FOREWORD

Biochemical Pharmacology is an international journal which will be devoted to "research into the development of biologically active substances and their mode of action at the biochemical and sub-cellular level". Promising reasonable speed of publication, it will provide a forum for the discussion of all phases of pure and applied cellular pharmacology.

One has many times pondered on the inexpressible debt owed by our subject to the pioneering endeavours of A. J. Clark, on his continuing influence, and on the key advances which have occurred during the relatively short space of time since his premature and lamented death—advances which he would certainly have greeted with characteristic delight. I believe he would have welcomed this journal, as furthering our knowledge of what he called the action of drugs on cells, and the modern approach thereto.

Special emphasis is to be given to cancer chemotherapy and related studies, and is already apparent in this first issue. Over ten years ago, R. A. Peters predicted the usefulness for cellular pharmacology of the radiomimetic and related substances which were then emerging into our general view from the hitherto restricted field of chemical warfare. The prediction has been amply fulfilled. During this period swords have been beaten into ploughshares, and the process is reflected in many succeeding papers dealing, among others, with such topics as the reactivity of the biological alkylating agents, enzyme-activated mustards, and the physico-chemical aspects of the action of the sulphonic acid esters. Yet this is only a stage. The alkylating agents are, as it were, reactive molecular spanners which we can throw into the cell machinery, and which can send back information on its innermost secrets. Hence, their over-riding importance is probably fundamental, through the light they may shed on the carcinogenic process and on cellular mutation and variability—in themselves largely questions of the action of drugs on cells—with all that this may mean for the future.

Advances in this particular field are, however, only an example. Equally striking and indeed spectacular progress has occurred over the past ten years in many other branches of cellular pharmacology, and the omens are that the process must expand and accelerate. To encourage and record it will be the main function of *Biochemical Pharmacology*, as the latest medium, and one which can prove itself to be not among the least.

ALEXANDER HADDOW

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THE BIOCHEMISTRY OF 2-DEOXY-2-FLUORO-DL-GLYCERALDEHYDE WITH A NOTE ON THE TOXICITY OF 2-DEOXY-2-FLUOROGLYCEROL

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Abstract—The compound (FGA) is toxic to the mouse and rat, producing convulsions and accumulations of citrate, particularly in heart and kidney. It has little effect upon glycolysis, but is readily phosphorylated by glycerokinase. Evidence is presented that with kidney particles, some of the FGA is converted to fluoroacetate, some to a compound which can inhibit an enzyme acting somewhere between malate and citrate. In brain, FGA inhibits respiration in part by a non-specific aldehyde effect, in part by conversion to some other respiratory inhibitor. The toxicity of FGA is probably due to a conversion of at least a part of it to fluoroacetate. 2-Deoxy-2-fluoroglycerol was also found to be toxic to the mouse and to cause citrate accumulation.

The study of this compound (referred to below as FGA) was undertaken to aid in the elucidation of normal metabolic pathways and in the development of new pesticidal or therapeutic agents. FGA was first prepared by Taylor and Kent.[1] Glyceraldehyde has been known for some time on the one hand as a glycolysable compound and as a substrate for liver glycerokinase,[2,3] and on the other as a potent inhibitor of anaerobic (but not aerobic) glycolysis in brain, and of respiration in brain.[4,5] The inhibition of brain respiration occurs only when glucose is present, for endogenous respiration is not affected;[6] the inhibition cannot be prevented by pyruvate addition[7,8] although pyruvate prevents inhibition of glycolysis by glyceraldehyde in tumours.[9] Although L- and D-glyceraldehyde are utilized at equal rates in liver glycolysis, the L- is far more effective than the D- form as an inhibitor of brain glycolysis and respiration.[5] Breusch[10] has reported that cat liver contains two enzyme systems capable of oxidizing glyceraldehyde, one of which is inhibited by calcium ions.

MATERIALS AND METHODS

FGA and 2-deoxy-2-fluoroglycerol were synthesized by Drs. Taylor and Kent and generously presented to us by them. The FGA was dissolved in water and stored frozen at −19°C. No differences were noted between freshly prepared or frozen-stored FGA. Reagents, analyses and the techniques of glycolysis and evisceration were as described by O'Brien and Peters.[11]

Kidney and brain particles were prepared as follows: one whole pigeon brain or two kidneys from a female guinea-pig (about 400 g body weight) were removed from freshly killed animals, placed in iced 0·9 per cent KCl and weighed therein. All subsequent operations took place in the cold-room at 2°C. In the case of kidney, the medulla

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was cut out and discarded. The tissue was ground thoroughly in a cold mortar, then 0·9 per cent KCl was added dropwise with continuous grinding, to a volume of about 15 ml. As has been previously stressed in this laboratory, any attempt to add large volumes of KCl at the start leads to coagulated rather than dispersed particles. The suspension was filtered through muslin, and rubbed through with a glass rod, then centrifuged in a Servall for 15 min at 6000 g. The supernatant was discarded, and the precipitate taken up in 0·9 per cent KCl, which was added very slowly and with much stirring. The volume was adjusted to give a concentration of 20 per cent w/v with respect to the original tissue. An appropriate volume of the enzyme preparation was mixed with iced buffer, substrate and cofactors, and the iced mixture was pipetted into iced Warburg flasks containing the inhibitor (or other variable component). By this procedure the pipetting of very small volumes was minimized. The flasks were removed from ice, placed on the manometers and immersed in the bath (38°C) at zero time, using a stop-watch to ensure that each flask was immersed in the bath precisely 30 sec after the preceeding one. The manometers were closed off in turn, precisely 10 min after the given flask had been immersed in the bath. Four readings, at ten minute intervals, were usually taken. The flasks were then removed in turn in time to allow the addition of 1 ml of 25 per cent trichloracetic acid at 60 min from zero. After standing for about 15 min, the flask contents were filtered and washed with 5 per cent trichloracetic acid, with which the volume was then made to 25 ml. Citrate was determined on duplicate 5 ml samples.

The amounts present in each Warburg flask were: enzyme preparation 1·0 ml; phosphate buffer 0·25 M, pH 7·2, 0·5 ml; disodium adenosine triphosphate (ATP) 0·5 mg plus $MgCl_26H_2O$, 0·835 mg in 0·5 ml; substrate for kidney, sodium fumarate 3·5 mg (2·2 μmole) or sodium citrate 6·43 mg (2·2 μmole) in 0·5 ml; substrate for brain, sodium pyruvate 3·52 mg (32 μmole) plus sodium fumarate 0·7 mg (4·4 μmole) in 0·5 ml. The volume was made to 3·5 ml with inhibitor or 0·9 per cent KCl. Unless otherwise stated, the respiration figures given below are for the oxygen taken up between 10 min and 40 min from zero time.

The figures given for a number of the results show the cumulative inhibition of the total O_2 at that time, given as per cent inhibition of respiration at time t; and not of the increment at that time. "Zero time" was taken from the time of removal of flasks from ice to enable extrapolation to the first minutes. It is to be noted that the per cent inhibition e.g. in Fig. 7 is calculated between two time intervals, say between 5 and 10 min, it would therefore be strictly more accurate to plot at 7·5 min. This should be kept in mind in interpretation. The accuracy of the figures is lower for small values of t, and this is especially true for kidney, in which the first readings are $\pm$ 10 per cent, those at 60 min are $\pm$ 1 per cent.

Aconitase was assayed as follows: 0·1 ml of the activated enzyme[12] was added to 2·0 ml neutral DL-*iso*citrate 0·25 mM, then 0·3 ml of water or inhibitor was added, then 0·3 ml of phosphate buffer, 0·5 M, pH 7·8. The preparation was incubated at room temperature for 15 min, then 0·2 ml of neutral 50 mM DL-*iso*citrate was added and the optical density change followed, at 1 min intervals, at a wavelength of 240 mμ. The order of addition of reagents was extremely critical.

The studies on carboxylic acids in respiring kidney particles were carried out with amounts four times those given above for the Warburg experiments. The substrate was fumarate. Incubation was at 38°C in open flasks, with shaking. After 1 hr, 2·5 ml of 60 per cent perchloric acid was added. The precipitate was filtered and washed with

10 ml of 10 per cent perchloric acid; filtrate and washings were neutralized to phenol red with 20 per cent KOH, cooled to 0°C, filtered, and the precipitate washed with a little cold 1 per cent KCl. The filtrate was reduced to dryness on the steam bath and chromatographed on a silica gel column as described by Bulen, Varner and Burrell.[13] Five ml fractions were collected; only the 5, 15, 25 and 35 per cent butanol –chloroform solvents were used.

