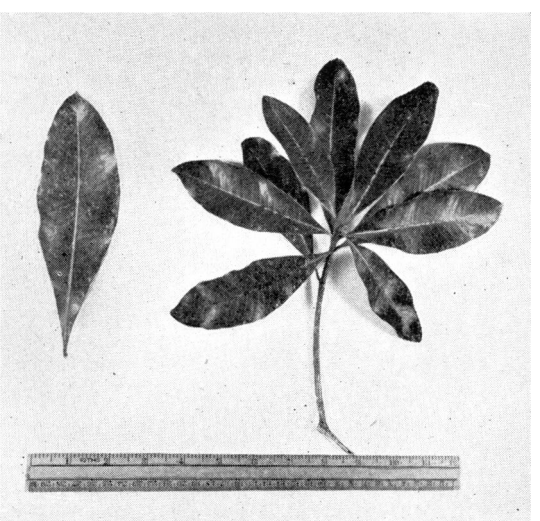

FIG. 1.—A single leaf (a) and a spray of leaves (b) of *Gluta renghas*.

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In some experiments a decoction was made by boiling the weighed, decimated leaves with distilled water for about 2 hr. The suspension was then filtered, centrifuged and neutralized in the manner described for the extract.

Dialysis of the extract or decoction was carried out, when required, by suspending about 50 ml. in a cellophane bag around which a stream of distilled water was passed for 48 hr. The dialysis bag had an average pore diameter of 48×10^{-7} mm. and it was freely permeable to trypan blue (mol. wt. 960).

Pharmacological Tests

Guinea-pig Ileum.—Freshly isolated segments of guinea-pig ileum were suspended in a 5 ml. bath filled with Tyrode solution at 37°. The solution was aerated with either a gas mixture of 95% oxygen and 5% carbon dioxide or air.

Rat Colon.—Segments of ascending rat colon were set up in a bath of 8 ml. Locke solution containing 1/8th of the usual amount of calcium chloride, at room temperature (28 to 30°). Atropine sulphate, sufficient to abolish the response of the colon to acetylcholine, was added to the bath before each addition of extract.

Rat Stomach.—Strips of muscle from the rat stomach were prepared according to the method described by Vane (1957). Each strip was suspended in an 8 ml. bath filled with Locke solution at 37°. The solution was aerated with air and hyoscine hydrobromide was added to the Locke solution to give a final concentration of 10^{-7}.

Isolated, Perfused Toad Heart.—Toad hearts were perfused through a Symes cannula with Ringer solution at room temperature.

Isolated, Perfused Mammalian Heart.—The hearts of guinea-pigs and rabbits were perfused with McEwen solution (1956), aerated with 95% oxygen and 5% carbon dioxide, at 37°. The solution was passed through a cannula inserted into the aorta and in some experiments the coronary outflow was measured.

Cat Blood Pressure and Respiration.—The effect of intravenous injections of the extract was investigated in cats anaesthetized with pentobarbitone sodium (45 mg./kg.) or chloralose (100 mg./kg.). In some experiments arterial blood pressure was measured by means of an arterial cannula inserted into the left common carotid artery and connected to a mercury manometer. In others, a heparinized polythene catheter filled with 2% sodium citrate solution was inserted into the right femoral artery and the blood pressure was recorded by means of a Statham strain-gauge pressure transducer, and a direct writing, Sanborn, 4-channel, physiological recorder. Central venous pressure was similarly recorded from a catheter threaded down the right external jugular vein, so that its tip lay at or near the junction of the superior vena cava with the right atrium. Lead II electrocardiograms were displayed on another channel of the recorder.

Respiratory movements were recorded either by a tambour and writing lever connected to a tracheal cannula or by a strain-gauge pressure transducer and catheter attached to a balloon inserted into the abdominal cavity.

Estimation of the Anticholinesterase Activity of the Extract.—This was done in two ways and the general procedure for both techniques has been described before (Lin, 1955). (*a*) The leaf extract was incubated at 38° for 1 hr. in a 0.134 M phosphate buffer solution (*p*H 7.2) together with a homogenate of guinea-pig brain and acetylcholine. The acetylcholine remaining at the end of the incubation was assayed on the toad rectus abdominis muscle suspended in eserinized Ringer solution at room temperature and aerated with air. For comparison, eserine salicylate was used in place of the extract. The brain homogenate was prepared by removing the cerebral hemispheres from a freshly killed guinea-pig and homogenizing 5 g. of tissue with 2 ml. phosphate buffer for about 10 min. in a Waring blender. The homogenate was made up to 30 ml. with phosphate buffer and thoroughly mixed. (*b*) The anticholinesterase activity of the extract was estimated manometrically in a Warburg apparatus at 38°. The reaction mixture in each vessel had a total volume of 5.0 ml. The cholinesterase was incubated with the extract for 45 to 60 min. before acetylcholine was added to the reaction mixture and hydrolysis of the acetylcholine was followed for a period of 120 min. The cholinesterase was provided either by a rat brain, homogenized and suspended in 24 ml. of bicarbonate Ringer solution, or by guinea-pig plasma diluted 1 in 10 with bicarbonate Ringer solution.

RESULTS

Effect of the Latex on the Skin

The vesicant properties of the sap were demonstrated on one of the authors by gently rubbing the cut end of a young stem on the forearm skin for approximately 10 sec. An irritant dermatitis resulted which persisted for three weeks and had to be treated clinically. In contrast, the leaf extract had no effect when applied topically to the forearm skin or to the shaved skin of the guinea-pig abdomen.

Pharmacological Actions of the Leaf Extract

Guinea-pig Ileum.—The extract caused a powerful contraction of the longitudinal muscle of the gut. The onset of contraction was delayed and occurred approximately 30 sec. after the extract was added to the bath. The contraction was usually well maintained even after the extract had been washed out, but it was inhibited completely by the addition of atropine to the bath (Fig. 2). After the extract had been washed out of the bath and the contraction had subsided, the response of the gut to acetylcholine was greater than it was

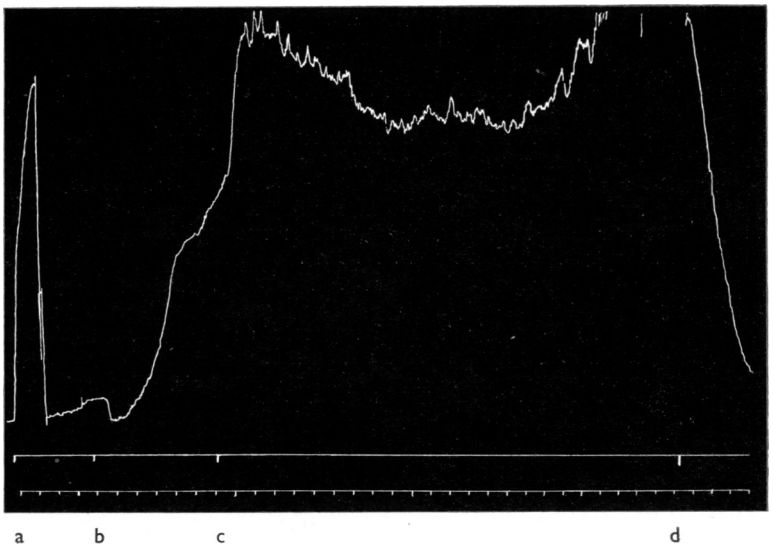

FIG. 2.—Effect of *Gluta renghas* leaf extract on the isolated guinea-pig ileum. At (a) 0·04 μg. acetylcholine and at (d) 2·0 μg. atropine sulphate were added to the bath. The effect of 0·5 ml. leaf extract (100 mg. fresh leaf tissue) is shown at (b), and at (c) the leaf extract was washed out of the bath. Time, 30 sec.

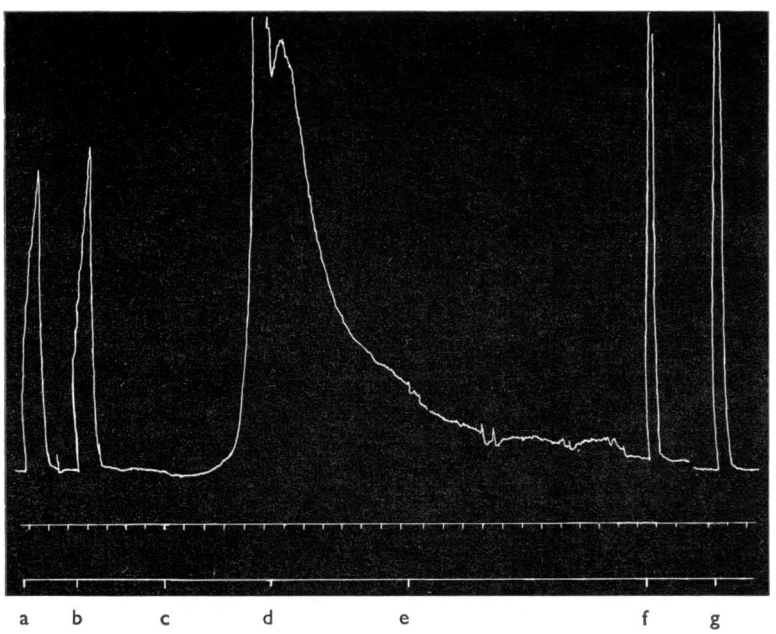

FIG. 3. — Response of the guinea-pig ileum to acetylcholine before and after addition of *Gluta renghas* leaf extract to the bath. Acetylcholine 0·2 μg. was added at (a) and (b), and 0·1 μg. at (f) and (g). At (c) 0·2 ml. extract (100 mg. leaf tissue) was added, and at (d) and (e) the bath was washed out with Tyrode solution. Between (f) and (g) there was an interval of 30 min. Time, 30 sec.

before the addition of the extract (Fig. 3). The agent, present in the extract, which caused contraction of the smooth muscle and which potentiated the effect of acetylcholine was stable to heat. A decoction of *Gluta renghas* leaves produced the same effect as the extract. The activity of the extract was not affected by boiling with N/3 sodium hydroxide for 1 min. and it was not lost during dialysis against distilled water for 48 hr.

Rat Colon.—Both an extract and a decoction of *Gluta renghas* leaves brought about a contraction of the atropinized rat colon and this response was reduced or abolished by the addition to the bath of (+)-2-bromolysergic acid diethylamide or methylergometrine tartrate (Methergin) in doses which abolished the response of the gut to 5-hydroxytryptamine (Fig. 4). The dialysed extract produced relaxation of the colon.

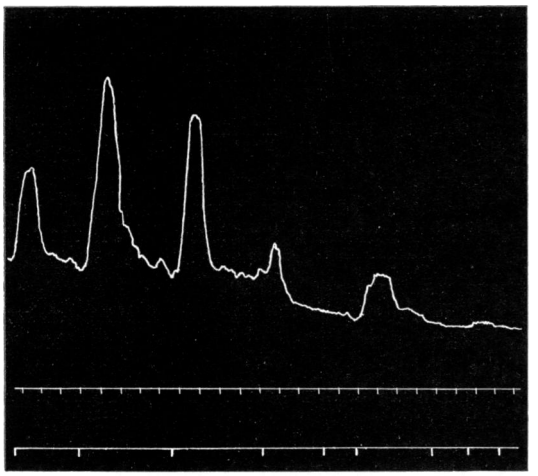

FIG. 4.—Contractions of the rat colon on the addition to the bath of the following: at (a) 0·2 ml. *Gluta renghas* leaf extract (10 mg. fresh leaves); at (b) 2·0 ng. 5-hydroxytryptamine; at (c) 0·3 ml. leaf extract (15 mg. leaf tissue); at (d) 10 μg. (+)-2-bromolysergic acid diethylamide which remained in the bath until it was washed out at (i); at (e) 2·0 ng. 5-hydroxytryptamine; at (f) 0·3 ml. leaf extract; at (g) 0·2 μg. atropine sulphate which remained in the bath until (i); at (h) 0·3 ml. leaf extract. Time, 1 min.

Rat Stomach.—The extract caused a contraction of the rat-stomach muscle, but this effect was not seen after the addition of 20 μg. methylergometrine to the bath. When assayed on the rat stomach preparation 1 g. leaf tissue was found to contain the mean equivalent activity of 110 ng. 5-hydroxytryptamine.

Toad Heart.—The experiment of Fig. 5 shows that 40 mg. leaf tissue had a marked stimulant action on the isolated toad heart. This effect could also be shown in the atropinized heart. The heart-stimulating substance was heat stable and resistant to the action of acid and alkali. It slowly passed through the wall of the dialysis bag into the surrounding water, but an extract which had been dialysed until it no longer caused contraction of the rat colon still stimulated the toad heart. Amounts of extract equivalent to as little as 4 mg. leaf tissue produced a large increase in the amplitude of the heart immediately after relatively large doses of 5-hydroxytryptamine (>5 μg.) had been shown to have no effect on the preparation. The action of the extract on the heart was not altered by the addition of methylergometrine to the perfusate either immediately before, or together with, the injection of the extract. Repeated, large doses of the extract (40 to 80 mg. leaf tissue) brought about a progressive increase in the tone of the heart.

Mammalian Heart.—Injection of the extract into the cannula of the isolated perfused guinea-pig or rabbit heart first increased the amplitude of contractions, then reduced it and finally stopped the heart in a state of systolic arrest (Fig. 6). During the period of cardiac stimulation the coronary outflow was increased.

Cat Blood Pressure and Respiration.—Intravenous injection of either the extract or the decoction, dialysed or undialysed, produced apnoea in the expiratory phase, a rise of venous pressure and an initial decrease in the heart rate (Fig. 7). The effect on arterial blood pressure was usually triphasic, consisting of a small rise, followed by a fall and then a rise above the pre-injection level. The secondary rise of arterial blood pressure was accompanied by an increased heart rate. After the injection of atropine, or when the extract was injected for the first time into the atropinized animal, the over-all response was similar but there was no slowing of the heart. When the vagi were sectioned, inhibition of respiration was no longer observed upon injecting the extract or decoction, and the rise of venous pressure was much reduced in magnitude. The response of the arterial blood pressure was either abolished or considerably

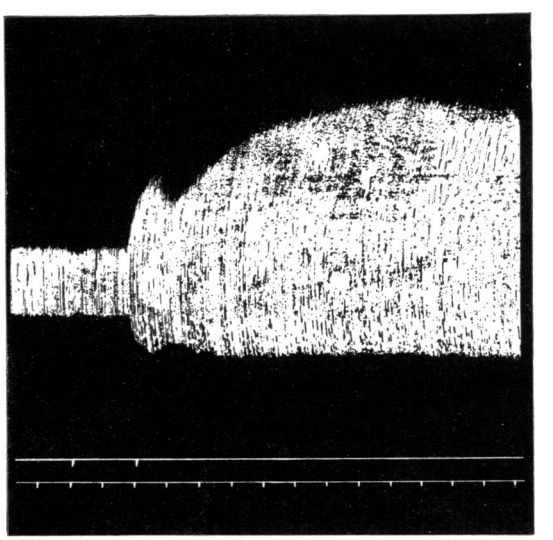

FIG. 5.—Stimulation of the isolated, perfused toad heart by 0·1 ml. *Gluta renghas* leaf extract (40 mg.) injected at (b). The effect of injecting 0·1 ml. Ringer solution into the Symes cannula is shown at (a). Time, 20 sec.

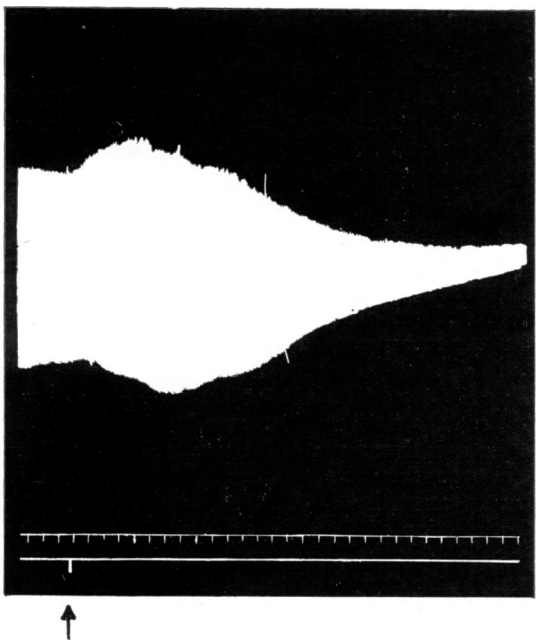

FIG. 6.—Stimulation of the isolated, perfused rabbit heart by 0·1 ml. *Gluta renghas* leaf extract (40 mg. leaf tissue) injected at the arrow. Time, 30 sec.

reduced, but an increased heart rate could still be observed. Injection of the extract into the previously vagotomized animal, which had not received any prior treatment with extract, produced a response similar to that shown in Fig. 7e. A dose slightly larger than that required to produce the apnoeic and depressor responses caused death from respiratory and circulatory failure in the anaesthetized animal with the vagi intact. Successive injections of the extract caused progressively greater disturbances of the rhythm of the heart beat and in the vagotomized animal the deterioration of the condition of the heart led to the death of the animal. The electrocardiogram showed that the disturbances of the heart rhythm consisted of periods of ventricular extrasystoles alternating with periods of complete cardiac arrest for about 6 sec. at a time.

Rabbit Eye.—No effect could be demonstrated on the diameter of the rabbit pupil when 1.0 ml. of the extract (750 mg. fresh leaves/ml.) was dropped into the eye, and the extract did not produce any lesion of the corneal or conjunctival surface.

Tachyphylaxis.—Tachyphylaxis was encountered with the guinea-pig ileum preparation, and on some occasions only a single response to the extract could be elicited from any one piece of gut. Re-

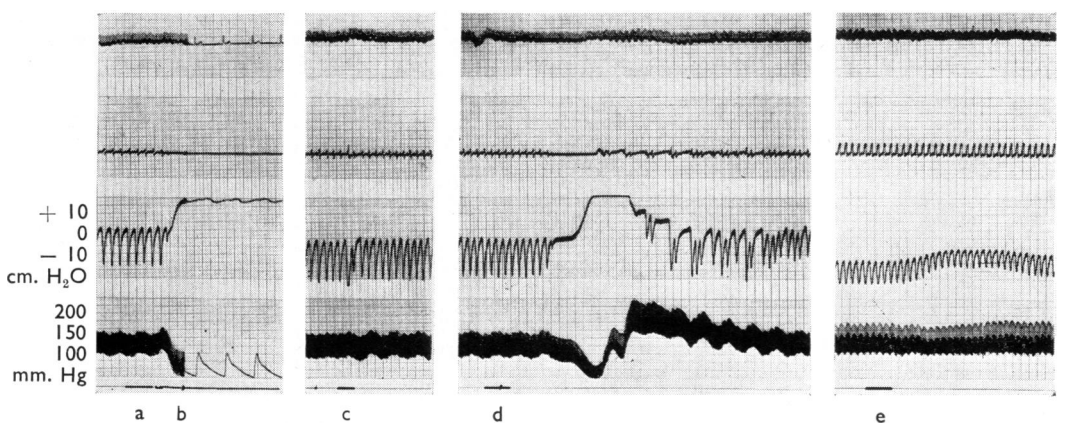

FIG. 7.—Cat, 3 kg., anaesthetized with chloralose. Records, from above downwards: electrocardiogram (lead II), intra-abdominal pressure (respiratory variations; inspiration ↑), central venous pressure, arterial blood pressure and signal/time-marker (1 sec.). Intravenous injection of 1·0 ml. *Gluta renghas* leaf extract (500 mg. fresh leaves) at (a), (d) and (e). At (b) the paper speed of the recorder was temporarily increased from 1 mm./sec. to 25 mm./sec. to show the electrocardiogram, and at (c) 1·0 μg. acetylcholine was injected intravenously. Between (b) and (c) 1·0 mg. atropine sulphate was injected intravenously and between (d) and (e) the vagi were cut in the neck.

peated injections of the extract at short intervals also produced tachyphylaxis of the blood-pressure and respiratory responses of the anaesthetized cat.

Pharmacological Activity of Stem Extracts

Extracts of young, green stems (30 mg. tissue) and also of old woody stems (50 mg. tissue) caused the isolated guinea-pig ileum to contract and this contraction was inhibited by the addition of atropine to the bath. The response of the gut to acetylcholine was enhanced by the extract. Stem extracts (170 mg./ml. stem tissue) also contracted the rat colon and the contraction was abolished by (+)-2-bromolysergic acid diethylamide.

Anticholinesterase Activity of the Leaf Extract

Effect of Incubation of Acetylcholine with Guinea-pig Brain Cholinesterase in the Presence of the Extract.—The experiments carried out on the isolated guinea-pig ileum suggested that the leaf extract might possess anticholinesterase activity. Table I presents the results of one of five experiments which were designed to test this. The Table shows that 1.0 g. leaf tissue was approximately half as effective in preventing hydrolysis of acetylcholine as was 250 μg. eserine salicylate; 250 mg. leaf tissue conferred no measurable protection against hydrolysis, and in other experiments 500 mg. leaf tissue was also without effect.

Manometric Estimation of the Anticholinesterase Activity of the Leaf Extract.—Five experiments were carried out using the Warburg apparatus, and in all instances inhibition of brain-cholinesterase activity was produced by the extract. An example of the results obtained is given in Table II, which shows that 818 mg. leaf tissue had almost the same inhibitory effect as 250 μg. eserine salicylate and that 409 mg. caused only slightly less inhibition (93% as opposed to 99%) than did 818 mg. leaf tissue. Similar results were obtained when a decoction of leaves was used in place of the extract.

TABLE I

INHIBITORY EFFECT OF *GLUTA RENGHAS* LEAF EXTRACT ON THE HYDROLYSIS OF ACETYLCHOLINE BY GUINEA-PIG BRAIN CHOLINESTERASE

Flask Contents	Flask No.							
	1	2	3	4	5	6	7	8
Guinea-pig brain homogenate, 1·0 ml.	+	+	+	+	+	+	+	+
Phosphate buffer, 2·0 ml.	+	+	+	+	+	+	+	+
Acetylcholine (50 μg.), 1·0 ml. of 0·005% solution	+	+	+	+	+	+	+	+
Eserine salicylate (250 μg.), 0·05 ml. of 0·5% solution	0	0	+	+	0	0	0	0
Gluta renghas leaf extract (250 mg./ml.), ml.	0	0	0	0	4	4	1	1
Distilled water, ml.	4·0	4·0	3·95	3·95	0	0	3·0	3·0
Acetylcholine recovered; estimated by assay on frog rectus abdominis muscle, μg.	<1·0	<1·0	45·0	45·0	25·0	23·0	<1·0	<1·0

TABLE II

RATE OF HYDROLYSIS OF ACETYLCHOLINE BY RAT-BRAIN CHOLINESTERASE IN THE PRESENCE OF *GLUTA RENGHAS* LEAF EXTRACT

Flask Contents	Manometer No.					
	1	2	3	4	5	6
Rat brain homogenate, 0·5 ml.	+	+	+	+	+	+
Bicarbonate buffer, 2·0 ml.	+	+	+	+	+	+
Acetylcholine (5·0 mg.), 0·5 ml. of 1·0% solution	+	+	+	+	+	+
Eserine salicylate (250 μg.), 0·05 ml. of 0·5% solution	0	+	0	0	0	0
Gluta renghas leaf extract (409 mg./ml.), ml.	0	0	1·0	1·0	2·0	2·0
Distilled water, ml.	2·0	1·95	1·0	1·0	·0	0
Rate of release of carbon dioxide, μl./hr.	318·1	7·3	28·0	28·5	11·8	12·1
Inhibition of enzymic hydrolysis, %	0	100	93·3	93·2	98·6	98·5

TABLE III

RATE OF HYDROLYSIS OF ACETYLCHOLINE BY GUINEA-PIG PLASMA CHOLINESTERASE IN THE PRESENCE OF (a) AN EXTRACT AND (b) A DECOCTION OF *GLUTA RENGHAS* LEAVES

Flask Contents	Manometer No.					
	1	2	3	4	5	6
Guinea-pig plasma, 0·5 ml.	+	+	+	+	+	+
Bicarbonate buffer, 2·0 ml.	+	+	+	+	+	+
Acetylcholine (30 mg.), 0·5 ml. of 6·0% solution	+	+	+	+	+	+
Eserine salicylate (250 μg.), 0·05 ml. of 0·5% solution ..	0	+	0	0	0	0
(a) Extract (400 mg./ml.), 2·0 ml.	0	0	0	0	+	+
(b) Decoction (350 mg./ml.), 2·0 ml.	0	0	+	+	0	0
Distilled water, ml.	2·0	1·95	0	0	0	0
Rate of release of carbon dioxide, μl./hr.	128·7	22·2	40·8	43·5	23·7	24·3
Inhibition of enzymic hydrolysis, %..	0	100	82·5	80·0	98·6	98·0

In three separate experiments guinea-pig plasma was used as the source of cholinesterase and the results of one of these experiments are shown in Table III. In all three experiments the rate of hydrolysis of acetylcholine by plasma was reduced by both the extract and the decoction of leaves. In the example given (Table III) the decoction was prepared from dried leaves which had been kept in the laboratory for one month after their collection from the tree.

The effect of 0.5 ml. of a 2.0% solution of catechol on the rate of hydrolysis of acetylcholine in the presence of rat-brain cholinesterase was tested in two experiments. In one of them catechol reduced the rate of enzymic hydrolysis of acetylcholine by 14%, and in the other experiment by 30%.

DISCUSSION

The evidence collected shows that the leaves of *Gluta renghas* contain an anticholinesterase substance. Aqueous extracts of the leaves reduced the rate of hydrolysis of acetylcholine by both " true " and " pseudo " cholinesterase *in vitro*, and experiments with the isolated guinea-pig ileum suggested that the extract potentiated the action of acetylcholine present in the gut wall, rather than caused a direct contraction of the smooth muscle. The present experiments have not provided any information about the nature of the anticholinesterase agent, other than that it is heat stable, resistant to the action of alkali and not readily dialysable. 409 mg. fresh leaf tissue reduced the rate of enzymic hydrolysis of acetylcholine by 93%, whereas 10 mg. pure catechol produced, at the most, only 30% inhibition of cholinesterase activity. The fresh leaves consisted largely of water (57% by weight) and fibrous tissue, and it is unlikely that as much as 10 mg. of pure catechol could be contained in 409 mg. fresh leaves. However, it is possible that the leaves contain glutarenghol or a related substance and that the alkylated catechols have a greater anticholinesterase activity than catechol itself.

The leaf extract also contained a powerful toad-heart stimulating substance which was resistant to the action of heat, acid, and alkali. The effect of the extract on the heart was not altered by atropinization, which suggests that acetylcholine was not involved in the response. The presence of a similar substance in the Malayan Jack-fruit seed has been reported by Lin (1955), but, in the present experiments, the leaf extract also stimulated the isolated mammalian heart. The increased tone of the toad heart, brought about by the addition of large doses of leaf extract to the perfusate, and the arrest of the mammalian heart after an initial phase of stimulation, by relatively small doses of extract, are actions which resemble those of some cardiac glycosides.

In the anaesthetized, intact animal, the leaf extract produced a fall of arterial blood pressure and also inhibition of respiration when injected intravenously. The afferent pathway for the depressor and apnoeic responses appeared to be the vagi, and in this respect the effects of the leaf extract resemble those produced by veratridine, 5-hydroxytryptamine, the amidines, and a number of other substances (Dawes and Comroe, 1954). The bradycardia which accompanied the fall of arterial blood pressure must have been associated with the release of acetylcholine at vagal nerve endings in the heart because it was abolished by atropine. It seems likely that this latter action

of the extract can be identified with the anti-cholinesterase activity described above, but the alternative possibility, that the bradycardia was brought about reflexly through an increased vagal tone, cannot be excluded by the experiments reported here. Since the depressor response could be demonstrated in the atropinized animal, in which the extract did not cause bradycardia, it could not have been entirely a consequence of the slowing of the heart. The increased heart rate which accompanied the secondary rise of arterial blood pressure and which could be shown to occur after vagotomy can be compared with the stimulation of the isolated mammalian heart by the extract. The disturbances in the electrocardiogram and the general deterioration of the condition of the heart, which caused the death of the vagotomized animal, are also reminiscent of the effect of the extract on the isolated mammalian heart. An interesting feature of the present results has been the rise of central venous pressure which occurred simultaneously with the cardiac slowing, fall of arterial blood pressure, and inhibition of respiration. It seems unlikely that this was a secondary effect due to either slowing of the heart, fall of blood pressure or inhibition of respiration, because Fig. 7 shows that the venous pressure remained high after respiration had started again and when the arterial blood pressure and heart rate had returned to or above their pre-injection levels. It is unlikely also that tachyphylaxis could account for the effects of vagotomy and atropine on the blood pressure, heart rate, and respiratory responses of the anaesthetized animal to the extract, because initial injection of the extract into the atropinized, vagotomized animal did not produce the responses shown in Figs. 7a and 7d.

Experiments with the rat colon and the rat stomach showed that the leaf extract possessed 5-hydroxytryptamine-like activity. It is possible that the 5-hydroxytryptamine-like activity of the extract contributed to its stimulant action on the isolated amphibian heart, although a direct comparison of the effects of the extract with those of 5-hydroxytryptamine, on the same heart preparation, suggested that this was not the case. The fact that methylergometrine did not alter the action of leaf extract on the toad heart, and that dialysed extracts could still stimulate the heart, also speaks against the identification of the heart-stimulating substance with 5-hydroxytryptamine. The participation of 5-hydroxytryptamine in the production of the apnoeic and depressor responses can be excluded since dialysed extracts, which had no effect on the rat colon or rat stomach preparation, were able to elicit these responses when injected intravenously into the anaesthetized cat.

The experiments described here have confirmed that *Gluta renghas* produces a vesicant latex (Backer and Haack, 1941), and it is not unreasonable to suppose that the action of the extract on isolated segments of mammalian gut could account, in part, for earlier claims that the fruit of this tree causes severe gastro-intestinal irritation (Burkill, 1935). The finding that relatively small doses of leaf extract caused the death of the anaesthetized cat from circulatory and respiratory failure is in accord with reports that extracts of the leaves and stems are lethal to man (Burkill, 1935).

We wish to record our gratitude to Professor D. W. Gould, of the Department of Physiology, University of Malaya, for making the electro-manometric measurements of blood pressure and respiration for us. We are also very grateful to Professor H. B. Gilliland, of the Department of Botany, for identifying the tree and for assistance in the collection of material for analysis. Our thanks are also due to Mr. J. Chua, who gave us technical assistance in all experiments.

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THE RESPONSE OF ISOLATED RABBIT ATRIA TO ACONITINE

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The arrhythmias observed in isolated rabbit atria appear to be due to two actions of aconitine. One is an apparent direct stimulant effect on the sino-atrial node and the other is a depressant effect on the processes involved in intra-atrial propagation of impulses. Acetylcholine has either an inhibitory or stimulatory effect on atria pretreated with aconitine because it antagonizes the actions of aconitine both on the sinus node and on conduction. When conduction block is not apparent, acetylcholine depresses the atrial rate. On the other hand, when conduction is markedly depressed, acetylcholine increases the atrial rate.

Atrial flutter and fibrillation can be produced *in vivo* by the topical application of aconitine to the atria of the dog. It is believed that these arrhythmias are due to the rapid discharge of an ectopic focus of activity (Scherf, Romano, and Terranova, 1948; Prinzmetal, Corday, Brill, Oblath, and Kruger, 1952).

Acetylcholine is known to favour the establishment of atrial fibrillation after the local application of aconitine to atrial tissue. For instance, Scherf *et al.* (1948) showed that vagal stimulation converts aconitine-induced flutter to fibrillation, and Burn, Vaughan Williams, and Walker (1956) found that it was necessary to add acetylcholine to the blood to produce fibrillation with aconitine in the dog heart-lung preparation.

This study was undertaken to define the nature of atrial arrhythmias induced by aconitine in isolated mammalian atria and to determine the effects of acetylcholine on the response. A preliminary report of these results was made at the meeting of the American Society for Pharmacology and Experimental Therapeutics, Philadelphia, Pennsylvania, April, 1958.

METHODS

Male rabbits of the New Zealand strain were used. Immediately after stunning an animal by a blow on the back of the head, the thorax was opened and the entire heart removed and placed in oxygenated nutrient fluid at room temperature. The atria were carefully dissected from the ventricles by cutting along the atrio-ventricular junction and through the inter-auricular septum, and then were cleared of adjacent tissue and suspended vertically in a muscle chamber which was located in a larger thermostatically controlled water bath. The tip of the left atrium was anchored to the tissue holder, and the tip of the right atrium was attached to a silk thread leading to a force displacement transducer. The diastolic tension was arbitrarily set at 1 g. Action potentials were obtained with two unipolar silver electrodes. One electrode was placed on the right atrial appendage, another on the left atrial appendage, and an indifferent electrode was placed in the bath remote from the atria. The action potentials and isometric contractions were recorded on a four-channel Sanborn Polyviso Recorder. The working volume of the muscle chamber was 100 ml.

The composition of the tissue medium in mmol./l. was as follows: NaCl, 119.7; KCl, 5.6; $MgCl_2$, 2.1; $CaCl_2$, 2.1; $NaHCO_3$, 17.0; and glucose 9.0. The *p*H of this fluid aerated with 95% oxygen and 5% carbon dioxide at 37.5° was 7.4.

Solutions of aconitine were prepared by dissolving 5 mg. of pure aconitine crystals (S. B. Penick and Co., New York, N.Y.) in 50 ml. of distilled water containing one drop of N hydrochloric acid. Since aconitine solutions are unstable, fresh solutions were made on the day of each experiment.

RESULTS

General Response to Aconitine

Control experiments carried out for periods of from 5 to 6 hr. showed that the spontaneous rate of the isolated atria remained the same or showed a slight progressive decrease, and that there was a one-to-one relationship between the action potentials and the isometric contractions throughout the test period (Fig. 1). When a basis for comparison had been established, aconitine was added in various concentrations. In a concentration of 0.01 γ/ml. aconitine had no effect on the atria in 6 tests. With

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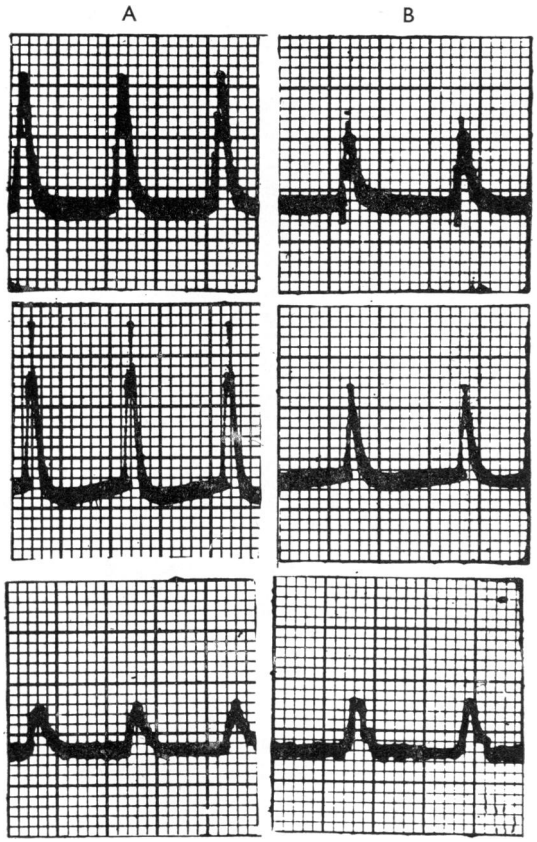

FIG. 1.—*Recordings from an Untreated Spontaneously Beating Isolated Rabbit Atria.* Top tracing, electrograms from the right atrial appendage; middle tracing, electrograms from the left atrial appendage; bottom tracing, mechanograms. (Paper speed—25 mm./sec.). (A) Record obtained at the beginning of the observation period; (B) record obtained at the end of 5 hr.; (B) shows a decrease in the spontaneous rate and in the size of the action potentials.

higher concentrations of aconitine, a triphasic response was produced. The first phase usually consisted of a progressive acceleration of the spontaneous rate. Intermittent bouts of tachycardia were occasionally observed before the maximal rate was reached. The second phase consisted of (*a*) an overall slowing in the rate, (*b*) intermittent periods of rapid and slow activity, (*c*) a loss of a one-to-one relationship between the action potentials recorded from each atrium, and between the action potentials and the contractions, (*d*) variations in the amplitude of the contractions, and (*e*) variations in the amplitude and shape of the action potentials. The onset of the third phase was manifested by apparent electrical and mechanical arrest of the atria. Four atria were observed for 40 min. after activity stopped. Two atria remained quiescent throughout this period, while occasional transient bursts of electrical activity were recorded from the right atrial appendage in the other two.

The effects of aconitine on the atria were reversed by numerous replacements of the nutrient fluid.

Aconitine (0.04 μg./ml.) produced only changes described as the first phase. When the concentration was increased to 0.16 μg./ml. the first and second phases were always seen and in 2 of 4 experiments the third phase was also observed. A concentration of 1 μg./ml. always produced a triphasic response (Fig. 2). The results are summarized in Table I.

Site of Action of Aconitine

A second series of experiments was carried out to determine whether aconitine acts during the initial phase by establishing an ectopic pacemaker, or by an action involving the sino-atrial node. To distinguish between these possibilities, experiments were conducted on isolated left atria, which did not beat spontaneously but responded to electrical stimuli. When stimulation was discontinued, the

TABLE I

EFFECTS OF GRADED DOSES OF ACONITINE ON ISOLATED RABBIT ATRIA

To estimate onset the aconitine response was considered to have begun when the atrial rate was increased by at least 20 beats/min.

No. of Atria	Aconitine μg./ml.	Control Rate Beats/Min.		Incidence of Response	Onset of Response Min. Range	Max. Rate Beats/Min.		Incidence of Arrest	Time for Arrest—Min.	
		Mean	Range			Mean	Range		Mean	Range
6	0·01	140	128–160	0/6				0/6		
4	0·02	130	120–140	2/4	5–10	340	320–360	0/4		
5	0·04	126	120–140	5/5	4–35	288	210–380	0/5		
4	0·16	145	120–160	4/4	2–10	365	340–420	2/4	50	40–60
5	1·0	135	120–160	5/5	1–5	360	300–480	5/5	36	20–52

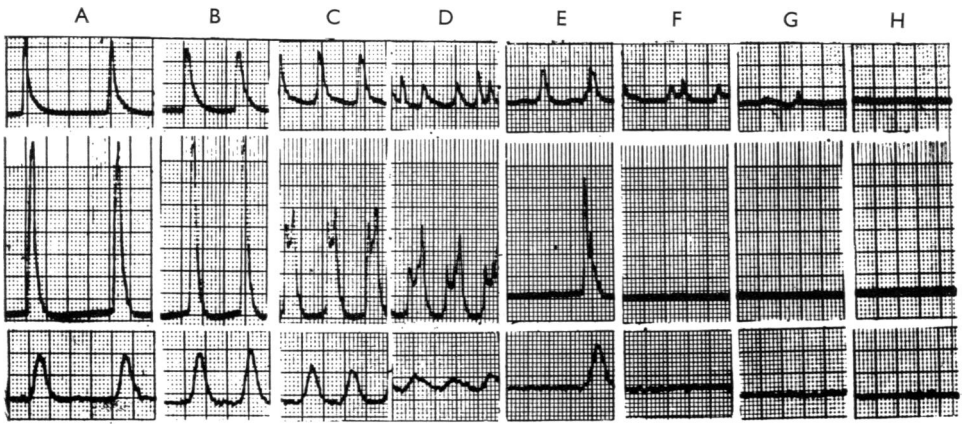

FIG. 2.—*Response of Isolated Rabbit Atria to Aconitine.* Top tracing, electrograms from the right atrial appendage; middle tracing, electrograms from the left atrial appendage; bottom tracing, mechanograms. (Paper speed—50 mm./sec.). (A) Control; (B, C) records taken 2 and 4 min. after the addition of aconitine (1.0 μg./ml.) show the tachycardia characteristic of the first phase of the aconitine response; (D, E, F, G) records taken 6, 8, 10, and 15 min. after aconitine show interference with the propagation of action potential which characterizes the second phase of the aconitine response; (H) shows electrical and mechanical arrest of the atria characteristic of the third phase.

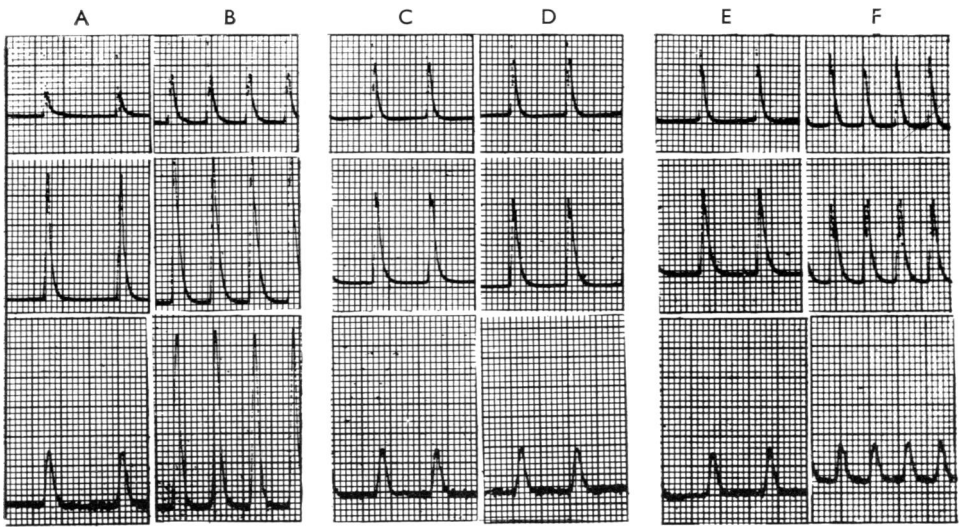

FIG. 3.—*The Effect of Hexamethonium on Aconitine-induced Atrial Tachycardia.* Top tracing, electrograms from the right atrial appendage; middle tracing, electrograms from the left atrial appendage; bottom tracing, mechanograms. (Paper speed—25 mm./sec.). (A) Control; (B) tachycardia induced by nicotine (9 μg./ml.); (C) record taken 10 min. after the removal of nicotine and the addition of hexamethonium chloride (0.06 mg./ml.) ; at this time, nicotine (9 μg./ml.) was again added to the bath; (D) block of the nicotine response by hexamethonium; (E) record taken after 9 washes and the subsequent addition of hexamethonium chloride (0.06 mg./ml.) ; at this time aconitine (0.04 mg./ml.) was added to the bath; (F) record taken 15 min. later shows hexamethonium does not block the tachycardia induced by aconitine.

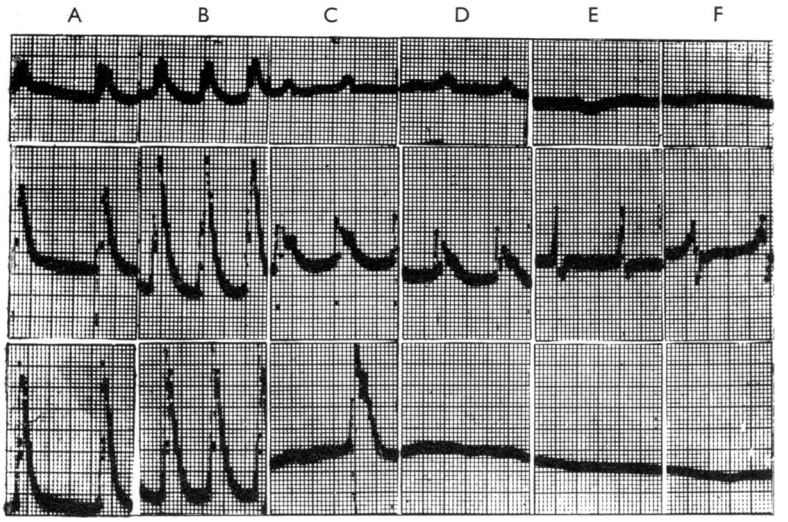

FIG. 4.—*Persistence of Electrical Activity in Atria Exposed to Aconitine.* Top tracing, electrograms from the right atrial appendage; middle tracing, electrograms from tissue in relatively close proximity to the sinus node; bottom tracing, electrograms from the left atrial appendage. (Paper speed—25 mm./sec.). (A) Control; (B) shows aconitine-induced tachycardia; (C, D, E, F) show the persistence of electrical activity in tissue near the sinus node and the progressive failure of the action potentials to be propagated to the appendages.