RESULTS

Effects in vivo

The intraperitoneal injection of FGA into mice and rats produced a condition substantially similar to that described for DL-1-deoxy-1-fluoroglycerol[11] which in several ways was like that produced by fluoroacetate. At a dose of 25 mg/kg, there were no deaths in the mice (males); but the citrate levels of kidney and heart were raised. At 50 mg/kg the animals died in about 30 hr, and as Table 1 shows, the citrate levels in the heart and kidney at death were extremely high. In the male rat, 22 mg/kg of FGA was non-toxic, but 100 mg/kg was lethal and produced citrate accumulation of the same order as reported in Table 1 for mice.

TABLE 1. THE EFFECT OF FGA UPON CITRATE LEVELS IN THE MOUSE

Dose (mg/kg)	Number of animals	Time to death (hr)	Citrate* (μg per g)			
			Heart	Kidney	Liver	Brain
0	3	—	119± 18	83± 9	42± 3	153± 34
20	2	—	256	301	156	211
			233	554	75	—
50	4	36±6	1233±846	889±119	114± 16	414±116

Mice were male albinos, injected intraperitoneally.
Figures are means±standard deviations.

Effects on kidney particles

Fluoroacetate and fluorocitrate, which resemble FGA in being toxic and causing citrate accumulation in tissues, induce large citrate accumulations with kidney particles respiring in the presence of fumarate.[14,15] FGA was observed to inhibit the oxygen uptake of such particles, the effect increasing with concentration; but citrate accumulation was only substantial at 100 μg per flask (0·33 mM) and was less at higher or lower concentrations (Fig. 1). The inhibition of respiration increased linearly with time, being negligible at zero time (Fig. 2).

It seemed possible that the reduction of citrate accumulation at high FGA levels was due to an inhibition of an enzyme concerned in the conversion of fumarate to citrate, superimposed upon an effect due to aconitase inhibition. This would give a reduced supply of citrate at high FGA levels, and since the observed citrate level is a function of the difference between the rates of supply and removal of citrate, could produce low citrate levels in spite of substantial aconitase block. This possibility was substantiated by observing the effect of FGA upon citrate disappearance from kidney particles, i.e. with citrate rather than fumarate as a substrate. The results (Fig. 3)

* Calculated as citric acid.

showed that citrate disappearance was inhibited, and therefore aconitase was blocked, to an extent proportional to the FGA concentrate. Further substantiation was obtained by showing that with fumarate as substrate, the citrate accumulation caused by fluoroacetate or fluorocitrate (known to inhibit aconitase specifically) is reduced by high levels of FGA (Table 2).

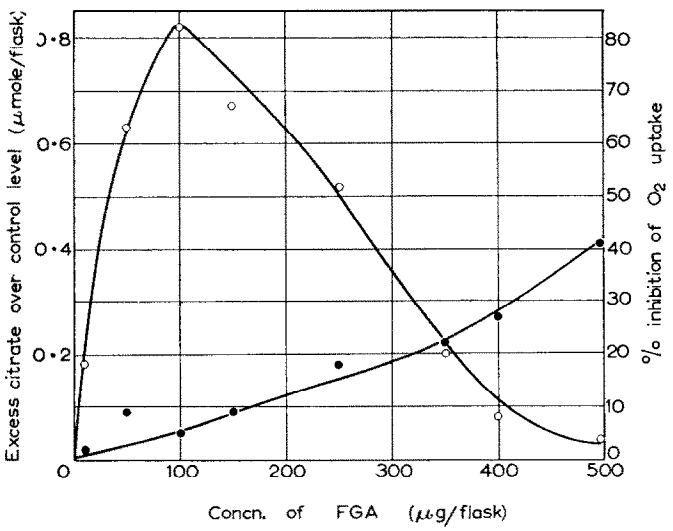

FIG. 1. Effect of concentration of FGA upon citrate accumulation (○) and respiration (●) of kidney particles.
Substrate: fumarate.

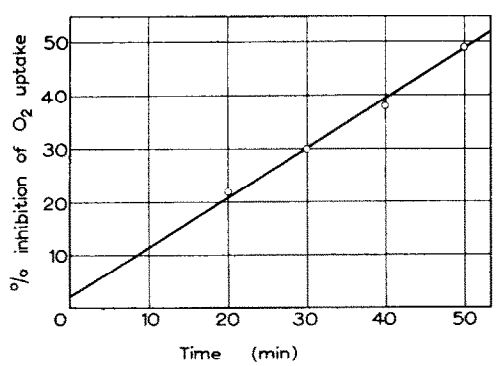

FIG. 2. Variation, with time, of inhibition of kidney particle respiration by FGA.

Experiments were carried out to compare the effects observed above with those given by fluoroacetate. In this case a considerable variation was encountered: at one extreme, the respiration of the kidney particles was very markedly inhibited by the compound (e.g. 1 mg per flask of fluoroacetate gave 73 per cent inhibition) and the citrate level in flasks treated with 1 mg was up to 1 μmole less than in those treated with 0·1 mg. Fig. 4 gives the mean values for three such experiments. In other preparations, the respiratory inhibition was much less (e.g. 30 per cent by 1 mg of fluoroacetate) and the citrate level remained at the same high level for 1 mg as was given

TABLE 2. EFFECT OF FGA UPON THE CITRATE ACCUMULATION CAUSED BY FLUOROACETATE OR FLUOROCITRATE IN RESPIRING KIDNEY PARTICLES

Inhibitors	Per cent inhibition of respiration	Citrate (μmoles per flask)
Nil	—	0·31
FGA 500 μg	62	0·33
Fluorocitrate 5 μg	51	0·71
Fluorocitrate 5 μg + FGA 500 μg	79	0·47
Fluoroacetate 250 μg	70	0·72
Fluoroacetate 250 μg + FGA 500 μg	77	0·45

Substrate: fumarate, 22 μmoles per flask.
Enzyme 1·0 ml; phosphate buffer 0·25 M pH 7·2, 0·5 ml; Na_2ATP 0·5 mg plus $MgCl_2 6H_2O$ 0·835 mg in 0·5 ml; Na fumarate 2·2 μmole ± inhibitor. Total volume made to 3·5 ml with 0·9 per cent KCl.

by 0·1 mg. This variability in response (which was never observed with FGA) is probably connected with the fact that some kidney samples gave very low or negligible respiratory rates. The results obtained with such samples are not given in this paper. Some 5 per cent of the kidneys taken were deficient in this way. Such poor kidneys were usually pale in colour and sometimes mushy in texture. They were found most commonly in old male guinea-pigs, least in young females. This kidney condition is due to

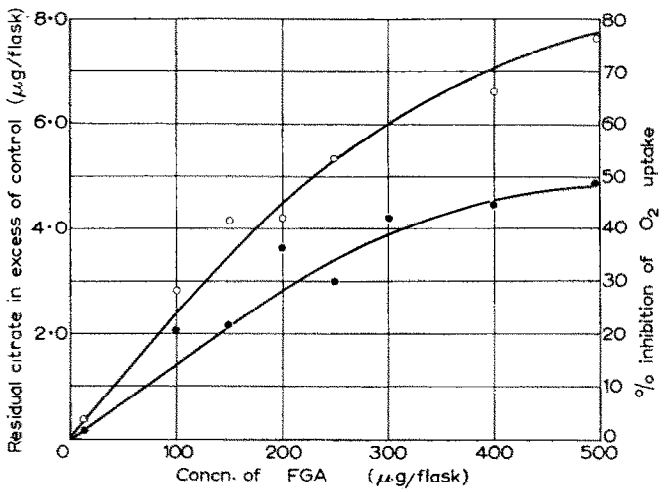

FIG. 3. Effect of concentration of FGA upon citrate utilization (○) and respiration (●) of kidney particles.
Substrate: citrate.

a nephritis endemic in this and possibly other guinea-pig populations.[16] The kidneys in best condition have the highest respiratory rate, which is least inhibited by fluoroacetate, and do not show the phenomenon of lowered citrate levels with increasing fluoroacetate concentration.

Fig. 5 shows how the respiratory inhibition, caused by fluoroacetate, varies with time. The kidney sample was in good condition, judged by the criterion of small

respiratory inhibition With less excellent specimens, the curves corresponding to Fig. 5 do not flatten out so rapidly.