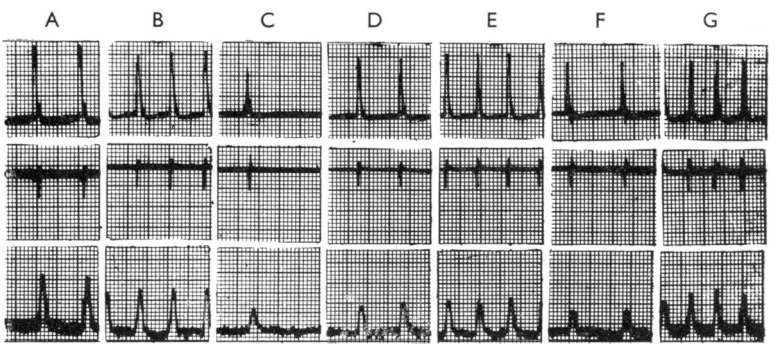

FIG. 5.—*Effects of Acetylcholine in Aconitine-induced Tachycardia.* Top tracing, electrograms from the right atrial appendage; middle tracing, electrograms from the left atrial appendage; bottom tracing, mechanograms. (Paper speed—25 mm./sec.). (A) Control; (B) tachycardia induced by aconitine; (C) shows the slowing of the rate immediately after the addition of acetylcholine (0.5 μg./ml.); (D) shows partial recovery of the rate 15 min. after acetylcholine was added; (E) shows complete recovery of the rate 20 min. after acetylcholine; (F) shows slowing of the rate after a subsequent addition of acetylcholine (0.5 μg./ml.); (G) shows recovery of the rate 5 min. after acetylcholine was added.

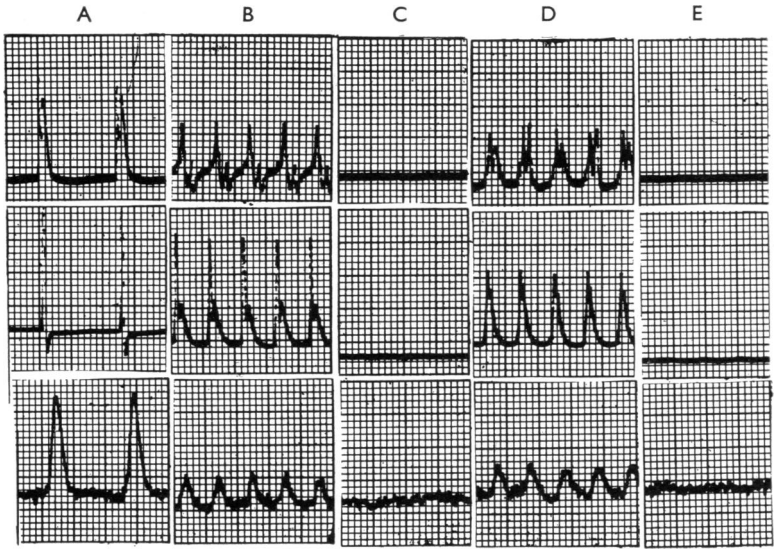

FIG. 6.—*The Effects of Acetylcholine in Atria Arrested by Aconitine.* Top tracing, electrograms from the right atrial appendage; middle tracing, electrograms from the left atrial appendage; bottom tracing, mechanograms. (Paper speed—25 mm./sec.). (A) Control; (B) shows aconitine-induced tachycardia; (C) shows arrest of the atria; (D) shows the immediate restarting of the atria by acetylcholine (0.05 μg./ml.); (E) shows arrest of the atria after a single replacement of the bath fluid.

atria stopped contracting. Aconitine (0.04 μg./ml.) produced no detectable electrical or mechanical activity in these atria during a 1 hr. observation period. At the end of the hr. the nutrient fluid was replaced, and after 5 min. the concentration of aconitine in the bath was increased to 1.0 γ/ml. Even so, no activity was observed in the next 25 to 30 min. The results suggest that aconitine was not establishing an ectopic pacemaker and so was affecting the sino-atrial node directly or indirectly.

It was possible that aconitine accelerated the atria by stimulation of sympathetic ganglia or atrial chromaffin tissue. The presence of sympathetic ganglia or chromaffin tissue in rabbit atria has been demonstrated by Kottegoda (1953), who reported that nicotine produced an increase in the rate of isolated atria which could be abolished by ganglionic blocking agents. It was found in 5 experiments that hexamethonium chloride (0.06 mg./ml.) or tetraethylammonium chloride (0.3 mg./ml.), which were shown in each test to block the tachycardia induced by nicotine (9 μg./ml.), did not modify the atrial tachycardia induced by aconitine. The results of one of these experiments are shown in Fig. 3.

It can be seen in Fig. 2 that conduction is impaired during the second phase of the aconitine response. The inability of the atria to conduct impulses well was found to be the cause of the relative inactivity of the atria during the third phase. This was determined in 4 experiments in which action potentials were recorded from each atrial appendage and from tissue in close proximity to the sino-atrial node. Fig. 4 shows that action potentials were recorded from the region of the node when no activity was recorded from the appendages.

The Effects of Acetylcholine on the Aconitine Response

During the first phase of the aconitine response, when the spontaneous rate was greater, acetylcholine (0.5 to 2.0 μg./ml.) depressed, for varying periods of time, the fast auricular rates induced by aconitine. A subsequent addition of acetylcholine was not as effective in depressing the rate and the effect did not last as long (Fig. 5). This antagonism by acetylcholine was seen when the concentration of aconitine in the bath was 0.04 or 1.0 μg./ml. Acetylcholine (0.05 to 2.0 μg./ml.) also antagonizes the depressant effects of aconitine on conduction. This can be seen in Fig. 6. In most instances, restarting of the atria was characterized by chaotic electrical activity and irregular contractions.

Physostigmine sulphate (0.05 μg./ml.) added during the third phase of the aconitine response

produced after a latent period of 4 to 15 min. the same effect as added acetylcholine. The latent period was presumably the time required to build up an effective concentration of acetylcholine. Atropine sulphate (0.1 to 0.2 μg./ml.) abolished the effects of acetylcholine or physostigmine. The results indicate that atropine can block the excitatory effects of endogenous acetylcholine in atria pretreated with aconitine. Webb (1950) showed that the depressant effect of endogenous acetylcholine could also be blocked by atropine; it was therefore assumed that pretreating atria with atropine would reveal whether or not the acetylcholine normally present in the atria plays a role in the genesis or maintenance of the aconitine response. In tests on 6 auricles, aconitine (0.04 to 1.0 μg./ml.) was added to the bath 10 min. after the addition of atropine (0.1 to 0.2 μg./ml.). The aconitine response was not modified. These experiments strongly suggest that the acetylcholine normally present in isolated atrial tissue is not at all concerned with the response of rabbit atria to aconitine.

Discussion

The experimental evidence obtained in this study indicates that aconitine-induced arrhythmias in isolated rabbit atria are due to stimulation of the sinus node and to interference with the propagation of impulses. The tachycardia observed after aconitine is attributed to an effect on the pacemaker, since isolated left atria which are presumably devoid of sinus nodal tissue do not respond to aconitine in concentrations that stimulate the paired atria. Furthermore, this effect on the pacemaker appears to be a direct action and not due to a nicotine-like stimulation of sympathetic ganglia of chromaffin tissue, since the response to aconitine is unaltered by ganglionic blocking drugs.

The depressant effects of aconitine on conduction are shown by the loss of a one-to-one relationship between the action potentials recorded from each atrial appendage and by the persistence of electrical activity in tissue in close proximity to the sinus node at a time when action potentials cannot be recorded from either appendage. This effect on conduction occurs with higher doses of aconitine than are required to cause atrial tachycardia and can be reversed by washing out the aconitine or by acetylcholine. The ability of acetylcholine to facilitate intra-atrial conduction when intra-atrial block is present has long been recognized (Lewis and Drury, 1923; Drury, 1925–26).

When atria depressed by aconitine were reactivated by acetylcholine, the electrical activity and contractions were irregular. It is evident that aconitine has a disproportionate depressant effect on the ability of atrial cells to conduct impulses, as shown by notching of the action potentials and the appearance of action potentials from one part of the atria when they are not being recorded from another part. Therefore, the arrested myocardium can be considered to be comprised of cells which have been depressed to varying degrees by aconitine. It would be expected that myocardial cells so depressed would vary at any one instance in their ability to propagate an impulse after the addition of acetylcholine. Under these conditions, re-entry of impulses is favoured. Furthermore, because of the marked acceleration of the repolarization process due to acetylcholine (Brooks, Hoffman, Suckling, and Orias, 1955; Hoffman and Sucking, 1953), the myocardial units would be capable of responding to greater rates of stimulation, therefore electrograms consisting of irregular action potentials could be expected.

This investigation was supported in part by a research grant from the U.S. Public Health Service and in part by a grant from the Schering Corporation, Bloomfield, New Jersey.

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ANAESTHESIA IN NEW-BORN ANIMALS

BY

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(RECEIVED APRIL 20, 1960)

Pentobarbitone was more toxic to new-born than to adult rabbits and rats, produced a longer loss of righting reflex in new-born animals but did not anaesthetize them effectively in less than toxic doses. Urethane did not anaesthetize new-born animals in doses which anaesthetized adults. Ether produced loss of righting reflex at lower concentrations for new-born than for adults, but the new-born animals became anaesthetized more slowly.

The responses of new-born animals to various drugs are known to differ from those of adults of the same species (Reiss and Haurowitz, 1929 ; Eddy, 1939 ; Chesler, Labelle and Himwich, 1942 ; Chen and Robbins, 1944) and it is not surprising, therefore, to find that they differ from adults in their responses to anaesthetic drugs. An appreciation of these differences is of importance to those working with new-born animals.

Homburger, Etsten and Himwich (1947) found that doses of pentobarbitone (40 to 50 mg./kg.), which killed rats less than 24 hr. old, produced a loss of righting reflex for only a few hours in adult rats. The sleeping time of new-born mice (Jondorf, Maickel and Brodie, 1959) which had received hexobarbitone was much longer than that of adult mice which had received the same dose. Fazekas, Alexander and Himwich (1941) found that new-born rats took longer to die than adults when breathing nitrous oxide or cyclopropane, although the time of arrest of respiration when breathing air containing an unstated concentration of chloroform vapour did not differ between newborn mice and mice older than 18 days (Reiss and Haurowitz, 1929).

While much of the evidence quoted above refers to the lethal effects of anaesthetic drugs, this paper is concerned with a comparison of some non-lethal effects of urethane, pentobarbitone and ether in new-born and adult rats and rabbits.

METHODS

Animals were exposed to ether in a 30 l. observation chamber at a controlled environmental temperature. Air was blown through the chamber at 6 l./min. by a Starling Ideal pump (C. F. Palmer). Ether vapour was added by passing the air through a calibrated E.M.O. Inhaler (Pentland Instrument) as described by Epstein and Macintosh (1956). The lower part of the inhaler was immersed in a water bath at 20°. With a flow of 6 l./min., the concentration of ether in the chamber could be changed by about 97% in 10 min., and this time was always allowed for equilibration after each change of ether concentration. All animals were kept in a warm chamber (30 to 35°) for not less than 30 min. before transfer to the observation chamber for exposure to ether. They were exposed to ether, either singly or in batches, and were not restrained. The chamber was closed by a perspex lid in which there were two holes 4 in. in diameter. One was covered by a soft rubber sheet with a slit, through which animals could be introduced or removed from the chamber. The rubber sheet minimized mixing of atmospheric air with the contents of the chamber. When not in use this hole was closed by a perspex shutter. The second hole had a long-sleeved rubber glove attached to its margin so that, when an animal began to stagger, the operator, with the hand inside the glove, could turn it on its back. The time was recorded at which the animal lost its righting reflex. The animal was regarded as being anaesthetized when it no longer responded to pinching of the hind paw.

Sleeping time was the interval during which the righting reflex was absent. Sleeping animals were kept at constant temperature. Deaths were recorded at 24 hr.

Sodium pentobarbitone B.P. (Abbott) was dissolved in distilled water not more than 2 hr. before use. Urethane (ethyl carbamate: M.P. 48 to 50°) solutions were prepared from B.D.H. laboratory reagent in distilled water. Injections of pentobarbitone or urethane were given intraperitoneally with the dose per kg. contained in 10 ml. fluid.

RESULTS

Pentobarbitone

Preliminary experiments are summarized in Table I, which shows that pentobarbitone was more toxic to new-born rabbits and rats than to adults. For example, a dose of 35 mg./kg. of pentobarbitone commonly given to adult rabbits to produce

light anaesthesia of about 1 hr. with subsequent recovery killed 5 of 6 rabbits less than 1 day old (Table I). These animals did not become anaesthetized until they breathed less than twice per minute and were cyanosed.

TABLE I

TOXICITY OF PENTOBARBITONE TO DAY-OLD RABBITS AND RATS

The drug was given intraperitoneally. Environmental temperature, 32°.

Dose (mg./kg.)	Deaths at 24 hr.
Rabbits 1 day old	
25	1/5
28	0/7
32	0/8
35	5/6
Rats 1 day old	
45	4/6
50	3/6
55	6/6
60	5/6
Adult rats	
60	3/7
100	4/7
140	6/7

The sleeping time of new-born rabbits which had received a given dose of pentobarbitone was much greater than that of adolescent or adult rabbits which had received the same dose per kg. (Table II).

TABLE II

THE SLEEPING TIMES OF RABBITS AFTER PENTOBARBITONE

The numerals in parentheses indicate the number of animals. Environmental temperature, 32°.

Dose (mg./kg.)	Range of Sleeping Times (min.)		
	1 Day Old	30–31 Days Old	Adult
6	49–56 (4)		
12	54–143 (4)	8–23 (3)	0 (3)
18	173–265 (4)	0–31 (3)	12–27 (3)
24	300 or more (4)	5–47 (3)	8–72 (2)

Urethane

The sleeping times of both new-born rats and rabbits treated with 400 and 600 mg./kg. of urethane was longer than those of adults (Table III). However, there was a pronounced difference between the depth of anaesthesia produced in young and in old rabbits and in rats with this drug. Only 1 of 4 rabbits, which had received 1.2 mg./kg. of urethane, less than 3 days old did not resist restraint on its back, whereas 4 of 4 adult rabbits which had received the same dose could be restrained on their backs. Young rats reacted to 1.2 g./kg. of urethane similarly to young rabbits.

TABLE III

SLEEPING TIME OF RABBITS AND RATS AFTER URETHANE INTRAPERITONEALLY

The sleeping time is given ± standard error. The numerals in parentheses indicate the number of animals used in each estimate. Environmental temperature 34°.

Dose (mg./kg.)	Sleeping Time (min.)	
	One Day Old	>90 Days
Rabbits		
400	68±13·1 (7)	15±4·5 (8)
600	90± 9·5 (7)	41±8·5 (8)
	Four Days Old	Adult
Rats		
400	28±5·4 (9)	0 (9)
600	43±2·7 (9)	0 (9)

Ether

A group of 54 rabbits of three different ages (1, 3, and 30 days) were exposed to one of three concentrations of ether (4.1, 8.3 or 12.8 vol. %) at one of three temperatures (20°, 30° or 40°). Animals were allocated to experimental groups according to a latin square design and litters were randomized. The interval between exposure to ether and loss of righting reflex became less as the concentration of ether was increased. It was 9 to 11 min. at 4.1 vol. % ether, 2 to 9 min. at 8.3 vol. % and 2 to 4 min. at 12.8 vol. %. This interval was affected neither by the temperature nor by the age of the rabbits.

There was a difference in the time taken for rabbits of different ages to regain their righting reflex after removal from ether (Table IV). Two groups each of 5 rabbits (4 days old and 28 to 32 days old) were simultaneously exposed to 12.8 vol. % ether at 32° for 3.75 min. At the end of this time all rabbits had lost their righting reflex. The concentration of ether was then reduced to 1.9 vol. % and the time taken to regain the righting reflex was observed. This experiment was repeated on a different day, and, in spite of divergence of the mean times, all older rabbits

TABLE IV

TIME TAKEN BY RABBITS TO REGAIN RIGHTING REFLEX WHEN EXPOSED TO 1·9 VOL. % OF ETHER AFTER EXPOSURE FOR 3·75 MIN. TO 12·8 VOL. % OF ETHER

Environmental temperature, 32°. The times are means ± standard error.

	Time (sec.)	
Age:	<4 days	28–32 days
Exp. No. 1 ..	> 60	30±3·8
,, ,, 2 ..	>180	69±6·0

regained their righting reflexes before any of the younger ones.

Two rats of different ages were placed simultaneously in an ether-air mixture at 30°. Five such pairs of rats were observed at each of three different concentrations of ether between 10.2 and 17.5 vol. %. Table V shows that there was little

TABLE V

TIME TAKEN BY RATS TO LOSE RIGHTING REFLEX AND TO BECOME ANAESTHETIZED WHEN EXPOSED TO ETHER.

Environmental temperature, 30°. There were 5 animals in each group. The times in min. are means±standard error.

Ether (Vol. %)	Time to Lose Righting Reflex		Time to Lose Response to Pinch	
	5 Days	24–28 Days	5 Days	24–28 Days
10·2	65±5·7	63±2·0	625±38	239±41
14·2	52±3·5	47±2·2	435±26	131±12
17·5	42±4·6	45±1·6	463±26	125± 9

difference between the times at which rats of different ages lost their righting reflex, but the younger rats took considerably longer before they became anaesthetized. The concentrations of ether used in these experiments can be regarded as high.

In other experiments, litters of rats of different ages were exposed simultaneously at 32° to lower concentrations of ether, either 1.9 or 4.1 vol. %. Table VI shows that all rats less than 1 day old had lost their righting reflex after 180 min. of exposure to 1.9 vol. % ether, whereas none of the older rats had lost their righting reflex after 240 min. of exposure to the same concentration of ether. In 4.1 vol. % ether, all the younger rats had lost their

TABLE VI

LOSS OF THE RIGHTING REFLEX IN NEW-BORN AND ADULT RATS EXPOSED TO ETHER

Environmental temperature, 32°.

Ether (Vol. %)	Age	No. of Rats in Group	Number of Rats Without Righting Reflex after Exposure for 10	20	30	60	90	120	180	240 (Min.)
1·9	<1 day	16	0	3	1	6	10	10	16	16
	9 weeks	17	0	0	0	0	0	0	0	0
4·1	4 days	11	11	11	11	11				
	4 weeks	6	0	5	6	6				
	9 ,,	5	0	1	5	5				

righting reflex after 10 min., but the older ones did not lose their righting reflex until after 20 min.

As with rabbits, the older rats also regained their righting reflex more quickly after ether. Groups of rats of several ages were exposed to 12.8 vol. % ether for 2 min., by which time all animals had lost their righting reflex. The concentration of ether was then reduced to 1.9 vol. % and the rats were examined after a further 20 min. By this time all the adults and a few of the older rats had regained their righting reflex, but none of the younger rats had righted themselves. These experiments were carried out at 20°, 30° or 40°, but, as the temperature did not appear to influence either the time to lose or to regain the righting reflex, the results have been combined in Table VII.

TABLE VII

DURATION OF LOSS OF THE RIGHTING REFLEX IN RATS EXPOSED TO 12·8 VOL. % OF ETHER FOR 2 MIN. AND THEN TO 1·9 VOL. % OF ETHER FOR 20 MIN.

For further explanation, see text.

Age (Days)	Number of Rats without Righting Reflex after 20 Min.
0–5	46/46
5–10	31/31
15–20	9/9
25–30	9/9
35–40	14/20
90–95	3/20
Adult	0/7

DISCUSSION

It is evident from these results that new-born rats and rabbits differ from adults in their reactions to anaesthetic drugs, but these differences are not

consistent. After small doses of urethane (400 or 600 mg./kg.) new-born rats and rabbits lose their righting reflex for longer than adults ; whereas a dose of urethane, which in both adult rats and rabbits produced relaxation sufficient for surgical manipulations, did not relax or anaesthetize new-born rats and rabbits. Similarly with ether, new-born rats did not become anaesthetized so quickly as adult rats although the new-born rats and rabbits lost their righting reflex as soon as or sooner than the adult animals and could be maintained, without their regaining a righting reflex, at lower concentrations of ether than could adult rats. Pentobarbitone, which produced longer loss of righting reflex in new-born rabbits than in adults, did not produce anaesthesia in new-born rabbits until the respiration was grossly depressed, and was more toxic to new-born rats and rabbits than to adults.

The finding that new-born rabbits were not anaesthetized by a dose of urethane sufficient to anaesthetize an adult rabbit has also been observed by Adamsons (personal communication) and by Downing (personal communication), both of whom used urethane to anaesthetize rabbits of different ages for surgical procedures (Adamsons, 1959 ; Downing, 1960). However, Cross, Dawes and Mott (1959) found that about 1/4 of the dose of chloralose required for an adult sheep was sufficient to produce light surgical anaesthesia in a new-born lamb.

The prolonged action of barbiturates in new-born mice has been described by Jondorf *et al.* (1959). These authors also found that all of an injected dose of hexobarbitone was present in mice less than 24 hr. old 3 hr. after injection, whereas in 21-day-old mice only 70% of the dose remained after 3 hr. The metabolism of amidopyrine and phenacetin was similarly reduced in new-born mice. The side chain oxidation of hexobarbitone by liver homogenates from rabbits 2 to 3 weeks old was only 5 to 37% of that from adult rabbits (Fouts and Adamson, 1959) : while new-born guinea-pig tissues were unable to form glycuronides (Brown and Zuelzer, 1958). Indeed there is much evidence that the concentrations of various enzymes in mammals are low at birth and increase during the first days of life (Potter, Schneider and Liebl, 1945 ; Reem and Kretchmer, 1957 ; Dawkins, 1959).

It is attractive to argue that the differences between the effects of urethane, pentobarbitone and ether in new-born and adult animals are due to functional differences in the central nervous systems. However, although there are known differences in enzyme concentrations in various parts of the brain at different ages, there is not sufficient known of the mechanism of action or of the distribution of the anaesthetic drugs compared in this paper to say how the observed differences occur. On the other hand it is probable that the prolonged action of pentobarbitone is due in part to the slow systemic breakdown of the drug by new-born animals. The same may be true of urethane, 90% of which in normal adult mice is metabolized in 24 hr. (Mitchell, Hutchison, Skipper, and Bryan, 1949). Ether is not metabolized to any appreciable extent but is eliminated through the breath and excreta (Haggard, 1924) so that slow metabolism of ether cannot explain its prolonged action in new-born animals.

The differences in the effects of these three anaesthetic drugs on new-born animals show that it is not possible to generalize about the greater " sensitivity to anaesthetics " of new-born animals as compared with adults, and with our present knowledge it is not possible to predict reliably how a new type of anaesthetic drug will act in a new-born animal.

I wish to thank the Sir Halley Stewart Trust for a personal grant during this work, Dr. H. G. Epstein for advice about administration of ether and for the loan and calibration of an E.M.O. Inhaler, and Dr. G. S. Dawes for his advice and his help in preparing the manuscript.

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Brit. J. Pharmacol. (1960), **15**, 458.

EXPERIMENTS ON THE PHARMACOLOGY OF HYDROXYDIONE SODIUM SUCCINATE

BY

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(RECEIVED APRIL 22, 1960)

The actions of hydroxydione sodium succinate (" Viadril ") have been studied in chloralose or decerebrate cats, or cats otherwise untreated. It is virtually devoid of action at the neuromuscular, ganglionic, or peripheral adrenergic synapse. After rapid intravenous injection, hydroxydione produced a transient hypotension with little bradycardia and stimulated the respiration. These effects were over in 1 to 2 min. and were reduced if injection was slow or the hydroxydione diluted. Vagotomy did not alter the depressor response, but reduced the respiratory stimulation. In the untreated or the decerebrate animal, as much as five times the anaesthetic dose of hydroxydione was required to depress the respiration or blood pressure. Chloralose potentiated these depressant actions 5 to 10 fold. Both the circulatory and the respiratory depression appeared to be mediated centrally. Decerebrate rigidity was readily lessened by hydroxydione, but the pinna reflex and conjunctival reflex were relatively resistant to its action, and the knee jerk outstandingly so.

Fine transient muscular fasciculations, not unlike those produced by suxamethonium, have occasionally been observed by one of us during hydroxydione administration in man, occurring shortly after the onset of unconsciousness. They appear first in the muscles of the face, spread to the upper limbs and trunk, and die away after a few seconds. Such an observation suggested that hydroxydione might have some neuromuscular action. The possibility became more important in the light of the relaxation which hydroxydione anaesthesia can produce, especially in the pharynx and the larynx. The experiments described below started, therefore, with an investigation, in cats under chloralose, of the neuromuscular action of the drug. It was found, however, that even large doses of hydroxydione had no neuromuscular action. But in the animal under chloralose a somewhat unexpected depression of the respiration by hydroxydione was observed which prompted a fuller investigation of its pharmacology. One interesting finding has been the great potentiation of the respiratory depressant action of hydroxydione by the presence of chloralose.

METHODS

Cats were used for most experiments. After induction with ethylchloride and ether, anaesthesia was maintained with chloralose 60 to 80 mg./kg. The blood pressure was recorded by a siliconed, heparinized cannula in one carotid artery. The respiration was recorded, after a tracheal cannula had been inserted, by the aid of respiratory valves and a respiration recorder on the expiratory side (Paton, 1949). When required, the twitches of the tibialis anterior muscle were recorded on the smoked drum with a steel spring myograph, in response to supramaximal excitation of the sciatic nerve through shielded electrodes in the thigh. Rectangular pulses of 0.5 m.sec. duration were delivered at varied frequencies, usually one every 10 sec. Action on an autonomic ganglion was tested by recording the contractions of the nictitating membrane with a lever of magnification about 5-fold and load about 2 g. writing frontally on the smoked drum, the membrane being excited to contraction by supramaximal preganglionic shocks through the cervical sympathetic nerve in the neck which had previously been separated from the vagus and divided from its central connexions. The contents of the eyeball were removed. Rectangular pulses of 0.5 m.sec. duration were applied, at 10 shocks per sec. either for a period of 15 sec. every min. or continuously, through platinum electrodes. To make injections a venous cannula was inserted into the femoral or jugular veins, and all injections were washed in with 2 ml. saline.

In some experiments decerebration was performed; a trephine hole was made in the parietal bone and, after incision of the dura, a transection made between the inferior and superior colliculi towards the pituitary fossa; the hemispheres were then removed. To reduce bleeding during the operation both carotid arteries were tied and a vertebral clamp adjusted to control bleeding as necessary. The animal was left for 0.5 hr. to 1 hr. before further dissection so that satisfactory clotting of the cerebral vessels could occur.

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In two cats induction of anaesthesia was performed with hydroxydione, the drug being made up in 1% procaine, and injected into a vein on the foreleg. When full anaesthesia had been produced one or more of the procedures just mentioned were used.

The doses of drugs are given in terms of their salts. These were hydroxydione sodium succinate (" Viadril," Pfizer), adrenaline tartrate, noradrenaline tartrate, acetylcholine chloride, histamine acid phosphate, succinylcholine di-iodide, *d*-tubocurarine chloride. The hydroxydione was normally made up as a 10% solution either in saline or, as described by Galley and Lerman (1959), in 0.25% or 0.5% procaine. The procaine made no detectable difference to the effects seen.

Results

Actions on the Neuromuscular Junction

In the cat under chloralose a dose of hydroxydione (5 to 10 mg./kg.), which can produce severe respiratory depression, leaves the twitch of the tibialis in response to excitation of its motor nerve completely unaffected (Fig. 1, *a* and *b*). There are neither

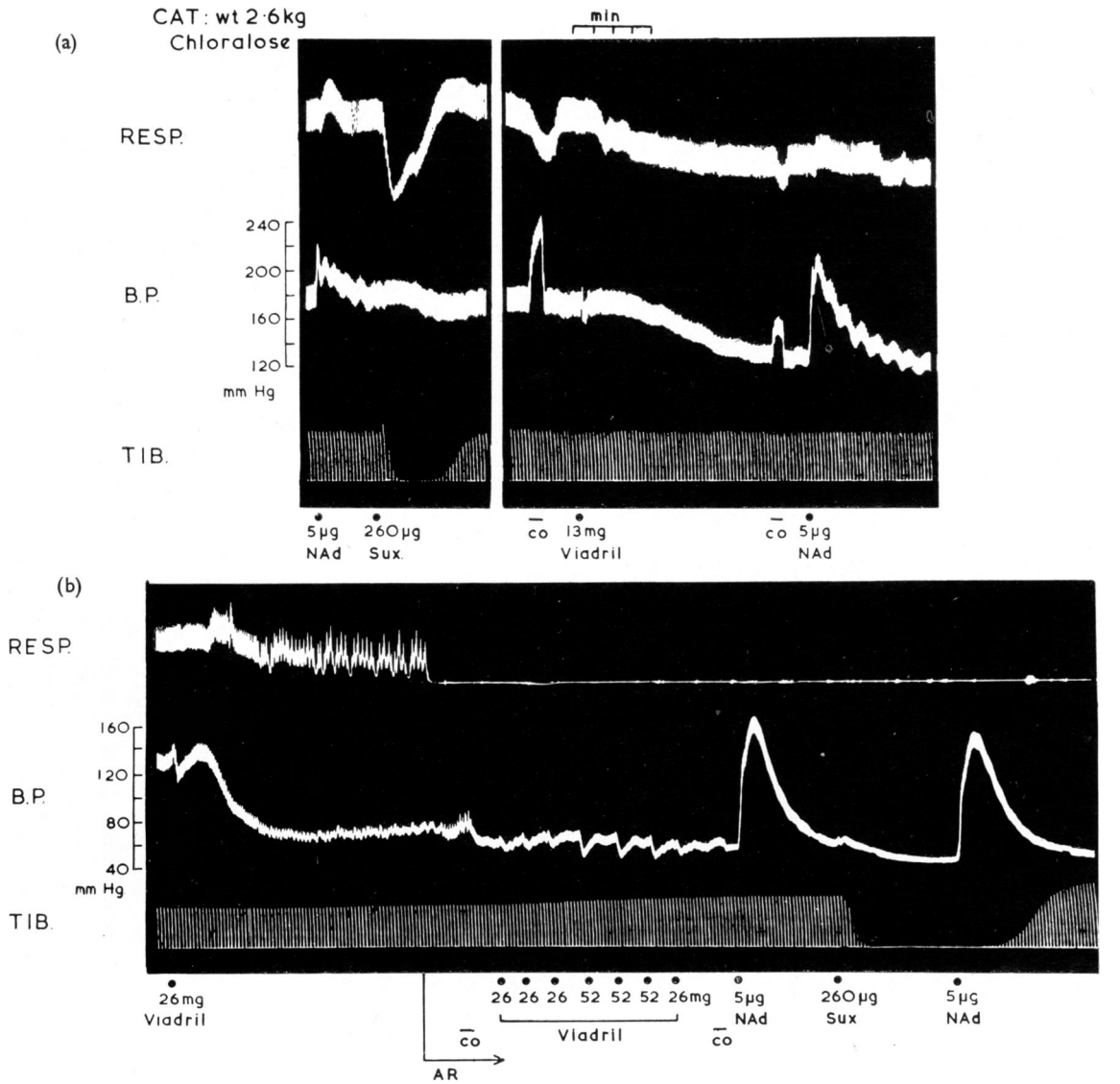

FIG. 1.—Cat, 2.6 kg., chloralose. Intravenous injections. Record of blood pressure, respiration, and tibialis twitch. CO, bilateral carotid occlusion for 30 sec. AR, artificial respiration. NAd, noradrenaline. Sux, suxamethonium.

preliminary fasciculations to be seen nor potentiation of the twitch nor any sign of neuromuscular block. Even doses up to 100 mg./kg. (Fig. 1*b*), which produce profound circulatory effects in the cat under chloralose, are either devoid of any effect on the muscle twitch or even slightly potentiate it. In addition, a test was made of the interaction of hydroxydione with succinylcholine and *d*-tubocurarine. Succinylcholine given to a cat which was under the influence of 10 mg./kg. hydroxydione did not vary from its usual behaviour; and a dose of 20 mg./kg. hydroxydione during recovery from succinylcholine paralysis did not influence the course of recovery in any way. After a massive dose of hydroxydione (100 mg./kg.) the fasciculations normally seen were abolished (Fig. 1*b*), and the neuromuscular block produced came on a little more slowly and was somewhat prolonged. If so small a change in the action of suxamethonium is produced by a dose 5 to 10 times the normal human anaesthetic dose, any such effect must be negligible in practice. With tubocurarine, corresponding observations were made; after 100 mg./kg. hydroxydione, it was possibly a little potentiated, and had a more prolonged action. These observations imply that, in a very large dose, hydroxydione has a slight ability to lessen specific depolarization of the endplate, a property shared, in much more intense form, by many anaesthetics.

Supporting evidence for the conclusion that hydroxydione had virtually no neuromuscular action was obtained indirectly in another way. In the decerebrate animal after total doses of hydroxydione up to 230 mg./kg., and with a dose of 500 mg./kg. after induction with hydroxydione, the knee jerk could still be elicited. The presence of a knee jerk necessitates intact neuromuscular transmission; this result is an even more cogent reason for rejecting any significant neuromuscular action.

Action on Autonomic Ganglia

Fig. 2 shows the effect of 10 mg./kg. hydroxydione on the response of the nictitating membrane to preganglionic excitation, a dose which produced the usual substantial sustained hypotension seen in chloralosed cats. Immediately after the injection, there was possibly a slight weakening of the response of the membrane to continued stimulation; and about 10 min. later, when the hypotension was fully developed, there was a doubtful reduction of the

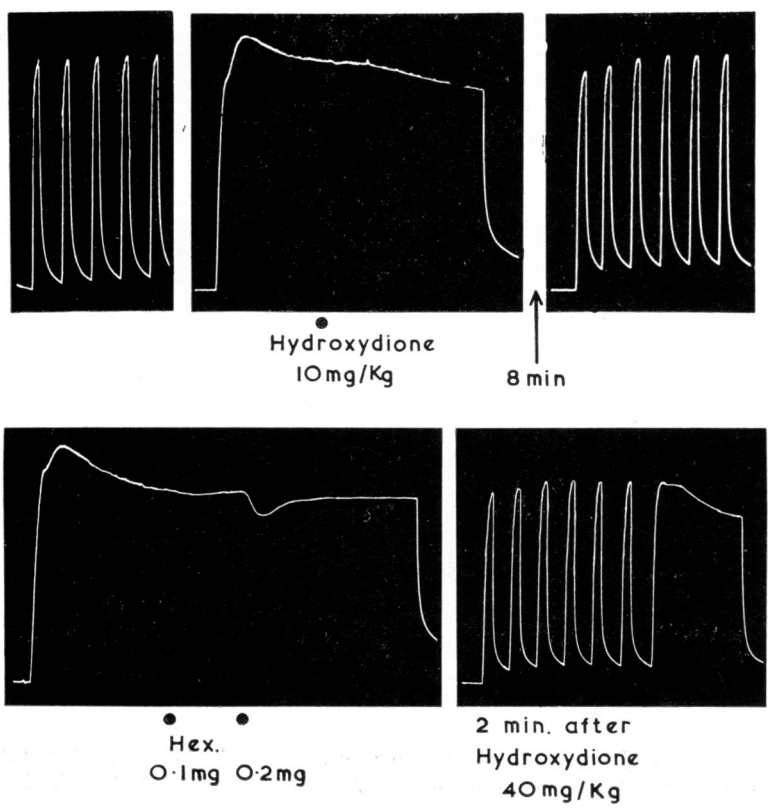

FIG. 2. — Cat, chloralose. Intravenous injections. Response of nictitating membrane to preganglionic excitation at 10/sec., either for 15 sec. every min., or continuously.

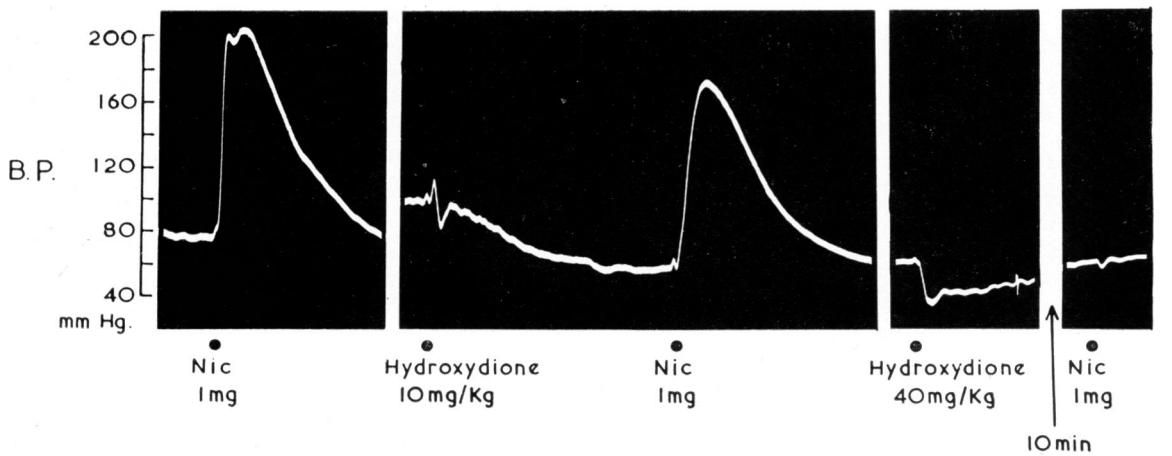

FIG. 3.—Cat, chloralose. Record of carotid arterial blood pressure. Artificial respiration. Responses to nicotine (Nic) before and after 10 and 40 mg./kg. hydroxydione.

response to intermittent excitation. With a higher dose, of 40 mg./kg., the response to intermittent stimulation was definitely reduced; and with continued excitation, the response was not quite as well maintained as usual. Hydroxydione thus has a slight ganglionic action, around 1/300 of the potency of hexamethonium, detectable but not sufficient to account for the hypotensive action.

A test was also made of the effect of hydroxydione on the pressor response to nicotine (Fig. 3). At 10 mg./kg., the rise in blood pressure was hardly altered, although its time course was modified slightly. With 40 mg./kg., however, the action of 1 mg. of nicotine was abolished. This anti-nicotine action is more pronounced than would be expected from the effect of hydroxydione on ganglionic transmission; but it must be remembered that nicotine, in addition to exciting autonomic ganglia, also stimulates chemoreceptors and exerts other central actions.

The fact that the nictitating membrane response to preganglionic excitation was little altered by hydroxydione also implies that hydroxydione has a negligible action at the junction between the sympathetic nerves and smooth muscle, and on the responses of smooth muscle to the sympathetic transmitter. The latter point was tested directly. A large dose of hydroxydione, indeed, increases the rise in blood pressure produced by noradrenaline (Fig. 1*b*), presumably because the blood pressure is lower and the buffer mechanisms paralysed. In short, hydroxydione has little peripheral synaptic effect, whether tested on the sympathetic effector junction, the autonomic ganglion or the neuromuscular junction.

Effects of Hydroxydione on Respiration and Blood Pressure

Animals under Chloralose.—In our first experiment, on a cat under chloralose, the somewhat startling observation was made that a dose of 10 mg./kg. of hydroxydione abolished the respiration in between 3 and 4 min. This is quite out of keeping with clinical experience (Lerman, 1956), so that a further analysis was made. In the chloralosed animal this dose always depressed respiration strongly, at least 40%. Even 5 mg./kg. had a distinct effect on respiration (Fig. 1*a*). Usually 20 mg./kg. would produce apnoea, but sometimes a higher dose was needed.

The immediate effect of hydroxydione on the respiration is usually a transient stimulation, occurring soon after the injection, increasing the minute volume sometimes as much as two-fold, sometimes only by 20 to 30%, with a rapid return to normal. It is after this that the depression of the minute volume occurs, accompanied by some slowing of respiration. Even with a large dose of hydroxydione there is some delay before respiratory paralysis ensues; and it probably cannot be produced in a shorter time than 2 or 3 min. The respiratory minute volume recovers again with time; after 10 to 20 mg./kg., recovery takes 30 to 60 min. During a reduction of respiration by hydroxydione, provided asphyxia is not produced, the minute volume often becomes more regular; spontaneous fluctuations, or reflex responses to rise or fall of blood pressure due to adrenaline or histamine or to stimulation of the cervical sympathetic trunk, are reduced. Thus, even in the experiment of Fig. 1*a* where the respiration was

already unusually stable, the respiratory effects of carotid occlusion and of 5 μg. noradrenaline were reduced by 10 mg./kg. hydroxydione.

Accompanying the respiratory action is a somewhat complicated response of the blood pressure. First, with a dose of 10 mg./kg. or more, is a pronounced transient fall in blood pressure; this may be as much as 50 mm. of Hg or more, according to the dose and speed of injection. It has a very short latency after the injection, and the blood pressure returns to the normal level in about 30 sec.

After this initial hypotension a second, pressor response up to 40 mm. and lasting 2 to 5 min. may be seen. There is no noticeable tachycardia during this time. The rise in blood pressure appears to occur only when there is a reduction of minute volume; but it is sometimes absent even when the respiration is depressed. This phase then passes into the third and main response, a slowly progressing hypotension which may reach its lowest point up to 15 or 20 min. after the injection; the blood pressure then slowly rises again, to return to normal at a time depending on the dose given.

The final prolonged hypotension appears to depend on a central vasomotor depression. The experiments on autonomic ganglia, mentioned earlier, exclude a peripheral paralysis of sympathetic effector organs as the cause, and this was confirmed by verifying that, for instance, the action of adrenaline and noradrenaline was unimpaired during the hypotension. (The depressor effects of histamine, and of histamine liberation, are also still demonstrable, although reduced as is usual when the blood pressure falls.) The hypotension is not accompanied by any marked bradycardia, and vagotomy did not influence it. The most reasonable explanation for it, therefore, is that it is due simply to reduction of central vasomotor tone. This conclusion was substantiated by finding that during the hypotension due to hydroxydione, the rise in blood pressure elicited reflexly by bilateral carotid occlusion was impaired despite the continuing effectiveness of autonomic transmission and of noradrenaline (Fig. 1*a*). With 5 mg./kg. hydroxydione (lowering the blood pressure by about 40 mm.) a considerable reduction of the carotid occlusion response occurred; with 10 mg./kg. (lowering the blood pressure to 60 mm.) it was virtually abolished (Fig. 1*b*) although 5 μg. noradrenaline had a larger pressor effect than normal. Three hours later the carotid occlusion response had recovered considerably; the blood pressure was still low, but, after 7 hr. anaesthesia, this is of doubtful significance.

Decerebrate Animals

If the animal is decerebrated under ether, and an interval of 1 hr. or more allowed after ceasing ether administration, one can test hydroxydione on the respiration and circulation in the virtual absence of any other anaesthetic. When this is done, the initial respiratory stimulant and depressor actions are still present, with the same time course; the respiratory stimulation, indeed, is more pronounced than in the presence of chloralose. But the subsequent depression of the respiration is absent, until relatively enormous doses of hydroxydione are given. Similarly, the blood pressure, after the initial hypotension, returns permanently to its original level, and there is little sign either of the pressor effect or of the slow prolonged hypotension. Thus, in one animal, 140 mg./kg. hydroxydione given in divided doses over a period of 40 min. had nothing but a stimulant action on the respiration. In another, a single dose of 40 mg./kg., following previous divided dosage of 70 mg./kg. (itself ineffective), only stimulated respiration. In a third animal, it required 40 mg./kg. to depress minute volume by 25%. The blood pressure responses are harder to interpret, since the pressure tends to decline from its initial level in any case; but it appeared that no persistent hypotension occurred with doses under 40 mg./kg. and in one experiment 110 mg./kg. failed to reduce the blood pressure.

The decerebrate animal appeared, in short, to be something like 5 to 10 times more resistant than the chloralosed animal to the depressant effect of hydroxydione on respiration and blood pressure. So large a difference raised the question whether decerebration had removed some nervous structure necessary for the action of hydroxydione. This was tested by giving chloralose to three decerebrate animals. In one, which had received 140 mg./kg. hydroxydione with no depression of respiration, 40 mg./kg. chloralose (half the standard dose) produced apnoea in 3.5 min. In the other two, 60 mg./kg. chloralose itself depressed the respiration considerably; after a period of recovery, 10 mg./kg. hydroxydione was now itself able to produce apnoea. There was thus a powerful synergism between the two drugs in the decerebrate animal, each potentiating the depressant action of the other; it was of such a magnitude that a dose of hydroxydione, a small fraction of that necessary by itself to exert respiratory depressant action, combined with a dose of chloralose much smaller than normally employed, could cause complete abolition of respiratory movement. The fact that giving chloralose to the decerebrate cat allowed the depressant action of hydroxydione to be reproduced as in the intact cat under chloralose makes it

unnecessary to suppose that decerebration removes some nervous structure requisite for the action of hydroxydione.