The finding of citrate accumulations following FGA treatment both *in vivo* and *in vitro* suggested that at least some of the FGA was metabolized fairly readily to

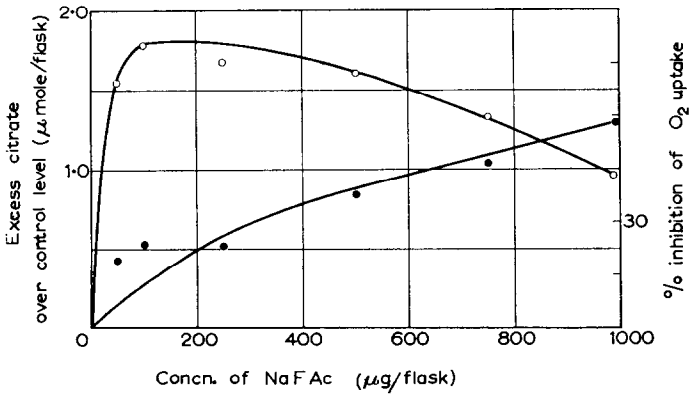

FIG. 4. Effect of concentration of Na fluoroacetate upon citrate accumulation (○) and respiration (●) of kidney particles.
Substrate: fumarate.
Na fluoroacetate 1000 μg = 10 μmoles.
Figures are means of three experiments.

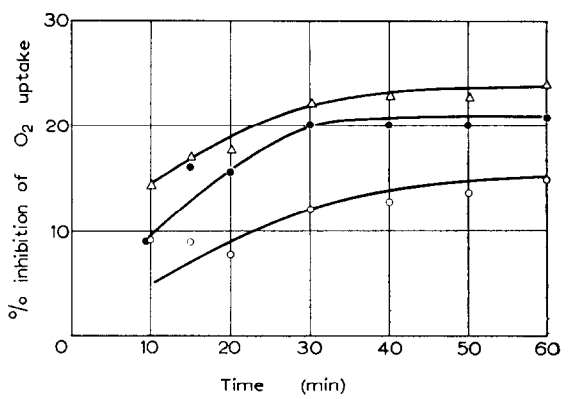

FIG. 5. Variation, with time, of inhibition of kidney particle respiration by fluoroacetate.
△ Top curve: 1000 μg fluoroacetate per flask (2·9 mM).
● Middle curve: 250 μg.
○ Lower curve: 100 μg.

produce fluorocitrate, which in turn blocked aconitase. Fluoroacetate or fluoroacetyl CoA might be intermediates in such a conversion. In order to test this hypothesis, kidney particles were incubated with FGA, then boiled and centrifuged. The supernatant was added to a preparation of respiring pigeon-brain particles (these accumulate citrate upon treatment with fluorocitrate, but not with fluoroacetate[17] nor with FGA, although FGA reduces the citrate accumulation caused by fluorocitrate (see results below)).

The results, given in Table 3, suggest that fluorocitrate was produced by FGA, and that more was produced by 400μg (25 per cent) than by 100 μg (13 per cent); this is in harmony with the finding (Fig. 3) that inhibition of aconitase in kidney particles, as assayed by citrate disappearance, increases with increasing FGA concentration. The findings are therefore those to be expected if some fluorocitrate were produced from FGA by the kidney particles via fluoroacetyl CoA. Nevertheless it was unsatisfactory that so much of the supernatant had to be used that there was substantial increase in citrate with the control A. Possibly this could be improved by much more controlled studies (cf. Peters and Wakelin[17]); but it is difficult to get reliable results, where as much as 1·0 ml of supernatant has to be used. There is the further uncertainty produced by the oxidation of fluoroglyceraldehyde, and the effect of any products on the subsequent brain test. In view of this, this experiment can only be regarded as indicative.

TABLE 3. PRODUCTION OF FLUOROCITRATE BY KIDNEY PARTICLES INCUBATED WITH FGA; AS ASSAYED BY SUBSEQUENT EFFECT UPON CITRATE PRODUCTION BY PIGEON-BRAIN PARTICLES

(i) Indicated levels of FGA incubated with kidney particles in triplicate for 1 hr in Warburg apparatus (38°C), then flasks chilled in ice. Contents of 3 flasks (9·75 ml) pooled. Three ml aliquot taken for citrate determination. Remainder boiled 5 min and centrifuged. Details of preparation were as given in Table 2.

Inhibitor	Citrate* (μmoles in 1 hr)	Designation of supernatant
Nil	0·12	A
FGA 100 μg	0·14	B
FGA 500 μg	0·18	C

(ii) One ml of supernatant incubated with pigeon-brain particles. Pigeon-brain particles (20 per cent) in 0·9 per cent KCl in 1·0 ml; 0·5 phosphate buffer M/4 pH 7·2, 0·5 ml (ATP 0·5 mg + $MgCl_2$) 0·1 ml Na fumarate (4·5 μmole); 0·4 ml Na pyruvate (32 μmole); 1·0 ml supernatant or 0·9 per cent KCl.

Additions	O_2 uptake (μmoles in 30 min)	Citrate*† (μmoles in 1 hr)
Nil	5·98	0·40
A	8·40	0·59
B	8·67	0·67
C	7·50	0·74

* Calculated per Warburg flask.
† Corrected for that added with A, B, and C.

In order to investigate the possibility that FGA inhibits respiration at some other point as well as at aconitase, a study was made of the levels of carboxylic acids in respiring kidney particles treated with the equivalent of 500 μg per Warburg flask of FGA, i.e. at the level of suppression of citrate accumulation. The results (Table 4) show that much less fumarate disappears if FGA is present. It also demonstrates the high malate level in the control preparation, and shows that the disappearance of this malate is reduced by the FGA. There appear to be no other important changes caused by FGA in the levels of known carboxylic acids.

Table 4. Effect of FGA upon carboxylic acids in respiring kidney particles: substrate was fumarate 88 μmoles. Other constituents as in Table 2

Maximum* (Tube no.)	Identity	Control		With FGA	
		μequiv.	μmoles	μequiv.	μmoles
4	?	2·3	—	2·9	—
10	acetate	1·5	1·5	2·4	2·4
25	fumarate	18·1	9·1	32·9	16·5
36	α-keto-glutarate	6·7	3·4	6·1	3·1
57	*cis*-aconitate	7·8	2·6	5·6	1·9
69	?	2·7	—	8·0	—
78	malate	62·6	31·3	82·4	41·2
104	citrate	9·7	3·2	10·2	3·4

* 5 ml fractions.
Analysis was by silica gel column chromatography. Identification was solely on basis of identity in position of maximum on the column, and is therefore tentative.
The unknown components are not: pyruvic, glutamic, lactic, succinic, *trans*-aconitic, oxalic, glycolic or *iso*citric acids, none of which were found in this preparation.
Substrate: fumarate, 88 μmoles.
Phosphate, ATP and ADP are not eluted from the column.
The μmoles are calculated on the assumption that the identity of the acids is as shown.
FGA at 1·6 mM (equivalent to 500 μg per Warburg flask).

Effect upon aconitase

The unambiguous demonstration that FGA was converted to fluorocitrate *in vivo* and in kidney particles *in vitro* required that FGA should be ineffective against aconitase. Aconitase was purified from pig heart according to Morrison[12] as far as the first ethanol fractionation. FGA was found to inhibit aconitase; the extent of the inhibition varied with the time of storage (at 0°C) of the activated enzyme, even though the activity of the uninhibited enzyme was unchanged. The effect is probably connected with progressive oxidation of the cysteine in the activated enzyme preparation. This was confirmed by the finding that acetaldehyde also inhibits aconitase, suggesting that the FGA effect is a non-specific aldehyde one. With freshly activated enzyme, 2·8 mM FGA gave 19 per cent inhibition, 4·7 mM acetaldehyde gave 63 per cent inhibition. Since acetaldehyde has no effect upon kidney particles, the direct effect of FGA upon pure aconitase may have no connection with the effect of FGA upon kidney particle aconitase.

Effect upon brain particles

Pigeon-brain particles are known to be unaffected by fluoroacetate; very small

Table 5. Effect of FGA and acetaldehyde upon pigeon-brain particles. Details of preparation were as given in Table 3 (ii)

Inhibitor	mM concn.	O_2 uptake (μmoles in 30 min)	Per cent inhibition	Citrate (μmoles in 1 hr)
Nil		6·16		0·54
FGA	0·036	5·89	4	0·55
	0·36	4·91	20	0·46
	1·80	2·68	57	0·44
Acetaldehyde	0·38	5·89	4	0·52
	1·90	4·37	29	0·43

quantities of fluorocitrate inhibit the oxygen uptake and cause large quantities of citrate to accumulate.[17] Table 5 shows that FGA was a strong inhibitor of the respiration of pigeon-brain particles; but that the citrate level was in fact a little reduced, presumably as a consequence of the respiratory inhibition. As a corollary, FGA was observed to reduce the citrate accumulation caused by the addition of fluorocitrate to respiring brain particles (Table 6), this effect being similar to that shown for kidney in Table 2.

TABLE 6. EFFECT OF FGA UPON THE CITRATE ACCUMULATION CAUSED BY FLUOROCITRATE IN RESPIRING BRAIN PARTICLES. DETAILS OF PREPARATION WERE AS GIVEN IN TABLE 3 (ii)

Inhibitors	O_2 uptake (μmoles in 30 min)	Citrate (μmoles in 1 hr)
Nil	6·00	0·40
Fluorocitrate 10 μg	3·12	1·48
FGA 500 μg	2·90	0·37
Fluorocitrate 10 μg + FGA 500 μg	2·14	1·04

Since certain other aldehydes have been reported to inhibit respiration in brain (e.g. Mann and Quastel[18]), a non-specific aldehyde effect was suspected, and acetaldehyde was therefore studied. As Table 5 shows, it too produced an inhibition of respiration and (to a small extent) of citrate production, but was only about one-tenth as effective as equimolar FGA.

It was still uncertain to what extent FGA owed its effect upon respiration to a non-specific aldehyde effect. A number of simple aldehydes were therefore examined and all

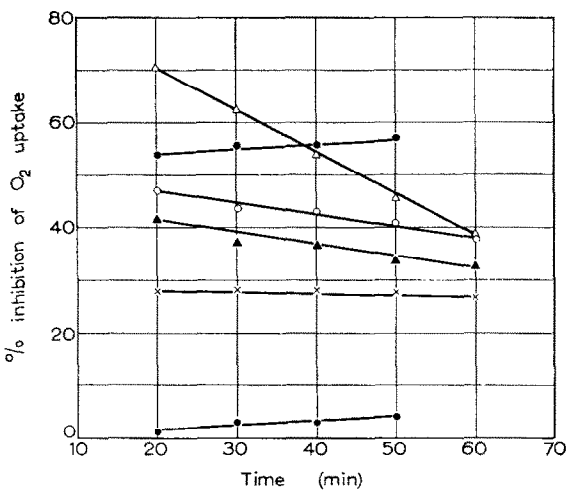

FIG. 6. Variation, with time, of inhibition of brain particle respiration by various aldehydes.
● FGA 1·6 mM (top) or 0·3 mM (bottom).
○ butyraldehyde 10 mM.
▲ acetaldehyde 3·2 mM.
△ propionaldehyde 50 mM.
× glyceraldehyde 20 mM.

were observed to have an inhibitory effect. One of the characteristics of all of the aldehydes except FGA and glyceraldehyde was that the inhibitory effect lessened with time of incubation, as shown in Fig. 6, and that the rate of reduction of inhibition increased with increasing aldehyde concentrations. Clearly the aldehydes were being oxidized, but quite slowly. This was confirmed by more extensive studies upon the effect of aldehyde concentration upon inhibition of respiration: thus inhibition of respiration was maximal at about 50 mM for propionaldehyde and butyraldehyde, and declined at higher levels; in fact with 1 M propionaldehyde there was a 35 per cent stimulation of oxygen uptake, clearly due to aldehyde oxidation. Because of this oxidation, the figures for inhibition by the aldehydes were calculated for zero time by extrapolation. These corrected figures were plotted and by interpolation the aldehyde concentration required to inhibit respiration of brain particles by 50 per cent was found. They are given in Table 7. The non-fluorinated aldehydes were observed to have no effect upon kidney particle respiration.

TABLE 7. INHIBITIONS OF BRAIN PARTICLES RESPIRATION BY VARIOUS ALDEHYDES. DETAILS OF PREPARATION WERE AS GIVEN IN TABLE 3 (ii)

Aldehyde	Concn. for 50 per cent inhibition of respiration (mM)
Formaldehyde	0·24
Acetaldehyde	4·5
Propionaldehyde	8·0
Butyraldehyde	8·0
DL-Glyceraldehyde	35
Glycolaldehyde	8·0
Benzaldehyde	1·3

Inhibitions were obtained by graphical estimation, from data obtained (for each of a series of concentrations) by extrapolating the plot of "per cent inhibition of respiration" against "time" back to $t = 0$.

FGA acted unlike the other aldehydes in that its inhibitory effect slowly increased with time, as shown in Fig. 6. A closer examination of this phenomenon was made, and by reducing the temperature equilibration phase to 5 min and reading at 5 min intervals the curves shown in Fig. 7 were obtained. Clearly there is a stimulation of respiration initially, with low levels of FGA. The significance of these curves is discussed below. From them the FGA concentration for 50 per cent respiratory inhibition was obtained: at $t = 10$ min, it was 2·8 mM; at $t = 30$, 1·8 mM; at $t = 65$, it was 1·6 mM.

If the effect of FGA and of acetaldehyde upon brain particles were a non-specific aldehyde effect and reversible, a protection of the brain by reagents that can combine with aldehyde would be expected. The effect of cysteine, at twice the molar FGA level, was examined, using FGA at 250 μg per flask.

The first experiments were upon the addition of cysteine some time after the addition of aldehyde. In the course of these experiments a curious observation was made. Often the per cent inhibitory effect of FGA seemed to pass through a maximum, e.g. at 30 min, and then to decrease. This was found to be always associated with the control preparations showing a levelling off in oxygen consumption at the time of the above maximum, whereas the consumption of the inhibited preparations did not level

off. This is shown in Fig. 8, which also shows that cysteine tipped in at 25 min had a negligible effect either upon the control or the FGA-treated system.

Two other procedures were investigated. It was found that if (a) the cysteine and FGA were incubated together for 5 min at room temperature, then iced and the other components then added, or (b) the FGA, enzyme, buffer, cofactors and substrate were iced, then cysteine added (i.e. cysteine added at zero time in an experiment otherwise equivalent to that reported in the paragraph above), then in both cases a substantial

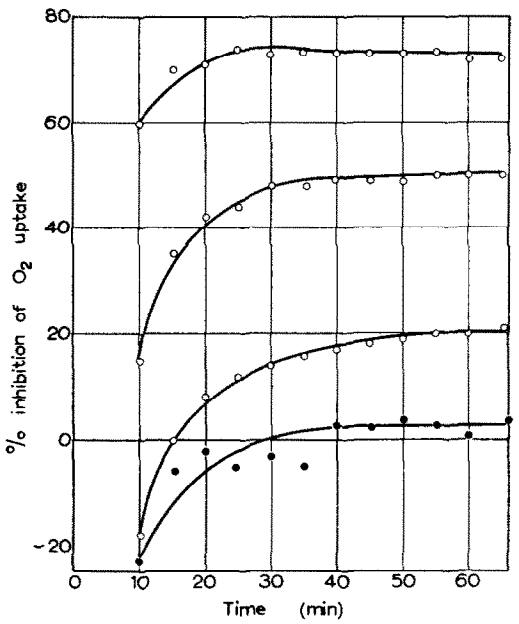

FIG. 7. Variation, with time, of inhibition of brain particle respiration by various levels of FGA
Top curve: 1000 μg FGA per flask (3·1 mM).
Lower curve: 500 μg.
Lower curve: 250 μg.
Bottom curve: 100 μg.

protection from inhibition was afforded, the respiratory inhibition at 30 min from zero being reduced from 35 per cent to 12 per cent with technique (a) and to 11 per cent with (b).

Effect of FGA upon glycolysis and glycerokinase

The effect of FGA upon glycolysis in guinea-pig brain was examined briefly using FGA at 7·2 mM (21·7 μmoles per flask). In a system glycolysing anaerobically in the presence of glucose, FGA inhibited lactic acid formation by 7 per cent (i.e. by 0·7 μmoles); but phosphorus incorporation was actually increased, by an average of 0·7 μmoles per flask (figures are means of four experiments). This apparent paradox would be explained if FGA inhibited glycolysis slightly, but was itself phosphorylated. The most probable enzyme that could account for FGA phosphorylation was glycerokinase. A purified preparation of this enzyme was made according to the method

of Bublitz and Kennedy,[3] using (as did these authors) rat livers. The resulting preparation phosphorylated FGA readily, as judged by disappearance (from ATP) of acid-labile phosphorus, and by acidogenesis in a bicarbonate buffer. In 20 min the reaction was complete and 50 per cent of the added FGA was phosphorylated (added 5·4 μmoles; acid released, 2·8 μmoles). Presumably only one optical isomer is phosphorylated. FGA (at 4·9 mM) was phosphorylated ten times faster than glycerol (at 9·9 mM). It has been reported[3] that DL-glyceraldehyde is phosphorylated at 1·5 times the rate of glycerol. One may therefore predict that FGA will inhibit glycerol

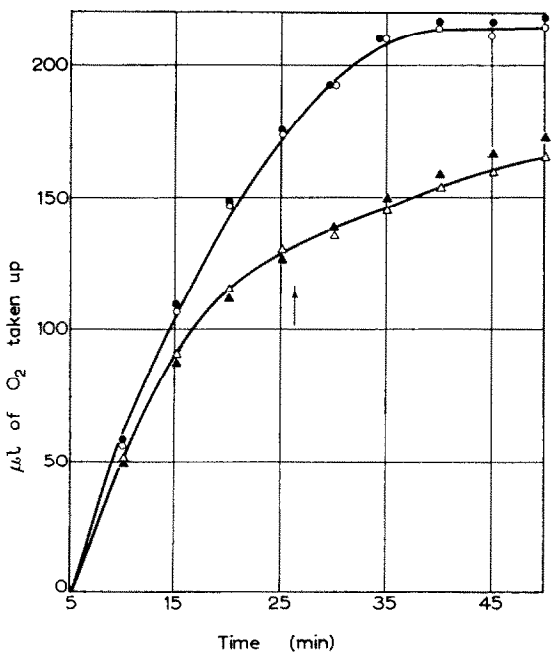

FIG. 8. Effect of FGA and cisteine upon brain particle respiration. Zero time is moment of taking from ice into Warburg bath.