The opportunity was also taken to make some qualitative observations in these decerebrate animals on muscle tone, the knee jerk, the conjunctival reflex and the pinna reflex. Decebrate rigidity, and the clonic character of the response to tapping the quadriceps tendon, were obviously reduced by doses of 20 to 30 mg./kg. hydroxydione; but it proved generally impossible to abolish the reflexes, although the conjunctival reflex was weakened by large doses. A similar observation had been made in chloralosed animals: that even when apnoea was produced with hydroxydione, the knee jerk remained.

Induction of Anaesthesia with Hydroxydione

Having found that hydroxydione in the decerebrate cat appeared to be a remarkably safe anaesthetic, an induction of anaesthesia with hydroxydione alone was performed. In one animal a dose of 10 mg./kg. intravenously sedated the animal, and a further 10 mg./kg. 20 min. later deepened the sedation, and after another 20 min. interval a further 20 mg./kg. produced surgical anaesthesia. In another, approximately 80 mg./kg. in divided doses over 45 min. produced a similar effect.

Some of the observations previously made were then repeated (Fig. 4). Under these conditions, a dose of 10 or 20 mg./kg. hydroxydione had little or no depressant effect on respiration, and did not cause any prolonged hypotension. The usual transient hypotension, with a marked accompanying respiratory stimulation at the time of injection, was still seen. The promptness and transience of these two responses suggest that they are due to the presence of a high concentration of hydroxydione in the blood during its first circulation after the injection. This was confirmed by finding that both responses are much attenuated, either by making the injection slowly, or by diluting the injected solution ten-fold (Fig. 4*a*). Our first impression was that the hyperpnoea was secondary to the hypotension: for a similar phenomenon is often seen with depressor drugs. But, recorded on a fast-moving drum, the respiratory response sometimes precedes, by a few seconds, the hypotension, so that the latter can hardly be the sole cause of the respiratory change.

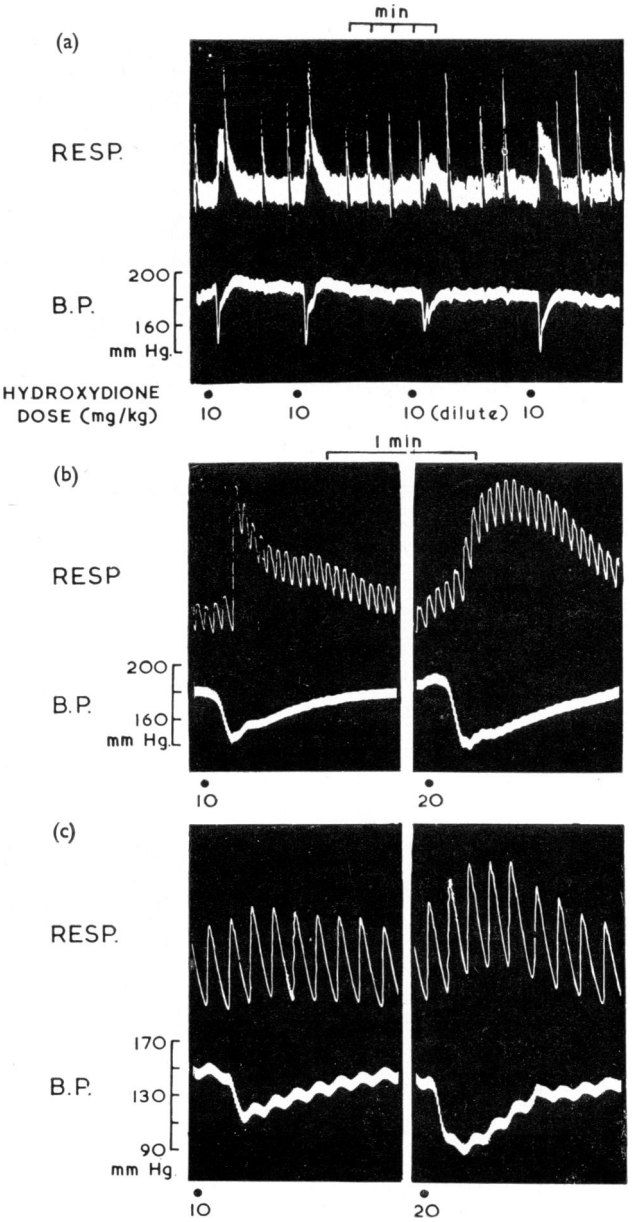

FIG. 4.—Cat, hydroxydione anaesthesia. Intravenous injections. Record of respiration and blood pressure. (*a*) Responses to hydroxydione 10 mg./kg. given in 0.23 ml. washed in with 2 ml. saline, except third injection in 2.3 ml. with 2 ml. washed in. (*b*) Responses to 10 and 20 mg./kg. hydroxydione on a 10 times faster drum. (*c*) Responses to 10 and 20 mg./kg. hydroxydione after cutting both vagi.

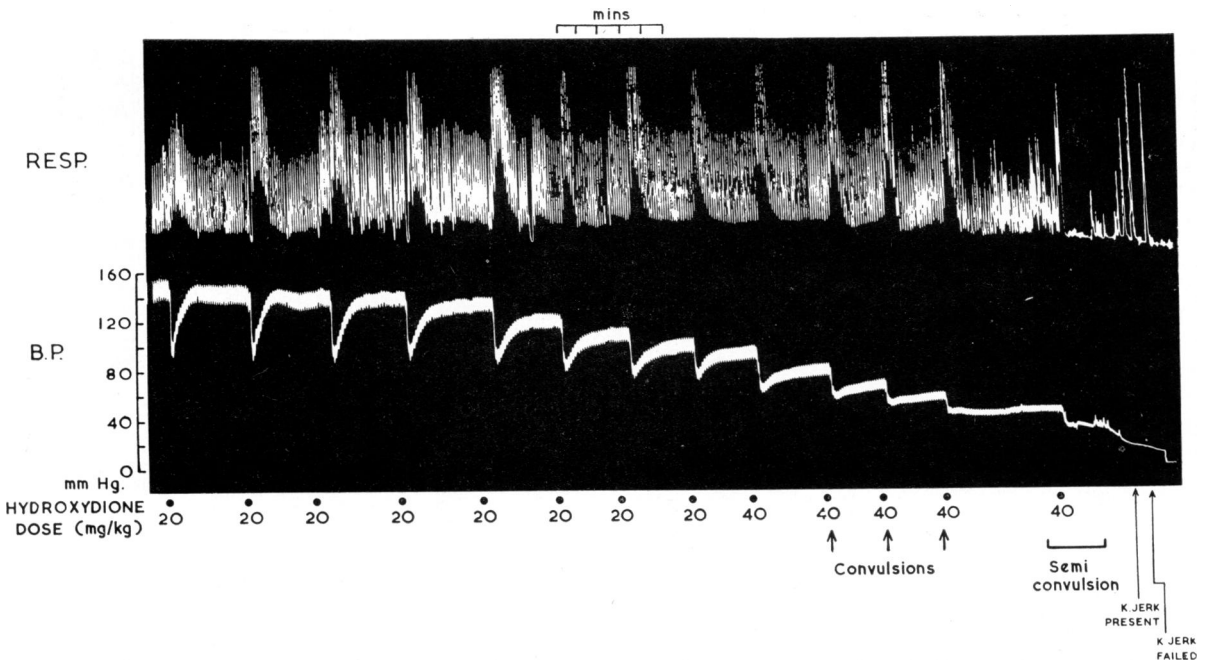

FIG. 5.—Cat. Hydroxydione anaesthesia. Record of respiration and blood pressure. Before the beginning of the record, 240 mg./kg. hydroxydione had been given over 2 hr. 40 min.

This analysis was confirmed by sectioning the vagi. As a result the hypotension produced by hydroxydione was reduced slightly, but its time course and the slight slowing of heart rate during the fall of pressure were unchanged. The respiration was more affected; the stimulant effect was much reduced; and in one experiment in which the first effect of hydroxydione had been to suppress one or two breaths before the stimulation of respiration, this transient apnoea disappeared. It appears, therefore, that the vagi play some part in the initial respiratory response to hydroxydione.

The experiment of Fig. 4 was terminated by giving repeated doses of hydroxydione at 5 min. intervals, to examine the response to massive dosage (Fig. 5). After around 320 mg./kg., the blood pressure began to fail to return to its original level after each injection. After 480 mg./kg., each injection gave rise to a species of transient convulsion, which, after the last dose (total 600 mg./kg.), was more persistent and was at last accompanied by respiratory failure. It was remarkable that the knee-jerk could still be elicited, and not until effective respiration had ceased for several min. did it disappear.

DISCUSSION

Hydroxydione deserves attention as representing a type of anaesthetic structurally quite distinct from those in general use. Although, as a rule, the chemical structure of the general anaesthetics is less important than their physical properties, it may well be that the steroid nature of hydroxydione is responsible for some of its novel features. In discussing these, comparisons with other anaesthetics will be made.

As with other drugs given intravenously, the concentration in the blood is very high during the first one, possibly two, circulation times after the injection. Gray and Paton (1949) showed that, in the cat, an injection of volume about 3 ml. was only diluted about ten-fold in the blood in its first passage from a femoral vein to carotid artery; and that the injection " slug " was still detectable during the second circulation. We have concluded that the first response to hydroxydione, a fall in blood pressure and a stimulation of respiration, is due to this " slug effect "; for the time relations are correct, and this first response is greatly reduced by diluting the injected solution or by slow injection. The hypotension is accompanied by only a slight bradycardia and survives vagotomy, so that it is

not of the Bezold-Jarisch type. The respiratory response, primarily stimulant but occasionally preceded by one or two " dropped breaths," appears to depend in part on vagal pathways; the remainder may well be secondary to the hypotension. We have not analysed this initial response further, but it is not altogether a surprising consequence of the sudden exposure of the heart and lungs to a concentration of around 10^{-3} hydroxydione. It is of little significance for clinical practice, where a much greater dilution of the injected material will occur. It compares not unfavourably with thiopentone, with which immediate cardiac depression may be severe, and accompanied by apnoea.

The subsequent responses of the blood pressure and respiration depend on the intensity of the depression of vasomotor and respiratory centres. In the animal under chloralose, after a latency of a few minutes a slowing of respiration and reduction of tidal air lead to a fall of minute volume. There is a fall of blood pressure usually of similar time course, although sometimes the blood pressure may rise initially for a few min. during the early stages of the respiratory depression. Both respiratory and blood pressure depression reach their peak in 10 to 20 min. At this stage, spontaneous fluctuations or changes of reflex origin are reduced or abolished, although the responsiveness of the blood vessels to directly acting drugs (adrenaline, noradrenaline, histamine) is retained. Recovery is gradual; the effect of a dose of 10 to 20 mg./kg. lasts 30 to 60 min.

Although the reactions of the animal under chloralose allow the type and duration of action of hydroxydione to be studied conveniently, they give an erroneous picture of their magnitude. In the decerebrate or normal animal, it was not possible to produce apnoea with doses less than 140 mg./kg., and hypotension was equally slight; but under chloralose, 5 mg./kg. both reduced blood pressure and minute volume substantially. The mutual potentiation between chloralose and hydroxydione is an interesting phenomenon, and may well extend to other anaesthetics; Lerman (1956) has already commented on a potentiation by hydroxydione of nitrous oxide. More important for practice, however, is an estimate of the safety of hydroxydione, expressed as the ratio of respiratory or circulatory depressant dose to anaesthetic dose. We have not made the systematic study necessary for this; but it is clear, from our own result (that roughly 50 mg./kg. will produce surgical anaesthesia) in conjunction with results reported elsewhere, that the safety factor is of the order of 5-fold, possibly higher. Some additional observations, which we have not described in detail, are relevant here. If apnoea is produced with hydroxydione, and allowed to continue for about 3 min. until circulatory failure occurs, it only requires artificial respiration and, if necessary, cardiac massage to restart quite a vigorous heart beat. At no time have we seen cardiac irregularities, even under such stringent conditions. It appears, in short, to be comparable with, or superior to, ether in its safety.

Finally, the lack of peripheral synaptic action is of interest. Ether is known to have quite a pronounced " curariform " action, shared in more or less degree by the other volatile anaesthetics. The barbiturates also have neuromuscular actions, and a power to block ganglia, which, with amylobarbitone, formed the basis of a test for neurogenic hypertension.

Hydroxydione thus presents features of considerable interest. It appears to be both safe and specific. Its time course of action differs from, say, thiopentone, but this merely means it must be used differently. If its principal disadvantage in practice (that of surface-activity, on which its local irritant effect in the tissues appears to depend) could be overcome, it, or similar compounds, would represent very valuable anaesthetic agents.

We are much indebted to Dr. H. R. Reinert, of Pfizer, for supplies of Viadril; and to Mr. D. A. Green and his staff for technical assistance.

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PERIPHERAL VASCULAR EFFECTS OF BRETYLIUM TOSYLATE IN MAN

BY

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(RECEIVED MAY 27, 1960)

After intra-arterial infusion of bretylium tosylate (12.5 mg.), the reflex changes in vasoconstrictor tone which normally occur in the forearm with body cooling, positive pressure breathing, the Valsalva manœuvre and postural change were greatly reduced or abolished. Reflex vasodilatation mediated by cholinergic fibres in response to body heating or to emotional stress was little affected. It was concluded that bretylium can selectively block the activity of sympathetic noradrenergic fibres without causing a similar block of sympathetic cholinergic fibres. As the responses to intravenous or intra-arterial infusions of adrenaline or noradrenaline were not reduced after bretylium, it was concluded that bretylium interferes with the activity of noradrenergic fibres rather than with the activity of the noradrenaline released at the nerve ending. After bretylium infusion, forearm and hand blood flow did not often rise to levels characteristic of full release of vasoconstrictor tone. As infusion of bretylium into a nerve-blocked forearm resulted in a pronounced reduction in flow, it is concluded that bretylium also has a constrictor effect on blood vessels. The state of the vessels following an infusion of bretylium appears to depend on the balance between this constrictor action and the longer-acting sympathetic blocking effect.

Bretylium tosylate ("Darenthin," Burroughs Wellcome) is one of a series of benzyl quaternary ammonium compounds described by Boura and Green (1959). Experiments in cats suggested that it blocks the peripheral sympathetic (adrenergic) nervous system selectively, without antagonizing the effects of released or injected adrenaline or noradrenaline, and without depression of the parasympathetic or central nervous systems. A preliminary trial in hypertensive patients by Boura, Green, McCoubrey, Laurence, Moulton and Rosenheim (1959) has shown that it lowers the supine blood pressure slightly and causes postural hypotension; and measurements of the circulatory response to the Valsalva manœuvre in a healthy volunteer by Dornhorst (1959) confirmed that the drug produces effects characteristic of block of the sympathetic system.

In the present experiments we have studied the effect of bretylium tosylate on certain vascular reflexes in the human hand and forearm. A brief account of this work has previously been published (Blair, Glover, Kidd and Roddie, 1959).

METHODS

The experiments were carried out on male normotensive subjects, who lay supine on a couch in a laboratory maintained at a temperature of 20 to 21°. The subjects were lightly clad but covered with a blanket. Blood flow in the hands or forearms was measured by venous occlusion plethysmography. A needle was inserted in the left brachial artery in the antecubital fossa, and through it 0.9% (w/v) saline was infused at a rate of 4 ml./min. using a mechanically driven syringe. In most experiments the dose of bretylium was 12.5 mg. infused over a period of 4 min. The preparation of bretylium tosylate contained 1% benzyl alcohol as a preservative. Infusion of the vehicle alone as supplied by the manufacturers did not produce any change in forearm blood flow. In some experiments adrenaline hydrochloride or (−)-noradrenaline bitartrate was infused intravenously (10 μg./min.) or intra-arterially (1 μg./min.) for 4 to 5 min. Ascorbic acid (0·001%) was added to these infusates as a preservative (Gaddum, Peart and Vogt, 1949).

The various stimuli used to elicit vascular reflexes in the forearm or hand were as follows:

Valsalva Manœuvre.—The subject blew into a rubber tube, raising a column of mercury to a height of 50 mm. and maintained this without closing the glottis for 10 sec. This causes an increase in vasoconstrictor tone in skeletal muscle (Sharpey-Schafer, 1955; Roddie, Shepherd and Whelan, 1958).

Emotional Stress.—The subject carried out a mental arithmetic test, a series of additions and subtractions of two or three digit numbers as quickly as possible, with a metronome ticking and with frequent distractions and criticisms. Emotional stress causes excitation of choliner-

gic vasodilator fibres to skeletal muscle (Blair, Glover, Greenfield and Roddie, 1959).

General Body Cooling.—The room temperature was quickly lowered to 16 to 18°. The subject's feet and calves were immersed in a water bath at 17° and as much of the body surface as possible was sponged or sprayed with water at the same temperature. After the first few min. shivering ceased, and the subject was reasonably comfortable during the period in which observations were made. Body cooling causes an increase in vasoconstrictor tone in forearm skin (Roddie, Shepherd, and Whelan, 1957a; Blair, Glover and Roddie, 1959) and in the hand.

General Body Heating.—The feet and calves were immersed in a water bath at 43 to 45° and the subject was wrapped in blankets. Body heating causes reflex vasodilatation in the skin of the forearm and hand. In the hand this is due to release of vasoconstrictor tone (Arnott and Macfie, 1948; Gaskell, 1956; Roddie, Shepherd, and Whelan, 1957b), but in the forearm it is due to an active vasodilator mechanism (Grant and Holling, 1938; Edholm, Fox, and Macpherson, 1957; Roddie *et al.*, 1957a; Fox and Hilton, 1958).

Positive Pressure Breathing.—The subject breathed through a mouthpiece supplied with air at a pressure of 15 mm. Hg above atmospheric for 2 min. This causes reflex vasoconstriction in the forearm (Blair, Glover, and Kidd, 1959).

Single Deep Breath.—The subject carried out a single maximal inspiration and then resumed breathing as

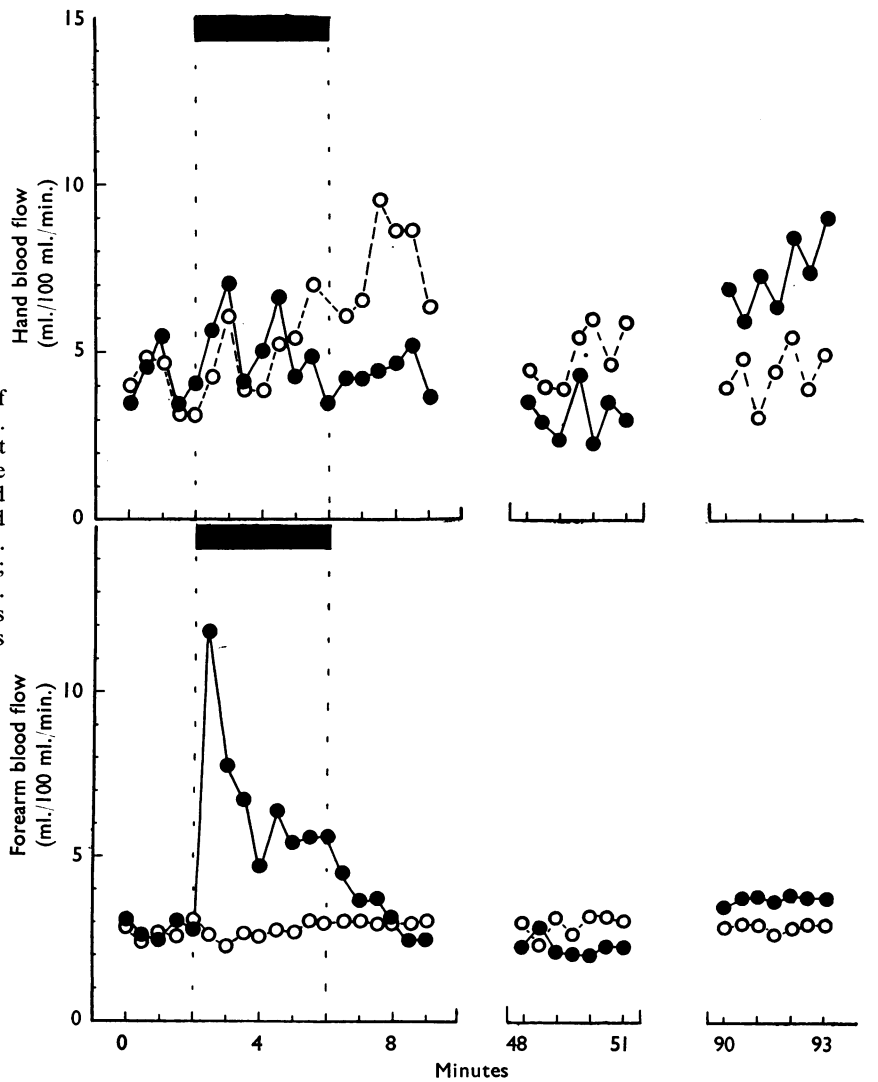

FIG. 1.—The effect of infusion of 12.5 mg. bretylium into the left brachial artery on the blood flow in the hand (upper panel) and forearm (lower panel). ●——● Left side; O——O right side. The black rectangles represent the periods of infusion.

normally as possible. This causes reflex vasoconstriction in the hand (Bolton, Carmichael, and Stürup, 1936).

Postural Changes.—The subject's legs were passively raised from the horizontal and maintained in a nearly vertical position for 2 to 3 min. This causes inhibition of vasoconstrictor tone in skeletal muscle (Roddie and Shepherd, 1956; Roddie, Shepherd, and Whelan, 1957c).

In one experiment the radial, median and ulnar nerves were blocked above the elbow with 3% ethocaine hydrochloride containing 0.002% adrenaline as described by Roddie, Shepherd, and Whelan (1957d).