○ control.
● cysteine 430 μg added at arrow.
△ FGA, 250 μg.
▲ FGA, 250 μg, cysteine 430 μg added at arrow.

phosphorylation substantially and competitively; the actual demonstration would involve analysis of the products of glycerokinase action in such a way as to distinguish between glycerophosphate and 2-deoxy-2-fluoroglyceraldehyde phosphate. If glycerokinase is present in guinea-pig brain, these results with glycerokinase account for the increased removal of inorganic phosphate observed above, when FGA was added to glycolyzing brain.

Importance of the liver in FGA poisoning of the whole animal

It has been shown for the rat that in the "eviscerated" and functionally hepatectomized animal, high levels of DL-1-deoxy-1-fluoroglycerol have no effect upon citrate levels of heart and kidney. In the intact animal, on the contrary, this compound pro-

accumulations are not as great as those observed in the intact animal. It therefore seems probable that the liver plays a role in poisoning by FGA.

The toxicity of 2-deoxy-2-fluoroglycerol

This compound was investigated because, like FGA, it is a 2-fluoro three-carbon compound; there is a possibility that FGA would be an intermediate if 2-deoxy-2-fluoroglycerol were oxidatively degraded.

Male mice were injected intraperitoneally with the compound. At doses up to 70

TABLE 8. EFFECT OF FGA UPON CITRATE LEVELS IN "EVISCERATED" AND FUNCTIONALLY HEPATECTOMIZED FEMALE RATS

	Citrate (μg/g)		Survival (hr)
	Heart	Kidney	
Controls	51	41	12
	30	18	12
	53	115	18
Injected (i.v.) FGA 100 mg/kg	563	75	13
	147	39	11
	157	920	13

The rats were anaesthetized with 1·5 g/kg of urethane. Treatment consisted of removing the alimentary canal after tying the coeliac and mesenteric arteries and portal vein. Penicillin (25,000 units) and glucose (200 mg every 3 hr) were given. Technique described by O'Brien and Peters[11].

mg/kg, no deaths were observed. At 100 mg/kg, three out of five survived and appeared completely normal. The other two died after 13 hr and 3 days respectively, after prolonged sprawling, then prostration and markedly reduced body temperature. The average citrate values (μg per g wet weight) in these five were: heart 320 ± 54, kidney 981 ± 544. The corresponding values for untreated mice were 119 ± 18 and 83 ± 9. At 200 mg/kg all mice died.

DISCUSSION

Though this is a subject which clearly requires more investigation, the results *in vivo* suggest the hypothesis that FGA produces its toxic effect by a preliminary conversion to fluoroacetate or to fluoroacetyl CoA. The poisoning of the animal would then follow by the usual conversion to fluorocitrate with consequent aconitase block. However at the biochemical level with kidney particles more complex phenomena were observed, inhibition both of aconitase and (at higher FGA levels) of some other enzyme being probable. This enzyme would be one interposed between fumarate and citrate in the tricarboxylic acid cycle, since its inhibition led to a reduced synthesis of citrate from fumarate. Since malate levels were actually increased by FGA (Table 4), fumarase is probably not inhibited. There is no other evidence upon the identity of this "other enzyme"; the accumulations of fumarate and malate caused by FGA in kidney particles could be caused by inhibition of malic dehydrogenase, condensing enzyme, malic enzyme, etc., since the accumulation of the metabolite

of the cycle could be caused by the inhibition of a fairly distant enzyme, if the relevant dissociation constants under biochemical conditions prevented the accumulation of the metabolite immediately preceding the inhibited enzyme. Since citrate levels in poisoned animals are greatly increased, it is unlikely that inhibition of the "other enzyme" is important *in vivo*, probably because high levels of FGA are not attained in the whole animal.

The linear production of respiratory inhibitor by kidney particles (Fig. 2), contrasted with the fluoroacetate type of inhibition (Fig. 5), shows that FGA *per se* is inactive as an inhibitor, but is converted to an active inhibitor. Since the curves show no suggestion of levelling off at the inhibitory level expected if all the FGA were converted to fluoroacetate, it is likely that the respiratory inhibition is not due to fluoroacetate production from FGA. The results with kidney particles shown in Fig. 1 suggest that an aconitase inhibitor is present as well as a more direct respiratory inhibitor. This aconitase inhibitor may be fluorocitrate; however, the fact that FGA inhibits pure aconitase is probably due solely to the compound's aldehyde group, and since acetaldehyde is also an aconitase inhibitor with pure aconitase but without effect on kidney particles, we may tentatively exclude a direct effect of FGA upon aconitase in the particles. If fluoroacetate or fluoroacetyl CoA is denoted FA, and the direct respiratory inhibitor is denoted X, two possibilities exist in kidney:

$$\text{FGA} \rightarrow \text{X} \rightarrow \text{FA, or FGA} \begin{cases} \nearrow \text{X} \\ \searrow \text{FA} \end{cases}$$

In either case other intermediates may occur.

In brain, a respiratory inhibition only is noted, which is further evidence that FGA cannot affect particulate aconitase directly. This respiratory inhibition may be associated with a non-specific aldehyde effect, since it is of an appropriate order or magnitude. The time relationships show a complex state of affairs (Fig. 7). One interpretation would be that FGA is readily oxidized itself (since negative "respiratory inhibitions" are observed), but that it also inhibits a system responsible for respiration. The strong inhibitory effect by, say, 1000 μg of FGA even at 10 min from zero suggests that FGA itself is a respiratory inhibitor (and this is probably due to its aldehyde grouping). It is clear that some process is going to convert FGA quite rapidly to an even more potent respiratory inhibitor, a process completed in 40 min in the case of high FGA levels. This inhibitor may be identical with the unknown "direct respiratory inhibitor" produced by the action of kidney particles on FGA.

The fact that cysteine added at or before zero time can combat FGA inhibition of brain respiration cannot be taken as evidence that FGA acts by complexing or combining with SH groups in brain. All that it shows is that cysteine forms with FGA a complex or compound that is not inhibitory. Acetaldehyde inhibition, however, is affected by cysteine just as is FGA inhibition, so that the findings with cysteine are compatible with the hypothesis that at least some of the inhibition of brain respiration by FGA is a non-specific aldehyde effect. Such a non-specific inhibition is to be expected from the finding of Schubert[19] that aldehydes combine with a great variety of SH compounds.

duces very high citrate accumulations in these tissues.[11] This and other evidence suggested that the liver was of primary importance in poisoning by this compound.

Using the same technique, FGA was injected intravenously into the "eviscerated" and functionally hepatectomized rat. The results (Table 8) show that considerable citrate accumulations were found in kidney and heart. It follows that citrate accumulations *in vivo* are not entirely dependent upon liver activity. However, survival time in these functionally hepatectomized rats was not affected by FGA and the citrate.

SUMMARY

(1) 2-Deoxy-2-fluoro-DL-glyceraldehyde (FGA) is toxic to the mouse and rat, producing convulsions and accumulations of citrate, particularly in heart and kidney.

(2) FGA is readily phosphorylated by glycerokinase, but has only a very small inhibitory effect upon glycolysis in brain.

(3) With kidney particles respiring in the presence of fumarate, FGA inhibits respiration and at low levels causes citrate accumulation; but this accumulation is eliminated at higher levels. Evidence is produced that this phenomenon is due to a conversion of FGA to fluoroacetate and also to a compound which can inhibit an enzyme acting somewhere between malate and citrate.

(4) With respiring brain particles, FGA inhibits respiration but does not affect citrate concentration. Evidence is produced that the inhibitory effect is due in part to a direct effect which FGA has in common with the several other aldehydes tested, in part to a respiratory inhibitor produced by the action of the particles upon the FGA.

(5) It is concluded that the toxicity of FGA is due primarily to the conversion of at least part of it to fluoroacetate.

(6) 2-Deoxy-2-fluoroglycerol was found to be toxic to the mouse though less so than FGA. It caused rises in the citric acid content of heart and kidney.

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THE INCREASED TUMOUR-INHIBITORY EFFECT OF ENZYME-ACTIVATED NITROGEN MUSTARDS

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Abstract—Studies have been made of the effect of acylation on various biological properties of the compounds NH_2⟨⟩$N(CH_2CH_2Cl)_2$ and HO⟨⟩$N(CH_2CH_2Cl)_2$. Acylation decreases the toxicity of the compounds to rats. The selective toxicity of the acylated derivatives for Walker sarcoma 256 may be strikingly higher or lower than that of the parent compounds. When the tumour contains an enzyme capable of splitting off the acyl group, selectivity is increased. When the tumour does not contain an appropriate enzyme, the selectivity is decreased.

THE success of non-humoral chemical agents in the therapy of neoplastic disease, generally speaking, has depended on their cytotoxic effect being greater towards dividing cells than towards normal, non-dividing cells. The tissues which are more susceptible to damage by these agents are usually those which possess a high mitotic index, e.g. malignant tissues, the haematopoietic system, the generative organs and the skin. The most striking instances of favourable clinical responses to chemotherapeutic agents are apparent with leukaemias. Similar treatment of many other malignant growths is unsuccessful, possibly since the dosage level has to be restricted because of the non-specific action of the drug on other tissues.

Theoretically, the selectivity of a drug can be increased by increasing the number of variables on which the drug depends for its activity.[1] The anti-mitotic effect of nitrogen mustards is dependent upon the ionization of the terminal halogen atoms to form carbonium ions. The substitution of electron-attracting groups into the molecule reduces the ionization rate, rendering the mustard inactive to an extent dependent upon the substituent groups. Administering the mustard in an inactive form will result in the mustard affecting only those tissues which are both able to activate the drug by enzyme action and which possess a high mitotic index. Thus the action of drugs of this type depends upon at least two variables.

The parent substances used in this investigation were I (N-N-di-(2-chloroethyl)-*p*-phenylenediamine) and II (N-N-di-(2-chloroethyl)-*p*-aminophenol).

NH_2⟨⟩$(CH_2CH_2Cl)_2$ I

OH⟨⟩$(CH_2CH_2Cl)_2$ II

The derivatives were obtained by substituting groups into the terminal amino- and phenolic groups. A comparative study of the effect of the parent mustards and their derivatives on the host and tumour was undertaken: it was also necessary to determine

whether there was a correlation between the drug action and the presence or absence of an activating enzyme in the tumour.

EXPERIMENTAL

The drugs were tested against the Walker carcinoma 256 growing subcutaneously in Wistar strain albino rats. The rats were bred on the premises and fed on a standard pellet diet consisting of No. 41 rat cubes (London Flour Co.) supplemented with cabbage and tap water *ad libitum*. The tumour, obtained from the ChesterBeatty Research Institute, was transplanted according to the technique of Haddow, as described by Walpole.[2]

Therapy was begun on rats bearing a small, well-established tumour (about 5 g wet weight), and consisted of daily intramuscular injections of a fraction of the median lethal dose for up to eight days. The effect on the tumour was deduced from changes in volume, calculated from the length measurement and two measurements taken at right angles to the long axis of the tumour. A comparison of the calculated tumour volume with the volume of water displaced by excised tumours indicated that the two estimations were directly proportional for all sizes of the tumour studied and that this method was reliable for estimating the progressive effect of a course of injections on tumour growth.

The main side effects of the drugs were noted throughout each course of injections. These were a progressive weight loss, diarrhoea which was occasionally haemorrhagic, an erythematous condition of the nostril, muzzle and Harderian gland region, a urinary upset, weakness, laboured breathing and coldness of the body and extremities. An investigation of the enzymes which would catalyse the hydrolysis of the substituent groups from the parent mustards was conducted on a quantitative basis using model substrates; qualitative confirmation of these results was obtained using the mustard derivatives as substrates. On the assumption that the specificity of the enzymes concerned was low (at the most a group specificity) the model substrates were derived from aniline and β-napthol for the two series investigated. The amount of aniline liberated from a substrate in the presence of a tissue homogenate was estimated colorimetrically after diazotization and coupling with a sulphonated naphthol to produce a water soluble dye. β-Naphthol was estimated colorimetrically after coupling with a stabilized diazonium compound under standard alkaline conditions and extracting into a chloroform–alcohol solution. The parent drug (compound I, table I) was estimated colorimetrically using a modification of the "Nadi" oxidation reaction.[3] The compounds used were described by Ross, Warwick and Roberts[4] and by Benn and Owen.[5]

RESULTS

The results are summarized in Table 1. The figures in column two are the median lethal doses as a measure of toxicity. A course of injections at the dosage levels stated in column three affected the tumour growth and the rat to an extent indicated in columns five, six and seven respectively.

The effect of the drugs on tumour growth is indicated graphically in Fig. 1. This was either negligible (when the growth rate was comparable with that of a normal untreated tumour (N)), a slowing down of the growth rate (S), a partial regression with subsequent recovery of the normal growth rate (PR) or a complete regression of the tumour (CR).

TABLE 1

1 Drug R= $(CH_2CH_2Cl)_2$	2 LD_{50} (mg/100 g rat)	3 Dose/100 g rat as fraction of LD_{50}	4 Activating enzyme* liver	ileum	tumour	5 Effect on tumour	6 Per cent weight change	7 Other symptoms of intoxication
Amine series								
I NH_2R	0·6	1/3	—	—	—	+	−20	+
III $CH_3CO.NH—R$	4·8	1/6	1·5	0·4	0·2	++	−20	+
IV $C_6H_5CO.NH—R$	35·0	1/3	0·3	0·1	0·0	—	+12	—
V $CCl_3CO.NH—R$	>100·0	1/20	37·4	3·8	0·2	++	−20	+
VI $CH_2F.CO.NH—R$	1·0	1/3	8·2	0·3	0·5	+	−14	+
VII $CF_3CO.NH—R$	9·0	1/9	7·0	—	0·1	++	−13	+
VIII $NH_2CO.NH—R$	0·9	1/9	0·0	0·0	0·0	++	−11	+
IX $CO_2Et.NH—R$	15·0	1/8	0·0	0·0	0·0	++	−13	+
X β-D-glucosyl—NH—R	6·0	1/6	0·0	0·0	0·0	++	−18	+
XI Tetracetyl-β-D-glucosyl—NH—R	30·0	1/3	†	†	†	++	−20	++
XII R′—N=N—R‡	15·0	1/15	0·2	—	0·02	++	−18	++
Phenol series								
II OH—R	1·0	1/5				++	−22	++
XIII $CH_3COO—R$	1·8	1/16	43·7	50·0	46·2	+++	−13	++
XIV $C_6H_5COO—R$	4·0	1/13	73·2	57·0	4·9	++	−25	++

Effect on tumour
+ decrease in growth rate
++ partial regression
+++ complete regression

Other symptoms of intoxication
+ slight, e.g. slight diarrhoea or weakness
++ severe

* Activating enzyme expressed as mg amine or naphthol liberated/g tissue (wet weight)/hr at 37°C and pH 7·3, substrate concentration being 0·014M
‡ 2-Carboxy-2′ methyl-4′-N, N-bis-(2-chloroethyl) azobenzene (CB 1414). See Ross and Warwick[6,7].
† Spontaneous hydrolysis of the glucoside linkage.

The symptoms of intoxication were broadly similar for all the drugs except VI, where symptoms of monofluoracetate poisoning developed. The ratio of severity of the symptoms differed with each drug.

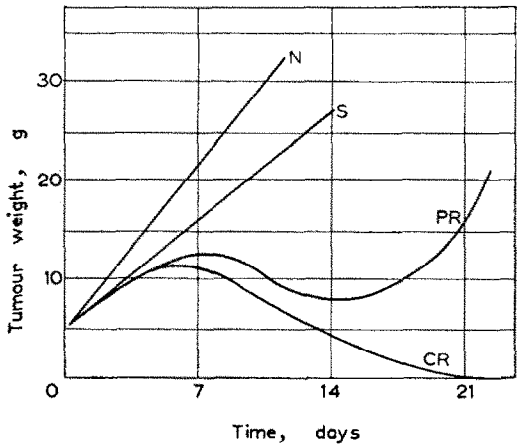

FIG. 1. Diagram to illustrate classification of results. Tumour weight is plotted against time in days from commencement of treatment. Drug administration was usually over four to ten days, daily from the time the implant reached a weight of about 5 gm. N = normal growth, S = slowing in growth rate, PR = partial regression, CR = complete regression.