Results

Effect of Bretylium on Forearm and Hand Blood Flow

The effect of an infusion of 12.5 mg. of bretylium on forearm blood flow is shown in Fig. 1 (lower panel). During the infusion there was a large transient and a smaller sustained increase in flow in the forearm infused with bretylium but not the control forearm. In one experiment 12.5 mg. bretylium was infused into the brachial artery on four occasions at 15 min. intervals. A quantitatively similar increase was seen on each occasion. After the infusion the flow fell to just below the level in the control forearm. There was little change in flow over the next 40 min. but after 80 min. the flow on the infused side was just above the control level. The results of 11 experiments illustrating the pattern of blood flow response following an infusion of bretylium are summarized in Fig. 2. Though there was considerable variation from subject to subject there was usually a fall in flow for about 30 min. but during the next hr. the flow returned to or above the previous resting level.

The response of the hand blood vessels to a similar infusion is shown by the experiment illustrated in Fig. 1 (upper panel) and the results of 7 such experiments are summarized in Fig. 3. During and immediately after the infusion flow was either little changed or reduced. About 1 hr. later, however, the blood flow on the experimental side had risen considerably above that on the control side. The size and time of onset of this increase varied greatly from subject to subject.

In one experiment forearm blood flow was measured in both arms, and the median, radial and ulnar nerves to one forearm were blocked above the elbow. This caused a large increase in blood flow on the blocked side (Fig. 4). Bretylium (12.5 mg.) was infused into the nerve-blocked side. A vasodilatation was seen during the infusion, but the flow after the infusion was much lower than the pre-infusion level.

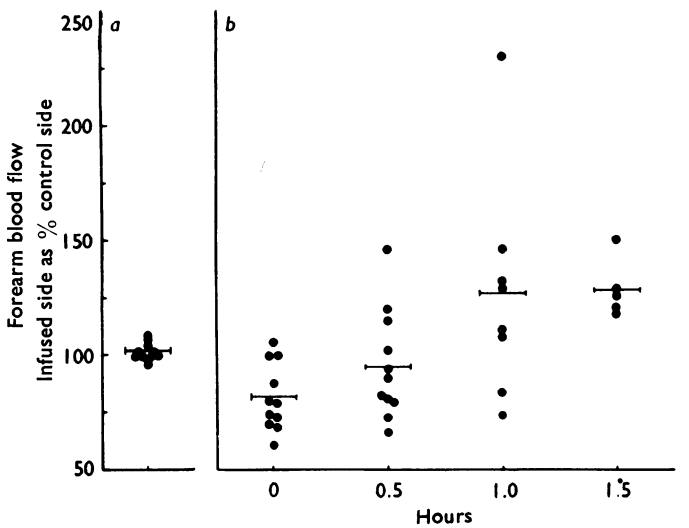

FIG. 2.—The effect of intra-arterial infusion of 12.5 mg. bretylium between *a* and *b* on blood flow in the forearm in 11 subjects. The blood flow on the infused side is expressed as a percentage of that in the control forearm. The dots represent the average values obtained over the 5 min. periods immediately before and after bretylium infusion and at subsequent 30 min. intervals.

Thirty min. after a second infusion the flows on the two sides were approximately equal, yet at this time muscular paralysis and skin anaesthesia were still complete.

Effect of Bretylium on Vascular Reflexes in the Forearm

Fig. 5 illustrates the effect of an intra-arterial infusion of 12.5 mg. of bretylium on some forearm vascular reflexes. After the infusion (1) the subject performed the Valsalva manœuvre (2). The usual vasoconstriction was seen on the control side, but there was a vasodilatation on the experimental side. During the period (3) emotional stress was produced by mental arithmetic; this caused an increase in flow in both the control and infused sides. The flows

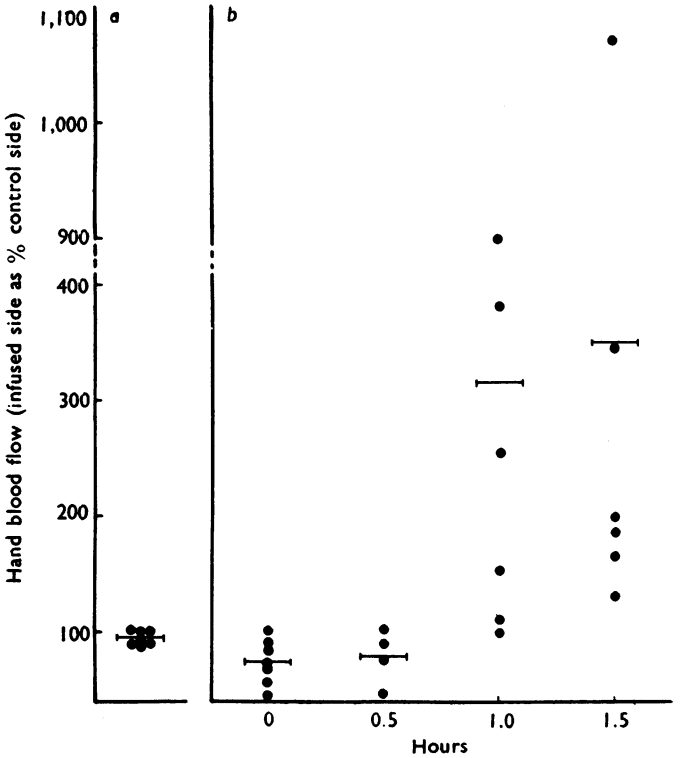

FIG. 3

FIG. 3.—The effect of intra-arterial infusion of 12.5 mg. bretylium between *a* and *b* on hand blood flow in 7 subjects.

FIG. 4.—The effect of intra-arterial infusion of bretylium on blood flow in the nerve-blocked forearm. At ↑ the radial, ulnar and median nerves to the left forearm were blocked. The black rectangles represent the periods when 12.5 mg. bretylium was infused into the left brachial artery. ●——● Left forearm blood flow; O——O right forearm blood flow.

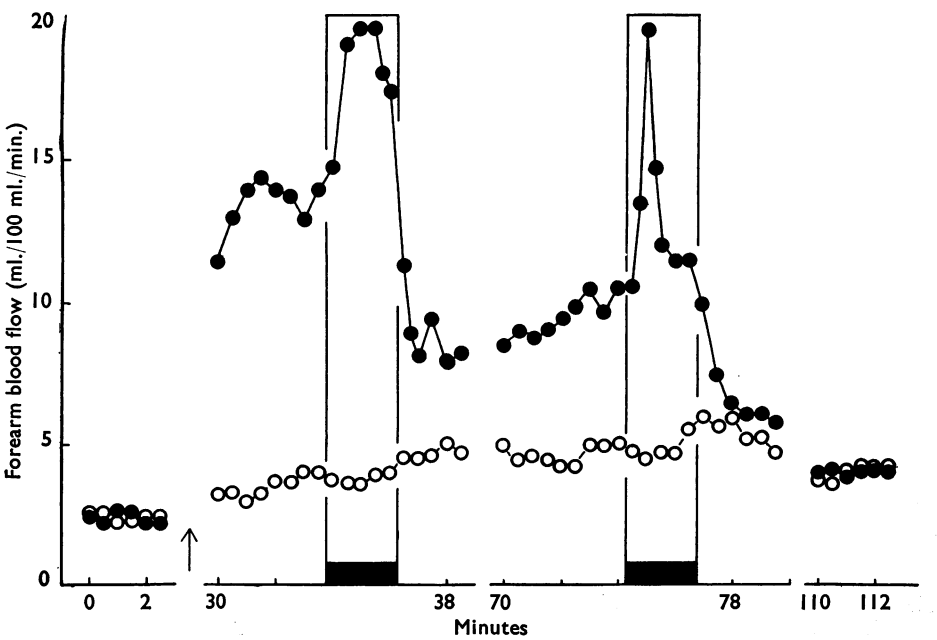

FIG. 4

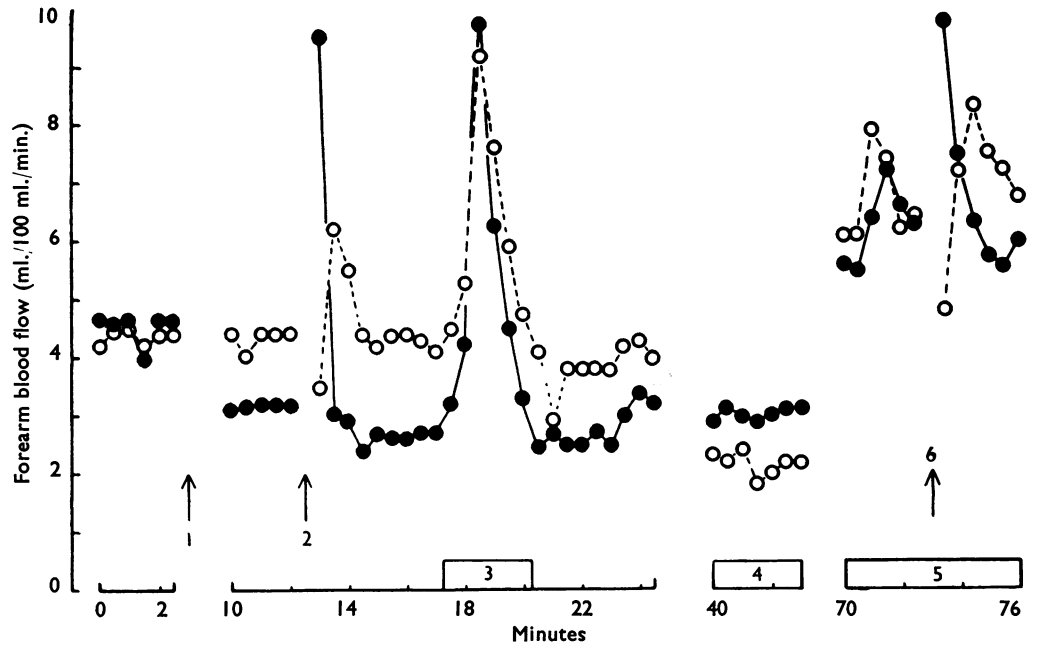

FIG. 5.—The effect of infusion of 12.5 mg. bretylium at (1) into the left brachial artery on the changes in blood flow in the forearm following the Valsalva manœuvre (2, 6) and during a mental arithmetic test (3), body cooling (4) and body heating (5). ●——● Left forearm blood flow; O——O right forearm blood flow.

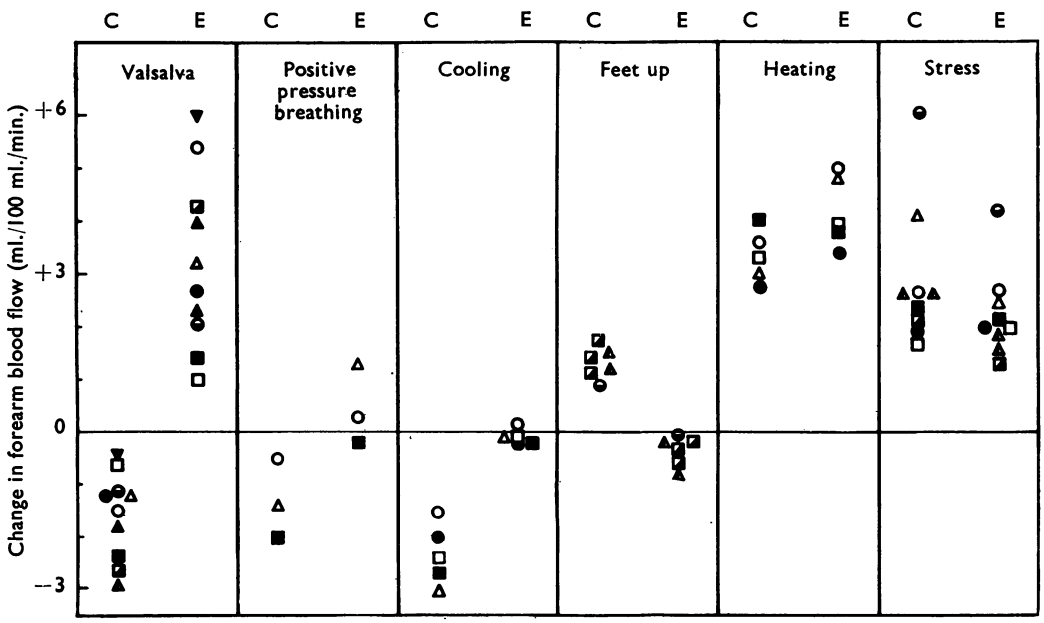

FIG. 6.—The effect of infusion of 12.5 mg. bretylium into the brachial artery on the changes in forearm blood flow following the Valsalva manœuvre and during positive pressure breathing, body cooling, body heating, emotional stress and passive raising of the legs of a recumbent subject. C, control forearm. E, experimental forearm.

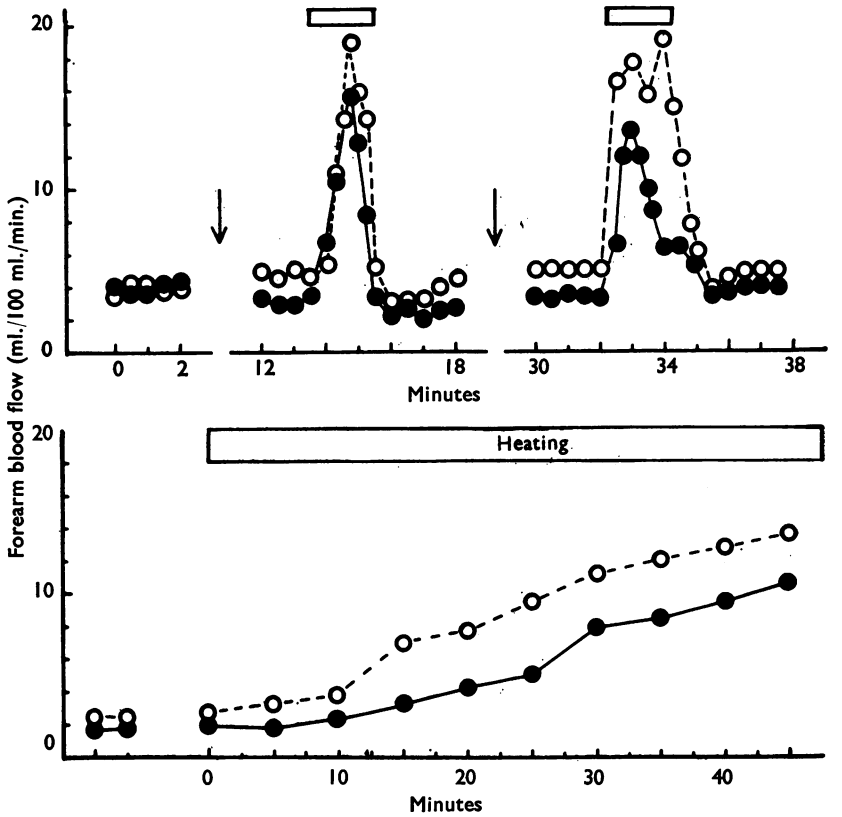

FIG. 7.—The effect of large doses of bretylium on the reflex responses in the forearm to emotional stress and body heating. Upper panel: 12.5 mg. bretylium was infused into the left brachial artery at first arrow and 37.5 mg. at the second arrow. During the periods represented by the open rectangles the subject was given a mental arithmetic test. Lower panel: 50 mg. bretylium was infused into the left brachial artery at the beginning of the experiment. The open rectangle represents the period when the body heating was carried out. ●——● Left forearm blood flow; O——O right forearm blood flow.

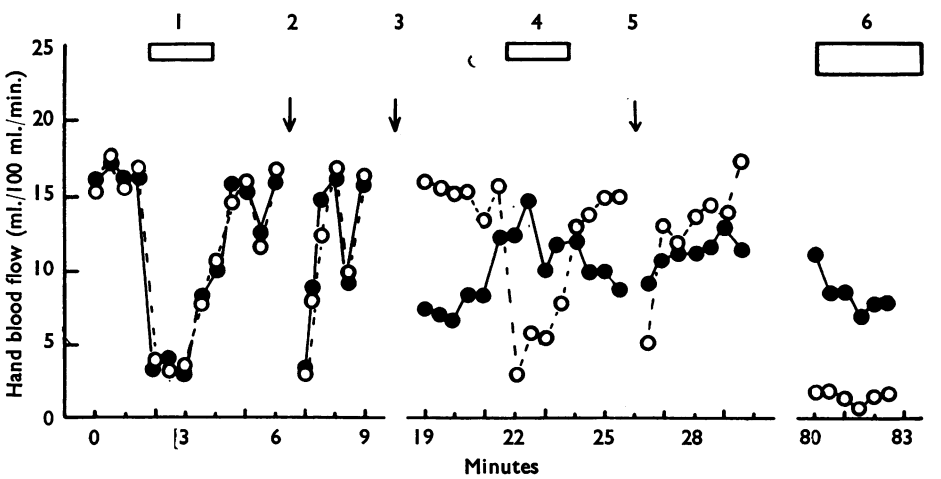

FIG. 8.—The effect of bretylium infusion (12.5 mg.) on vascular reflexes in the hand. During (1) the subject was given a mental arithmetic test. At (2) he made a deep inspiration and 12.5 mg. bretylium was infused into the left brachial artery at (3). At (4) and (5) the subject was given a mental arithmetic test and made a deep inspiration respectively. The flows during (6) were measured at the end of a period of body cooling.

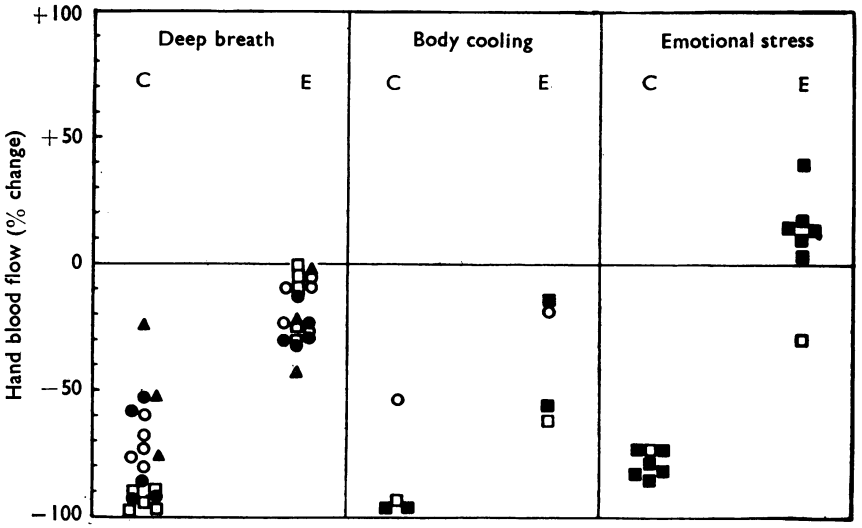

FIG. 9.—The effect of intra-arterial infusion of 12.5 mg. bretylium on the changes in hand blood flow during body cooling, emotional stress and following a deep breath. The mean blood flows during the tests in the control (C) and experimental (E) hands are expressed as percentages of the flow during the previous resting periods.

at (4) were measured after 15 min. of general body cooling. This caused a fall in flow on the control side but not the infused side. The flows at (5) were measured after a period of general body heating; there was an increase in flow in both forearms. The Valsalva manœuvre was repeated during this time (6), and the vasoconstrictor response was still abolished on the infused side. The results of all the experiments of this type on 10 subjects are summarized in Fig. 6. The vasoconstrictor response to the Valsalva manœuvre was reversed, and the responses to positive pressure breathing, leg raising and general body cooling were abolished on the infused side. However, this dosage of bretylium had little

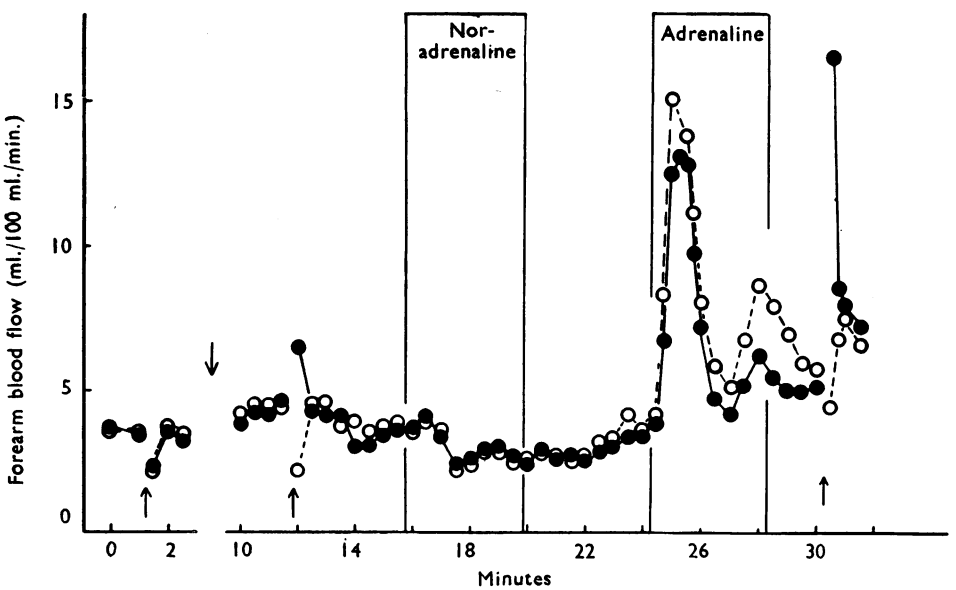

FIG. 10.—The effect of bretylium on the response of forearm blood flow to intravenous infusion of noradrenaline and adrenaline. Bretylium (50 mg.) was infused into the left brachial artery at the time indicated by the descending arrow. Valsalva manœuvres were performed at the times indicated by the ascending arrows. Adrenaline and noradrenaline (10 μg./min.) were infused during the periods represented between the vertical lines. ●——● Left forearm; O——O right forearm.