The level of activity of the enzymes capable of removing the inactivating groups from the derivatives is indicated in column four for three rat tissues.

DISCUSSION

Ross[8] has shown that substituted derivatives of N,N-bis-(2-chloroethyl) aniline which had a hydrolysis rate greater than about 20 per cent by his standard method were active as tumour growth inhibitors, whereas substances with a hydrolysis rate less than 20 per cent were inactive. The results expressed in Table 2 indicate that there is no correlation between spontaneous hydrolysis rate in water and action upon the tumour for the drugs used in this investigation. However, the derivatives used by Ross did not depend upon enzymic activation for their antimitotic activity.[4]

The substituent groups in drugs V, VI, VII, XII, XIII and XIV have reduced the hydrolysis rate to a considerable extent whereas drugs III, IV, VIII, IX and X have a moderately reduced hydrolysis rate. The drugs in the first group were active as tumour growth inhibitors although their hydrolysis rates were less than 20 per cent. Thus activation must have occurred *in vivo*: this is correlated with a significant amount of "activating" enzyme in the tumour cells. The drugs in the second group are sufficiently reactive chemically to exert an antimitotic effect without hydrolysis. However, drug IV, with a hydrolysis rate of 40 per cent is not active whilst drug III, with a hydrolysis rate of 42 per cent is active. III is capable of activation by the tumour whereas IV is not. Physico-chemical characteristics, e.g. molecular size and oil/water solubility which affect the ability of the molecule to penetrate the cell membrane are not the primary cause of the inertness of IV since there is a close physical similarity between IV and XIV (which is active). On the other hand, there is a complete correlation between the presence of an activating enzyme in the tumour and the inhibitory action of the drugs.

It is concluded that some degree of enzymic activation is necessary for this group of drugs to exert an anti-tumour effect.

We have not been able to obtain evidence *in vitro* of enzymic activation of VIII, IX and X by tumour tissue *in vitro*. Their hydrolysis rates are sufficiently high to allow for their anti-tumour effect but it is possible that the derivatives are activated *in vivo* (cf. IX with the *in vivo* hydrolysis of *p*-iodophenylurethane resulting in methaeoglobinaemia).[9]

TABLE 2.

Drug R=  $(CH_2CH_2Cl)_2$	Per cent hydrolysis rate	Effect on tumour	Activating enzyme in tumour
Amine series			
I NH_2R	100	+	
III $CH_3CO.NH—R$	42	++	+
IV $C_6H_5CO.NH—R$	40	—	—
V $CCl_3CO.NH—R$	13	++	+
VI $CH_2F.CO.NH—R$	19	+	+
VII $CF_3CO.NH—R$	7	++	+
VIII $NH_2CO.NH—R$	44	++	—
IX $CO_2Et.NH—R$	31	++	—
X β-D-glucosyl—NH—R	55	++	
XI Tetracetyl-β-D-glucosyl—NH—R		++	—
XII R′—N=N—R	<1	++	+
Phenol series			
II OH—R	60	++	
XIII $CH_3COO—R$	15	+++	+
XIV $C_6H_5COO—R$	15	++	+

The increased anti-tumour effect of the derivatives compared with the parent mustards may be due in part to the following considerations.

As the derivatives were administered in oily solution the rate of absorbtion from the injection site would be slower than that of I which was administered in aqueous solution. Consequently, the time during which a single dose was effective would be increased in the case of the derivatives. Also, since cells are more permeable to molecules in the unionized state, the lowered tendency of the derivatives to ionize would facilitate an easier penetration into the cells. It is possible that the substituent groups in VIII, IX, X and XI endow the molecule with the capacity for incorporation into an active transport mechanism into the cell.[10]

It is apparent that the selectivity for tumour cells is increased when I and II are converted into derivatives from which the parent substances can be released by enzyme action. If, instead of the simple inactivating groups used here, groups which are important in protein and nucleic acid synthesis are used to inactivate the drugs it is expected that a greater specificity of action towards tumour cells would result, since this may result in utilization of the high rates of active transport associated with tumour cells.

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THE REACTIVITY OF RADIOMIMETIC COMPOUNDS* II

SITES OF REACTION OF NITROGEN MUSTARDS, EPOXIDES, METHANE SULPHONIC ACID ESTERS AND AN ETHYLENEIMINE WITH BOVINE SERUM ALBUMIN.

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Abstract—Bovine serum albumin has been treated with the following biologically active (radiomimetic) alkylating agents; nitrogen mustards, epoxides, esters of methane sulphonic acid and an ethyleneimine. The extent of reaction with the different reactive side chains of the protein has been determined; all the agents esterify carboxyl groups and react with histidine although in every case only a fraction were accessible to the reagent. Combination with amino groups was only appreciable with the epoxides and none of the reagents reacted with tyrosine.

The most reactive groups towards all the alkylating agents were the —SH groups in denatured egg albumin. In the native protein these were not available for reaction.

SUBSTANCES which are mutagenic, carcinogenic and produce mitotic inhibition and true chromosome breaks, are called radiomimetic because they evoke the same end-effects in cells as do ionizing radiations. The usefulness of such compounds to cellular pharmacology was predicted by Peters[1] in 1947 and amply confirmed by their application to cancer research and chemotherapy. The most active compounds in this class of substances contain two or more groups which are capable of alkylating electrophilic centres under physiological conditions. The alkylating agents may be activated chlorethyl groups as in mustard gas ($S(CH_2CH_2Cl)_2$) or in the nitrogen mustards ($>N{\cdot}CH_2CH_2Cl$), epoxides (—CH(O)—CH_2), ethylene imines (—N<CH_2—CH_2>) or esters of methane sulphonic acid (—$R{\cdot}OSO_2CH_3$). In view of the enormous variation in the chemical structure of this type of biologically active agent it seems certain that they exercise their action by alkylating vital molecules in the cell. As they are capable of combining with a large number of sites a high proportion of the drugs is likely to be wasted in trivial reactions and it may not be possible to determine their mode of action by following their fate in the cell. Since the most potent compounds are active at 1 mg/kg or less it is clear that the "target" molecules they alkylate must play a very important role since only a few will be affected. Many enzymes for example are present in excess of requirement and alkylation of a few of these even if it led to their inactivation would not produce radiomimetic effects. These considerations suggest that their most likely site of action is on the few enzyme systems which act as metabolic pace

* Part I published in *Biochem. J.* **52**, 177 (1952).

makers[2] because the number present in the cell is only just sufficient to maintain the metabolism or on the genetic material (nucleoprotein) where the loss of a single macro-molecular unit may be serious.

For these reasons, we are studying the combination *in vitro* of a range of active substances with proteins, nucleic acids and nucleoproteins to see if by comparison some of the many possible reactions can be eliminated as biologically significant. On general chemical grounds one would expect that these alkylating substances will react readily in aqueous solution with unionized amino, and ionized acid groups. This has been fully confirmed with model substances (see review by Ross[3]), but only fragmentary data has been reported on the reaction of these substances with proteins (see review by Alexander[4]). Most of the previous work was done with mustard gas, usually present in vast excess as a second phase at the bottom of the protein solutions. There is general agreement that the carboxyl groups are esterified by this substance but there is complete conflict about the alkylation of amino and imidazol groups[5] in protiens. At pH values in the neighbourhood of seven, reaction with sulphydryl groups was observed by Peters and Wakelin[6], and by Banks, Bournsnell, Francis, Hopwood and Wormall[7] but is limited in extent. This is to be expected since these groups will be almost wholly in the non-reactive —SH form. Bacq[8] showed that as the pH was raised the number of SH groups blocked was increased presumably because more of the groups were in the $—S^-$ form which has a very high reactivity.[9]

Much less is known about the reaction of the other alkylating agents with proteins (see e.g., Burnop, Francis, Richards and Wormall[10]); for the nitrogen mustards there is only the observation of Fruton, Stein and Bergmann[11] that amino groups were alkylated and no other measurements were recorded. With epoxides there is agreement about the reaction with amino and sulphydryl groups[12] but evidence for esterification of carboxyl groups is ambiguous.[13] Ethylene imine derivatives and the methane sulphonate esters were shown to combine with proteins by Alexander, Fox, Stacey and Smith,[14] but the groups involved were not identified.

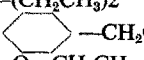
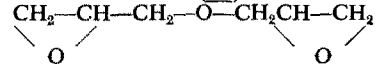
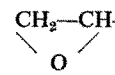
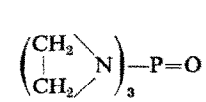
The object of the present investigation was to study the reaction of representative, biologically active nitrogen mustards, epoxides, ethylene imines and methane sulphonic acid esters with bovine serum albumin (abbreviated to BSA) under identical conditions so as to determine the similarities and differences of these various reagents, all of which produce very similar biological effects.