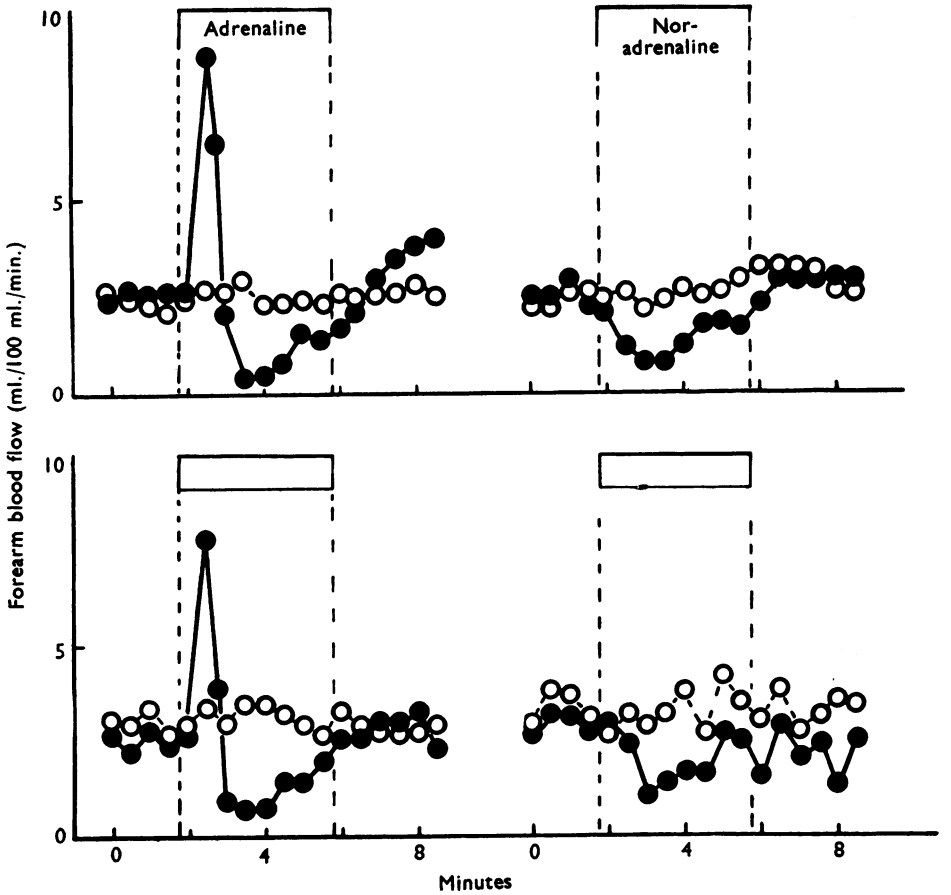

FIG. 11.—The effect of bretylium on the response of forearm blood flow to intra-arterial infusion of adrenaline and noradrenaline. Upper panel: before bretylium infusion. Lower panel: after bretylium infusion. Adrenaline and noradrenaline (1 μg./min.) were infused into the left brachial artery during the periods represented between the dotted vertical lines. ●——● Left forearm; O——O right forearm.

effect on the increase in forearm blood flow during emotional stress or general body heating.

Some experiments were performed to study the effect of large doses of bretylium on vasodilator reflexes in the forearm. In the experiment illustrated in Fig. 7a, 12.5 mg. of bretylium was infused into one forearm. This had no effect on the response to a mental arithmetic test. A further 37.5 mg. was infused into the same side, and the response to a mental arithmetic test was then reduced on the infused side. The same result was obtained in each of 2 similar experiments. In the experiment illustrated in Fig. 7b, 50 mg. bretylium was infused into one forearm, and general body heating was started at time 0. The vasodilatation was both delayed and reduced on the infused side. A similar result was obtained in one other experiment of this type. In neither case was sweating abolished on the infused side.

Effect of Bretylium on Vascular Reflexes in the Hands

Fig. 8 illustrates a typical experiment, and Fig. 9 summarizes the results of all of the experiments of this type on 5 subjects. The vasoconstrictor responses in the hands to mental arithmetic, a single deep breath and general body cooling were reduced or abolished following an intra-arterial infusion of 12.5 mg. of bretylium.

Effect of Bretylium on Response to Adrenaline and Noradrenaline

In the experiment illustrated in Fig. 10, 50 mg. bretylium was infused into the left brachial artery.

The formerly symmetrical Valsalva response was now reversed on the infused side, indicating successful sympathetic vasoconstrictor block. However, the response to the intravenous infusion of 10 μg./min. of noradrenaline and adrenaline respectively for 4 min. was similar on the two sides. Fig. 11 (upper panel) illustrates the response to an intra-arterial infusion of 1 μg./min. of adrenaline and noradrenaline. Bretylium (50 mg.) was then infused into the same side. The lower panel shows that the responses to subsequent adrenaline and noradrenaline infusions were unaffected by the bretylium. In 2 other experiments the response to noradrenaline only was tested in the same way. In one the vasoconstrictor response to noradrenaline was unchanged and in the other was slightly increased after bretylium.

Discussion

It is clear from the present experiments that the intra-arterial infusion of bretylium interferes with the reflex activity of vasoconstrictor nerves in the arm. Thus after infusion of bretylium there was no fall in forearm blood flow during body cooling or positive pressure breathing, or following the Valsalva manœuvre. Forearm blood flow did not increase when the subject's legs were passively raised. In the hand the vasoconstrictor responses to body cooling, mental arithmetic and a deep breath were either reduced or abolished. With such evidence of block of sympathetic vasoconstrictor nerves it was surprising that the blood flow often fell slightly following infusion of bretylium, for removal of sympathetic vasoconstrictor tone in the forearm (for example by acute nerve-block) is well known to increase the forearm blood flow 2 to 3 fold (Barcroft, Bonnar, Edholm and Effron, 1943). This suggested that bretylium had some action in addition to its blocking effect on sympathetic nerves. To study the direct effect of the drug, bretylium was infused into an acutely nerve-blocked forearm. Due to release of vasoconstrictor tone the blood flow through the nerve-blocked forearm was initially much higher than on the control side, but after the infusions of bretylium the flow fell to that in the control forearm, although at this time the nerve block was complete. This experiment indicated that in the absence of vasoconstrictor tone bretylium causes considerable vasoconstriction. This sympathomimetic effect could be due to the direct action of bretylium on the blood vessels or on the sympathetic nerve endings. The sharp initial vasodilatation and the smaller sustained vasodilatation seen during the infusion of bretylium also occurred in the nerve-blocked forearm, so these changes must be due to the direct action of the drug and not to release of vasoconstrictor tone.

The changes in flow after an infusion of bretylium were qualitatively similar in the hand and forearm. The flow was at first below that on the control side, but after 30 to 60 min. rose slightly above it. The state of the vessels at any time following infusion seemed to depend on the balance between the sympathomimetic action of the drug, and its sympathetic blocking effect. Immediately after infusion the sympathomimetic effect predominated but appeared to wear off over the next hour or so. This would explain why there is often an initial pressor response to bretylium (Boura and Green, 1959).

It is clear that the sympathomimetic effect is initially strong enough to compensate for the removal of the sympathetic tone present when in the supine position, since vasodilatation does not occur. The finding, that there is no great fall in peripheral resistance in the limbs, is in keeping with the observation that bretylium causes little fall in supine blood pressure (Boura *et al.*, 1959). However, because of the sympathetic blocking action, the usual increase in vasoconstrictor tone on adopting the erect posture is not possible; thus postural hypotension results.

In the animal experiments (Boura and Green, 1959) it was found that bretylium did not interfere with transmission at autonomic ganglia or at most caused only transient cholinergic block. It has been shown that the increase in blood flow that occurs in forearm skin during general body heating can be delayed and reduced by atropine (Roddie *et al.*, 1957a), and is therefore due, at least in part, to a cholinergic mechanism. With small doses of bretylium, the response to general body heating was little affected. After larger doses there was some reduction in the vasodilatation during body heating, but sweating was not obviously reduced. This suggests that cholinergic block, if it occurs at all, is very slight.

The vasodilatation in human skeletal muscle during emotional stress has lately been attributed in part to activation of cholinergic fibres (Blair, Glover, Greenfield and Roddie, 1959). In the present experiments the vasodilator response to a mental arithmetic test was often found to be unaffected at a time when other tests showed vasoconstrictor block to be present. These experiments provide mutual support for the findings that bretylium has little effect on cholinergic nerves, and also that vasodilatation can occur in the forearm during stress without involving the release of vasoconstrictor tone. In some experiments, particularly

with large doses of bretylium, there was some reduction in the response to stress on the infused side. This could be due to a slight degree of cholinergic block. Alternatively, reflex release of vasoconstrictor tone may occasionally contribute to the changes seen in the forearm during stress.

The present experiments indicate that the mode of action of bretylium is similar in man to that described in animals. Doses of bretylium which caused vasoconstrictor block did not reduce the responses to adrenaline or noradrenaline, given either intravenously or intra-arterially, and in one case the vasoconstrictor response to noradrenaline was increased. These results therefore provide further evidence that the action of the drug is sympatholytic rather than adrenolytic.

We wish to thank Dr. D. A. Long, of Burroughs Wellcome, for kindly supplying us with bretylium tosylate.

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THE ANTAGONISM OF MUSCLE RELAXANTS BY AMBENONIUM AND METHOXYAMBENONIUM IN THE CAT

BY

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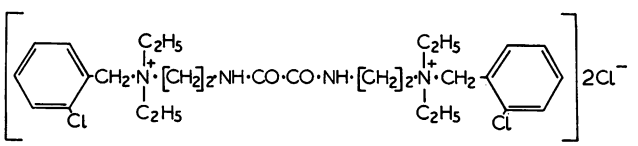
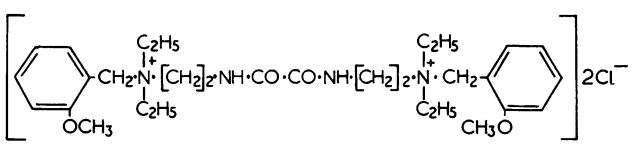
The ability of ambenonium and a methoxy analogue to antagonize paralysis produced either by tubocurarine or by decamethonium has been studied in the tibialis anterior muscle of the cat under chloralose anaesthesia. In small doses, both oxamides facilitated neuromuscular transmission, but in larger doses they depressed the sensitivity of the motor end plates to depolarizing substances and it is considered that this latter action is sufficient to account for their anti-decamethonium action. Although both compounds possess anticholinesterase activity, there was found to be no correlation between their relative abilities to antagonize tubocurarine paralysis and their abilities to inhibit muscle cholinesterase *in vitro*.

Ambenonium chloride (Mytelase; WIN 8077) and its methoxy analogue (WIN 8078) are members of a series of bisquaternary oxamides, synthesized in the Sterling-Winthrop Laboratories and first reported by Arnold, Soria, and Kirchner (1954).



Ambenonium chloride



Methoxyambenonium chloride

Both compounds have been shown to produce marked effects upon neuromuscular transmission (Arnold *et al.*, 1954; Lands and Karczmar, 1955; Lands, Karczmar, Howard, and Arnold, 1955; Lands, Hoppe, Arnold, and Karczmar, 1957; Karczmar, 1957; Lands, Hoppe, Arnold, and Kirchner, 1958) and were considered worthy of further study in the present experiments, particularly since one of them, methoxyambenonium, has been shown to be capable of antagonizing neuromuscular block produced in the tibialis anterior muscle of the cat both by tubocurarine and by decamethonium (Karczmar, 1957).

Methods

The experiments were carried out with 48 adult cats (1.6 to 4.9 kg.) anaesthetized with intravenous chloralose (80 mg./kg.).

For experiments on innervated muscles, shielded silver electrodes were placed on the sciatic nerve of one hind-limb and the nerve was ligated centrally to the electrodes. The limb was set up in a horizontal position on a Brown–Schuster myograph stand, and the tendon of the tibialis anterior muscle, and for certain experiments that of the soleus, were attached to flat steel spring myographs. Twitches, which were recorded on smoked paper, were excited by rectangular pulses of 0.2 msec. duration and of twice the strength required to evoke a maximal twitch. In a few experiments action potentials were simultaneously recorded from the tibialis anterior muscle by means of glass mounted platinum electrodes inserted through belly and tendon. After differential amplification by a Tektronix (type 122) battery-driven pre-amplifier, the action potentials were displayed on a Tektronix (type 502) double beam oscilloscope and photographed on 35 mm. film. For experiments on denervated muscle, the sciatic nerve was divided under pentobarbitone sodium anaesthesia and degeneration was allowed to proceed for from 17 to 19 days.

The drugs used were tubocurarine chloride, acetylcholine chloride, ambenonium chloride, methoxyambenonium chloride, decamethonium iodide and physostigmine salicylate. The doses quoted in the text refer to the salts. All drugs were dissolved in 0.15 M sodium chloride solution and were injected intravenously or intra-arterially. Since the main drugs under study

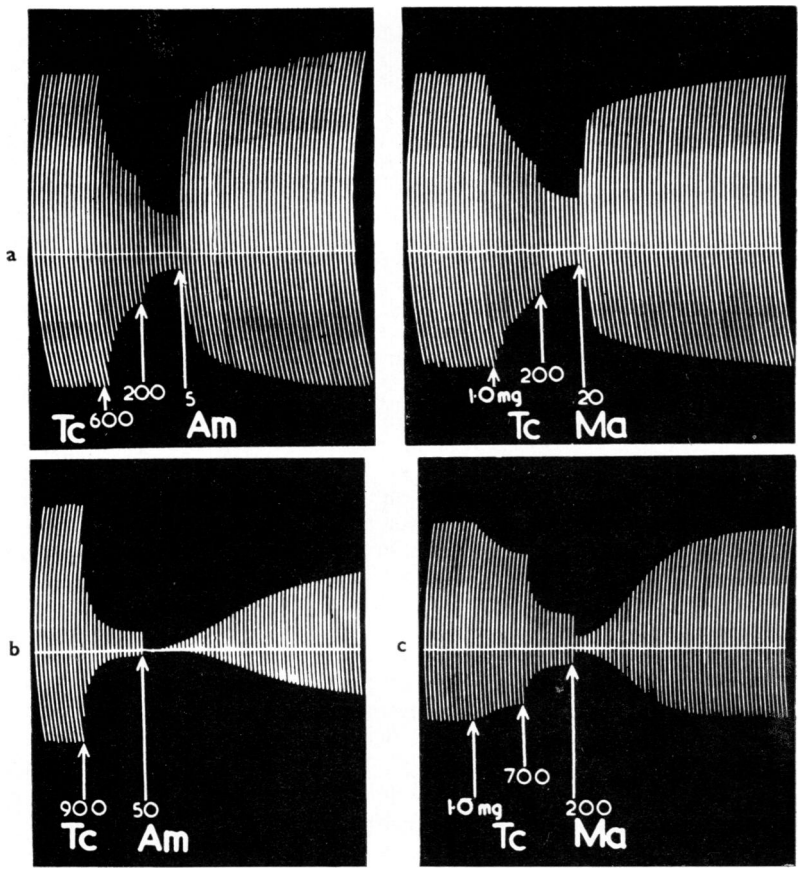


FIG. 1.—(*a*) Cat 1.75 kg.; (*b*) cat 3.3 kg.; (*c*) cat 4.9 kg. Maximal twitches of the tibialis anterior muscles were elicited indirectly once every 10 sec. At Tc, tubocurarine was injected intravenously. At Am, ambenonium, and at Ma, methoxyambenonium was injected intra-arterially. All doses in μg. unless otherwise stated.

were always injected intra-arterially close to the muscle, no adjustment for body weight was made. Intra-arterial injections were made towards the heart, in a volume of 0.2 ml. into the tibial artery below the tibialis anterior muscle, and at the moment of injection the femoral artery was occluded just peripheral to the saphenous branch; the injected drug therefore reached both the tibialis anterior and the soleus muscle. At the beginning of all experiments atropine sulphate (1.5 mg./kg.) was administered intraperitoneally, and arterial blood pressure was always recorded throughout experiments.

Cholinesterase was estimated manometrically using the Warburg apparatus. Cat tibialis anterior muscles were homogenized in an all-glass homogenizer to give a concentration of 150 mg./ml. in 0.04 M sodium bicarbonate (adjusted to *p*H 7.6 by the addition of hydrochloric acid) and 2.0 ml. of this homogenate were used for each estimation. The inhibitors were dissolved in 0.04 M sodium bicarbonate. Acetylcholine chloride was used as the substrate, the final concentration being 0.0138 M (Aldridge, 1950). The total volume of fluid in each flask was 3.0 ml. The flasks were gassed with a mixture containing 95% N_2 and 5% CO_2 for 10 min. and the manometers were read at 10 min. intervals for 30 min.

RESULTS

Effects on Tubocurarine Paralysis

In 6 experiments tubocurarine was administered intravenously in doses sufficient to reduce the indirectly excited maximal twitches of the tibialis anterior muscle by approximately 80% and ambenonium or methoxyambenonium was administered intra-arterially at the time of maximum block. The smallest dose of ambenonium necessary to produce a distinct anti-curare effect was of the order of

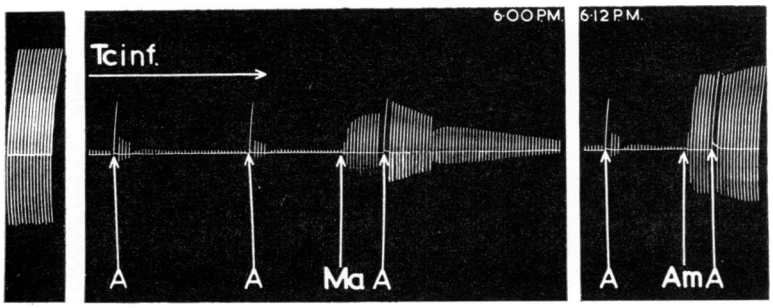


FIG. 2.—Cat 4.0 kg. Maximal twitches of the tibialis anterior muscle elicited indirectly once every 10 sec. A constant partial paralysis was maintained by a continuous intravenous infusion of tubocurarine (1.8 mg./hr.). At A, 100 μg. acetylcholine; at Ma, 10 μg. methoxyambenonium; and at Am, 3 μg. ambenonium injected intra-arterially.

0.05 μg. and the maximal effect was obtained with doses of 4 to 5 μg. Methoxyambenonium also antagonized tubocurarine paralysis,the corresponding doses being 0.2 μg. and 10 to 20 μg. respectively.

With further increase in the dosage of both compounds the antagonistic effect became progressively smaller until, with doses of the order of 50 μg. of ambenonium and 200 μg. of methoxyambenonium, the effect was reversed, both compounds then further deepening the paralysis produced by tubocurarine. Fig. 1 illustrates some of these results.

In 4 other experiments, a constant partial paralysis was maintained by a continuous intravenous infusion of tubocurarine. At intervals throughout the experiments, electrical stimulation was temporarily stopped and acetylcholine in a dose sufficient to produce a submaximal contraction was injected close-arterially. Ambenonium (3 to 5 μg.) or methoxyambenonium (10 to 20 μg.) was then injected and their effects on twitch tension and on the contraction produced by acetylcholine were studied. Both compounds caused a marked increase in the tension of the partially blocked twitches, but only ambenonium potentiated the contraction produced by injected acetylcholine. Fig. 2 illustrates these results.

Effects on Decamethonium Paralysis

Decamethonium blocks the tibialis anterior muscle of the cat by long lasting depolarization of the motor end plates (Zaimis, 1951; Burns and Paton, 1951). In the soleus muscle, on the other hand, the drug produces what Jewell and Zaimis (1954) called a dual mode of action block. That is, the substance starts its action by depolarizing the motor end plates, but during the blocking process the action changes and the block shows many of the characteristics of that produced by tubocurarine. For this reason, in 5 of these 15 experiments, maximal twitches of both muscles were recorded simultaneously. Decamethonium was administered intravenously in doses sufficient to reduce the twitch tension of the tibialis anterior muscle by approximately 80% and ambenonium and methoxyambenonium were injected intra-arterially at the height of the paralysis.

The effects produced by the oxamides in the soleus muscle closely resembled those already described for the tibialis anterior muscle when tubocurarine was the blocking agent used. Thus, small doses (5 μg. of ambenonium and 20 μg. of methoxyambenonium) antagonized the paralysis while larger doses (100 μg.) caused a further deepening. In the tibialis anterior muscle, small doses of ambenonium (0.2 to 1 μg.) often caused a small increase in the tension of the partially blocked twitches. With larger doses (5 to 10 μg.) the effect was reversed and a small decrease in tension was produced. Further increase in the dosage (20 to 50 μg.) again reversed the effect,with the result that a striking antagonism of the block occurred. With very large doses (200 μg. and above) the paralysis was again deepened, but later experiments showed that this effect was due to conversion of the decamethonium block to that of ambenonium itself.

Minimal effective doses of methoxyambenonium (4 to 5 μg.) antagonized the decamethonium block in the tibialis anterior muscle and this response increased with increase in dose, up to a maximum with doses in the range of 50 to 100 μg. Very large doses (400 μg. and above) caused a further deepening of the paralysis, but, as with ambenonium, this effect was shown to be due to a conversion from the

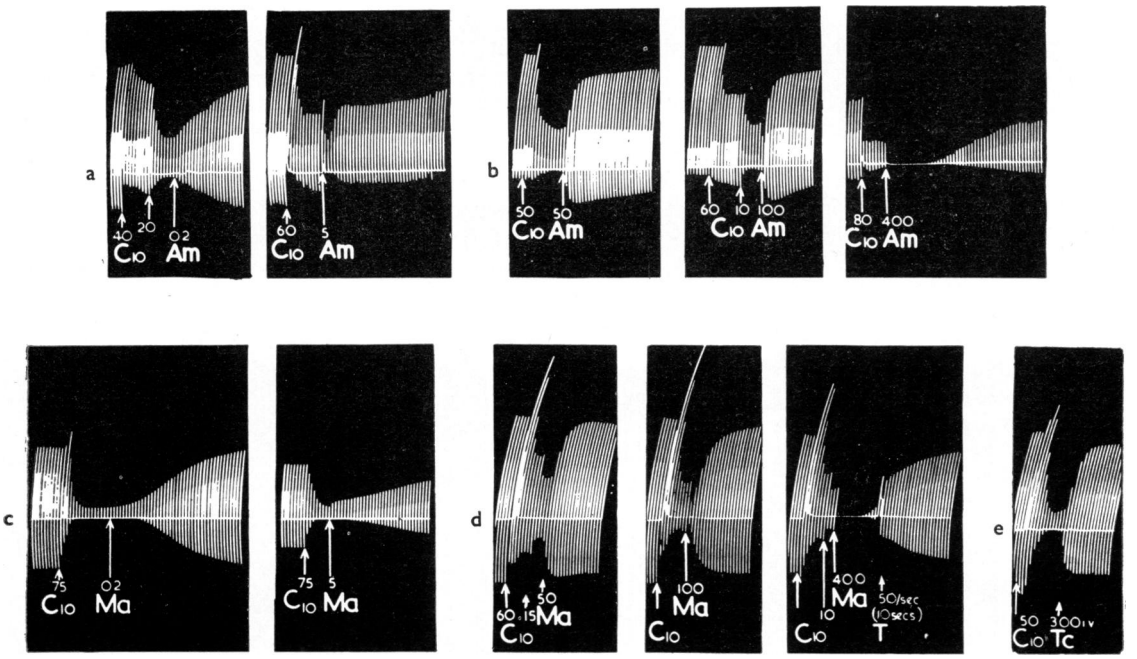


FIG. 3.—(*a*), Cat 2.8 kg.; (*b*) cat 3.1 kg.; (*c*) cat 3.1 kg.; (*d*) cat 2.3 kg.; (*e*) cat 3.0 kg. Maximal twitches of the tibialis anterior muscles elicited indirectly once every 10 sec. All doses in μg. At C_{10}, decamethonium was injected intravenously. At Am, ambenonium, and at Ma, methoxyambenonium were injected intra-arterially. At Tc, tubocurarine intravenously, and at T, tetanus 50/sec. for 10 sec.

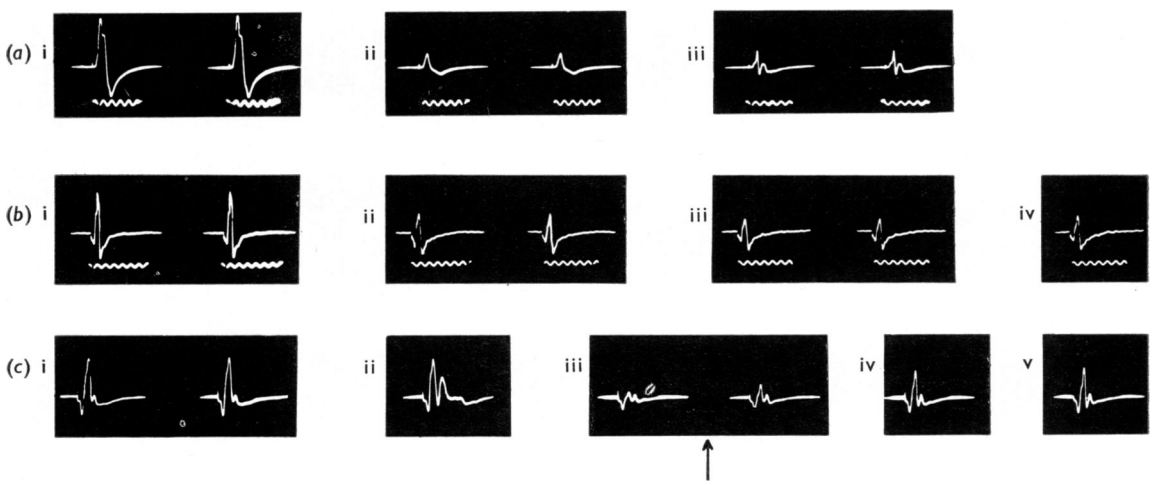


FIG. 4.—Maximal twitches of the tibialis anterior muscles were elicited indirectly once every 10 sec. (*a*) Cat 3.1 kg.; (i) normal muscle action potentials; (ii) action potentials recorded at the peak of the paralysis produced by the intravenous injection of 60 μg. decamethonium; and (iii) repetitive action potentials recorded 30 and 40 sec. after the intra-arterial injection of 1.0 μg. ambenonium. (*b*) Cat 5.0 kg.; (i) normal; (ii) at peak of paralysis produced by 75 μg. decamethonium; (iii) 20 and 30 sec. after intra-arterial injection of 10 μg. ambenonium; (iv) 100 sec. later. (*c*) Cat 3.2 kg.; (i) normal; (ii) repetitive firing elicited by 75 μg. decamethonium; (iii) at peak of decamethonium paralysis; at the arrow (↑) 100 μg. methoxyambenonium injected intra-arterially; (iv) 30 sec. later; (v) 100 sec. later. Time trace, 200 c/s.

decamethonium block to that of methoxyambenonium itself. These effects on the tibialis anterior muscle are illustrated in Fig. 3.

Recording of the gross action potential in tibialis anterior showed that repetitive firing initiated by decamethonium occasionally persisted in some muscle fibres throughout a partial neuromuscular block. The small increase in the twitch tension of the partially blocked muscle caused by very small doses of ambenonium (0.2 to 1 μg.) was accompanied by increased repetitive firing in some of those fibres which remained unblocked. The small increase in tension was not a result of more muscle fibres contributing to the gross tension, because the peak voltage of the action potential was not increased at this dose level. Larger doses of ambenonium (5 to 10 μg.), which deepened the paralysis produced by decamethonium, decreased the peak voltage of the action potential. Still larger doses (20 to 50 μg.) produced a true antagonism as shown by the fact that the peak voltage of the action potential was increased. With methoxyambenonium, the first two responses were absent and doses from 5 to 100 μg. increased the peak voltage of the gross action potential. Methoxyambenonium was therefore a more potent antagonist of decamethonium paralysis than ambenonium. These changes in the gross action potential are illustrated in Fig. 4.

Effects on Maximal Twitch and on Contractions Produced by Acetylcholine

In 9 experiments doses of ambenonium (3 to 5 μg.) previously shown to antagonize tubocurarine always potentiated both the indirectly excited maximal twitches and the contractions produced by intra-arterially injected acetylcholine (Fig. 5*a*). With larger doses (100 μg. and above), the maximal twitch tension was depressed and the responses to acetylcholine were abolished (Fig. 6*a*). In contrast, methoxyambenonium did not potentiate either response in any dose. The smallest effective dose (10 μg.) caused a depression of the contractions produced by acetylcholine (Fig. 5*b*). Large doses (200 μg. and above) depressed the maximal twitch tension and completely abolished the response to injected acetylcholine (Fig. 6*b*). During the depression of the indirectly excited maximal twitches produced either by ambenonium or by methoxyambenonium, direct stimulation of the muscle produced a normal contraction. Even in the maximum dose administered (800 μg.), neither of the oxamides by themselves caused a contraction of the muscle.

In 12 experiments on chronically denervated muscles, the only effect of the oxamides was to reduce the contracture produced by intra-arterially administered acetylcholine. Once again there was no evidence of a direct stimulant action of the compounds.

In a further series of 8 experiments, the characteristics of the block produced by large doses of the oxamides themselves were studied. Since ambenonium in particular is known to be a potent anticholinesterase (Lands *et al.*, 1955; Koelle, 1957) the blocking effects of both compounds were compared with that of physostigmine. During neuromuscular block produced by physostigmine, tetanic tension was not maintained and after the tetanus the paralysis was temporarily deepened. The administration of acetylcholine, decamethonium or tubocurarine also deepened the paralysis produced by physostigmine. In contrast, during partial paralysis produced by ambenonium or methoxyambenonium

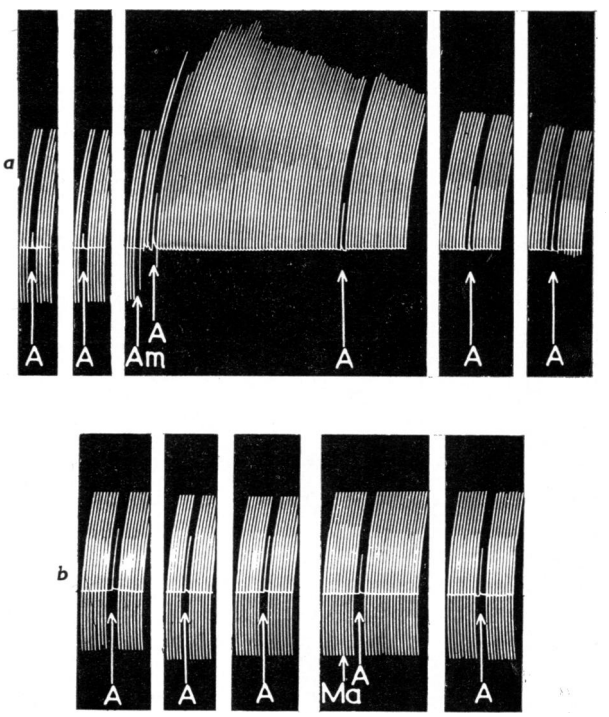


FIG. 5.—(*a*) Cat 3.2 kg.; (*b*) cat 2.6 kg. Maximal twitches of the tibialis anterior muscles elicited indirectly once every 10 sec. In (*a*) A=4 μg. and in (*b*) A=8 μg. acetylcholine. At Am, 3 μg. ambenonium, and at Ma, 10 μg. methoxyambenonium were administered. All injections intra-arterially.

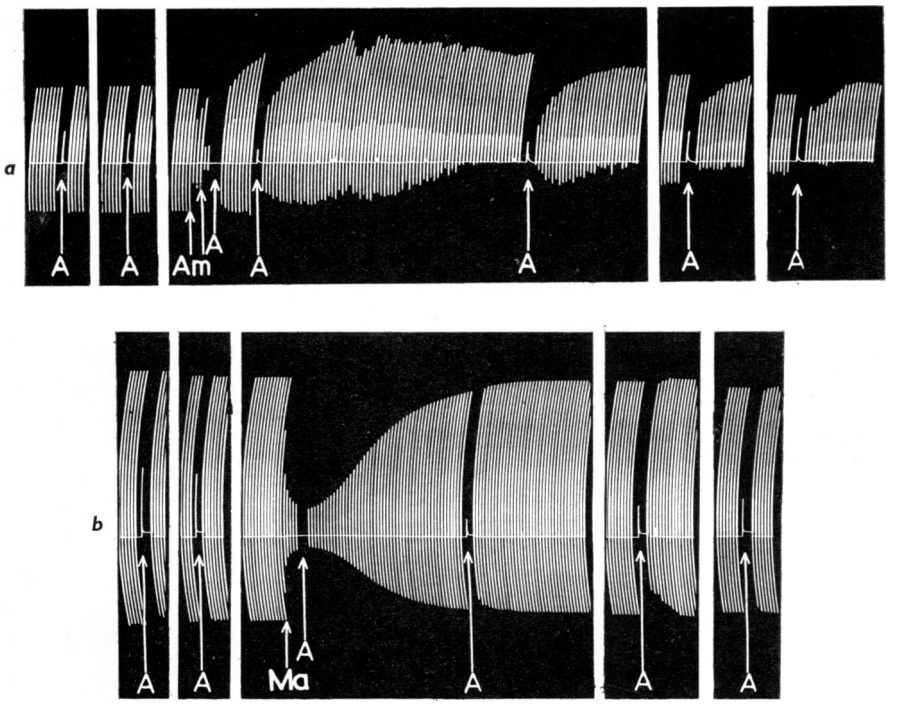


FIG. 6.—(*a*) Cat 2.15 kg.; (*b*) cat 3.6 kg. Maximal twitches of the tibialis anterior muscles were elicited every 10 sec. in (*a*), A=5 μg., and in (*b*), A=10 μg. acetylcholine. At Am, a total of 400 μg. ambenonium, and at Ma, 800 μg. methoxyambenonium was administered. All injections intra-arterially.

tetanic tension was well maintained and, after the tetanus, the paralysis was temporarily antagonized. Furthermore, although the block was deepened by the administration of tubocurarine, it was antagonized by the depolarizing substances, acetylcholine and decamethonium.

The experiments illustrated by Fig. 7 demonstrate the contrast between paralysis produced by physostigmine and that produced by methoxyambenonium.

Owing to the potent anticholinesterase action of ambenonium, antagonism of its blocking action was seen only with small doses of acetylcholine (approximately 10 μg.). With larger doses, the block was converted to that of acetylcholine itself. In contrast larger doses of acetylcholine (100 μg. and above) were required to antagonize the paralysis produced by methoxyambenonium. Such doses are similar to those necessary to antagonize tubocurarine paralysis (Hutter, 1952; Blaber and Bowman, 1959). The block produced by the oxamides was not antagonized either by edrophonium or by neostigmine.

Anticholinesterase Activity

The *in vitro* activity of the oxamides was estimated, using tibialis anterior muscle homogenates as the source of enzyme. Koelle (1957) has shown that ambenonium and methoxyambenonium are reversible inhibitors of cholinesterase and the enzyme activity was therefore estimated in the presence of the inhibitors. The results obtained are expressed in Fig. 8 and show that throughout the whole range of concentrations ambenonium was approximately 100 times more potent than methoxyambenonium in its ability to inhibit the enzyme. The pI 50 values were 8.2 for ambenonium and 6.0 for methoxyambenonium. These figures closely agree with those reported by Koelle (1957), who used cat brain as the source of the enzyme.

DISCUSSION

The results obtained confirm Karczmar's (1957) finding that methoxyambenonium is capable of antagonizing neuromuscular block produced in the tibialis anterior muscle of the cat either by tubo-

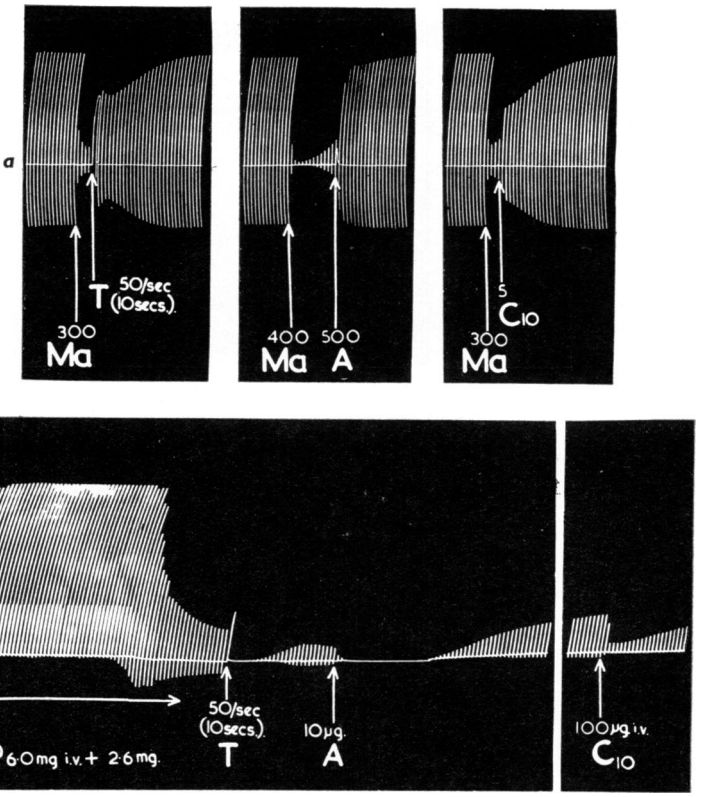


FIG. 7.—(*a*) Cat 4.9 kg.; (*b*) cat 3.2 kg. Maximal twitches of the tibialis anterior muscles elicited indirectly once every 10 sec. At Ma, methoxyambenonium; at A, acetylcholine; at C_{10}, decamethonium; and at P, physostigmine were administered. All doses are in µg. intra-arterially unless otherwise stated. At T, a tetanus was elicited by stimulation of the motor nerve.

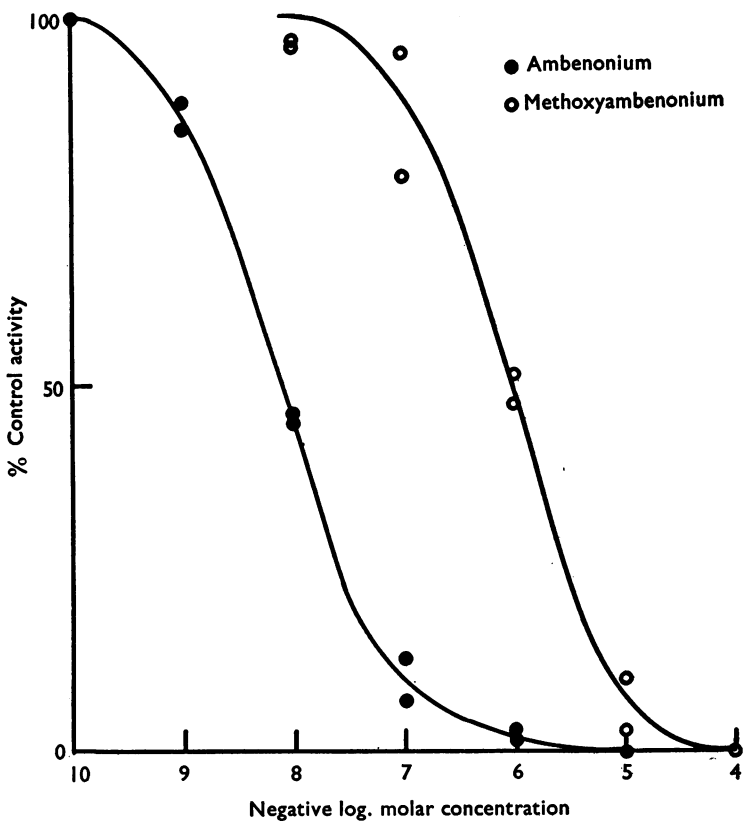


FIG. 8.—Inhibition of cholinesterase activity of cat tibialis anterior muscle homogenate by ambenonium and methoxyambenonium.

curarine or by decamethonium; in addition, they show that ambenonium also produces these effects. Tubocurarine and decamethonium are believed to cause paralysis in the tibialis anterior muscle of the cat by entirely different modes of action, but since the same compound could antagonize both, Karczmar concluded that his results cast serious doubt on the currently accepted theories of neuromuscular block produced by these drugs. However, as the present experiments emphasize, much larger doses than those required to antagonize tubocurarine are needed to produce a comparable relief of decamethonium paralysis, and at the higher dose level an entirely different action of the oxamides comes into play. Indeed the large doses capable of producing a marked antagonism to decamethonium paralysis actually deepened that produced by tubocurarine.

In the normal muscle, large doses of the oxamides themselves blocked both the response to injected acetylcholine and the indirectly elicited maximal twitches. Although ambenonium is a potent inhibitor of cholinesterase, an analysis of the neuromuscular block produced by the oxamides showed that it differed markedly from that produced by physostigmine. Rather, it resembled in many respects that produced by tubocurarine. Thus the block was antagonized by tetanus, by acetylcholine and by other depolarizing substances. Unlike that produced by tubocurarine, block produced by the oxamides was not antagonized by neostigmine or edrophonium and high frequency stimulation of the motor nerve during the paralysis produced a well sustained tetanus. These differences can probably be accounted for by the fact that the oxamides possess a facilitatory action on neuromuscular transmission, in addition to their blocking action at the motor end plates. Whatever the detailed mechanism of their blocking action, however, the important factor is that large doses of the oxamides raise the threshold of the motor end plates to the action of depolarizing substances. This effect is therefore sufficient to explain their antagonistic action to decamethonium paralysis since small doses of tubocurarine (see Fig. 3*e*) will produce the same effect (Dallemagne and Philippot, 1952). Although ambenonium was more potent than methoxyambenonium in other respects, it was less potent as an antagonist of decamethonium paralysis. This can probably be explained by the powerful anticholinesterase action of ambenonium, since persisting acetylcholine would tend to increase the decamethonium block and thereby mask its antagonistic action at the motor end plates to some extent. In fact, potentiation of decamethonium paralysis was seen with intermediate doses of ambenonium but not of methoxyambenonium.

Small doses of the oxamides produced a powerful antagonism to tubocurarine paralysis, but only ambenonium increased the contraction produced by injected acetylcholine and potentiated the indirectly elicited maximal twitch tension in the unblocked muscle. These differences between the two oxamides are again explainable by the finding that ambenonium is a much more powerful anticholinesterase than methoxyambenonium. Throughout the whole range of effective concentrations, methoxyambenonium was found to be about 100 times less powerful than ambenonium in its ability to inhibit muscle cholinesterase, but in spite of this, it was only 3 to 4 times less effective in its ability to antagonize tubocurarine. These results add some support to the suggestion (Bowman, 1958; Blaber and Bowman, 1959) that cholinesterase inhibition is not the main mechanism responsible for the anti-curare action of anticholinesterases but that such inhibition does play an important part in their ability to potentiate the maximal twitch tension of unblocked muscle.

Methoxyambenonium did not potentiate the response of the innervated muscle to injected acetylcholine and neither oxamide potentiated that of the chronically denervated muscle. These results make it unlikely that an increase in the sensitivity of the effector cell plays any part in the anti-curare effect. Results obtained with other anticholinesterases led Bowman (1958) and Blaber and Bowman (1959) to suggest that a pre-synaptic action, through which the amount of acetylcholine liberated by a nerve impulse is increased, might play a part in their anti-curare action and a similar mechanism would explain the results obtained with ambenonium and methoxyambenonium in the present experiments.

In conclusion, it can be said that, although most of Karczmar's results have been confirmed, they are capable of a different interpretation and can readily be explained on a basis of the current theories of neuromuscular block produced by tubocurarine and decamethonium. In small doses the oxamides facilitate neuromuscular transmission in some way, as shown by their antagonism of tubocurarine paralysis. In larger doses they depress the sensitivity of the motor end plates to depolarizing substances and this accounts for their ability to antagonize decamethonium. The oxamides are not alone in their ability to produce these responses; other facilitatory substances, which in large doses possess a weak curare-like action, may be capable of the same effects. For example, tetraethyl-

ammonium causes a pronounced antagonism to tubocurarine paralysis (Stovner, 1958; Koketsu, 1958; Blaber and Bowman, 1959), but very large doses themselves produce a curare-like block of the indirectly excited maximal twitches and at this dose level the substance deepens block produced by tubocurarine and antagonizes that produced by decamethonium (Blaber and Bowman unpublished).

I am indebted to Bayer Products and to Sterling-Winthrop Research Institute for their generous gifts of ambenonium and methoxyambenonium respectively. My thanks are also due to Dr. W. C. Bowman for his constant advice and encouragement throughout this work, and to Mr. D. Tulett for technical assistance.

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BRITISH JOURNAL OF PHARMACOLOGY AND CHEMOTHERAPY
SEPTEMBER, 1960. VOL. 15. No. 3.

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Printed in Great Britain by Fisher, Knight & Co. Ltd., Gainsborough Press, St. Albans, Herts.

ERRATUM

In the paper by M. J. Greenberg in the September issue, p. 369, Fig. 3, the concentrations of 5-hydroxytryptamine should read from left to right 3×10^{-9} 3×10^{-8} 3×10^{-7} 3×10^{-6} 3×10^{-5}.