EXPERIMENTAL

Materials

Crystalline bovine serum albumin (BSA) and crystalline egg albumin obtained from Messrs. Armour Laboratories, were used in these studies. The various polyfunctional radiomimetic agents used are listed in Table 1 and are all biologically active (i.e. exhibit radiomimetic properties). In almost every case a similar monofunctional compound was also examined since the much higher activity of the polyfunctional substances is one of the most interesting features of radiomimetic agents. The aromatic nitrogen mustard derivative of phenylbutyric acid (Compound 3, in Table 1) was included because of its very great biological activity[15] and the methane sulphonic acid esters (Compounds 9 and 10 were included because they behaved biologically somewhat differently from the widely studied "Myleran" (Compound 8).[16] All these substances were prepared in the organic chemistry department of this Institute under the

TABLE 1.

1. Methyl di-2-chloroethylamine (HN2)	$(ClCH_2CH_2)_2NCH_3$
2. Diethyl-2-chloroethylamine	$(ClCH_2CH_2)—N—(CH_2CH_3)2$
3. NN-di-2-chloroethyl-*p*-amino-phenyl butyric acid	$(ClCH_2CH_2)_2—N$  $—CH_2CH_2CH_2COOH$
4. Di-(2: 3-epoxypropyl) ether	$CH_2—CH—CH_2—O—CH_2CH—CH_2$ 
5. 1: 2-3: 4-Diepoxybutane	$CH_2—CH—CH—CH_2$ 
6. Propylene oxide	$CH_2—CH—CH_3$ 
7. Methane sulphonyloxy ethane	$CH_3—CH_2—OSO_2CH_3$
8. 1: 3-dimethane sulphonyloxy propane	$CH_3SO_2O(CH_2)_3—OSO_2CH_3$
9. 2: 5-dimethane sulphonyloxy hexane	$CH_3SO_2O(CH_3)C—(CH_2)_2C—(CH_3)OSO_2CH_3$
10. 1-chloro-2-methane sulphonyloxy ethane	$CH_3SO_2O—CH_2CH_2Cl$
11. N'N''N''' Triethyleneiminophosphoramide (TEPA)	

supervision of Dr. Ross and Messrs. Everett and Timmis, to whom we are indebted for making these compounds available to us.

Treatment of proteins

A 3 per cent solution of crystallized bovine serum albumin (BSA) in 0·5M sodium bicarbonate was made 0·12M with respect to the other bifunctional reagents used; monofunctional reagents were used at concentrations of 0·24M. The solutions were allowed to react in a thermostat at 37°C. until more than 75 per cent of the compound had combined with the protein or hydrolysed by the solvent. The methane sulphonyloxy esters and epoxides were allowed to react for 6 days, and the nitrogen mustards for 36 hr. After reaction, the solutions were dialysed to free them of hydrolysed and unreacted reagent and bicarbonate and then diluted to give a 1·8 per cent solution of treated protein. These solutions were then ready to be used, and the exact concentration of the solutions was determined by evaporating the solutions to dryness at 110°C.

The concentrations and ratios of proteins to reagent were selected after some preliminary experiments had been carried out. For purposes of comparison it was desirable always to maintain the same ratio of protein to reagent; a few trial experiments showed that increased ratios of reagent to protein or at concentrations of BSA greater than 3 per cent precipitation of protein was liable to occur during either the reaction or the dialysis, particularly when the reaction was with an epoxide or methane sulphonate.

A high ratio of reagent to protein was used in order to facilitate the measurement of changes in the protein produced by the reaction. A 0·12M solution of a bifunctional reagent gives an approximately four-fold excess over the concentration required for

complete reaction with all the functional groups of the protein assuming that all the reaction was with the BSA and none of the material hydrolysed.

Analysis of treated proteins

Esterification of carboxyl groups. The number of free carboxyl groups, side-chain as well as terminal, was determined from the titration curve which was obtained with a glass electrode by adding 0·1N HCl or 0·1N NaOH to 5 ml of a 1·8 per cent solution of the protein in 1M KCl. The solution was stirred by bubbling nitrogen through it and foaming was prevented by adding one drop of capryl alcohol to the solution. The titration curve was fully reversible.

The amount of free acid or base present at a given pH was deduced as follows. A "blank" titration was performed on the same volume of 1M KCl without any protein present. The difference between the "blank" curve and the protein curve at any given pH then gives the amount of acid or base bound to the protein. This assumes that the activity of the ions is the same in the presence and absence of protein, a procedure which has been found reliable by other workers.

The titration curves were plotted as moles of H^+ dissociated per 10^5 g of BSA. A typical curve is plotted in Fig. 1 showing results obtained from three different deter-

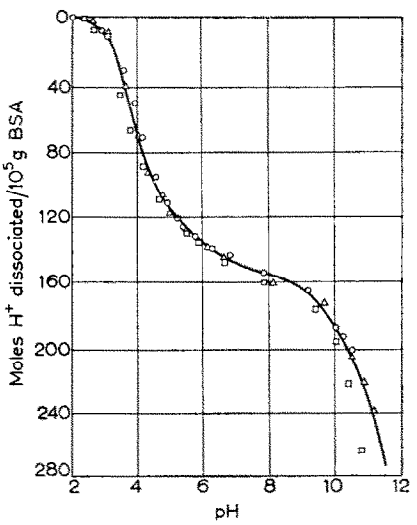


FIG. 1. Titration curve of bovine serum albumin at 20°C. Ionic strength 1.

minations. The reproducibility is good in the acid and neutral parts of the curve, but begins to fail at alkaline pHs.

From the pK values for the different ionizing groups present in the protein it is possible to decide over what pH range a given type of group can be expected to titrate. The range from pH 2–5·5 was found to be a satisfactory one for the estimation of carboxyl groups.[17] Fig. 2 shows a typical set of curves for a number of treated proteins from which the extent of esterification was estimated.

In the case of the nitrogen mustards this technique could not be used since the amine group of the mustard titrates in the same pH range. The extent of esterification was determined by measuring the amount of mustard liberated by alkaline

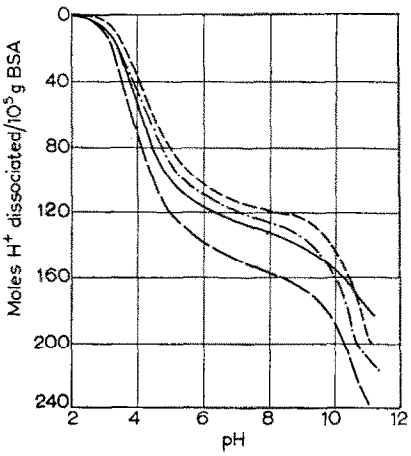


FIG. 2. Titration curve of bovine serum albumin treated with methane sulphonates. Ionic strength 1.
— — — — BSA untreated.
——— BSA treated with methane sulphonyloxyethane.
- - - - - - - BSA treated with 1:3-dimethanesulphonyloxypropane.
——————— BSA treated with 2:5-dimethanesulphonyloxy-hexane.

hydrolysis since only the esters formed are likely to be hydrolysed and any product from reaction with other groups is unlikely to be affected. A solution of BSA treated with the "mustard", p-$(ClCH_2CH_2)_2NC_6H_4(CH_2)_3COOH$, was allowed to stand for 2 hr at room temperature in 0·5M sodium hydroxide. The solution was then neutralised with hydrochloric acid and a 5 ml aliquot was dialysed against 25 ml of water. The u.v. absorption spectrum of the dialysate was measured after 16 and 72 hr dialysis. A control solution of untreated BSA was subjected to the same treatment, the spectra are plotted in Fig. 3. From the optical density of the dialysate it was possible to calculate how much mustard had been removed from the BSA by alkaline hydrolysis.

Reaction with primary amino groups. The conventional van-Slyke method of deamination with nitrous oxide was used to determine the total number of NH_2 groups. Untreated albumin gave 87 moles of amino groups per 10^5 g of protein in agreement with the values of 86 and 87 reported by Tanford[17] and Tristram[18] respectively. Reaction with the radiomimetic agents converts these amino groups into secondary or tertiary amines which do not liberate nitrogen on treatment with nitrous acid.

Reaction with phenolic groups of tyrosine. The u.v. absorption spectrum of bovine serum albumin changes from a peak at 2790Å at pH 7 to one at 2925Å at pH 13 due to the ionization of the phenolic hydroxyl group of tyrosine. In the present work this change in spectrum has been used in an effort to discover whether the hydroxyl group

of tyrosine has reacted with the alkylating agents to form an ether linkage. If such a reaction takes place, ionization becomes impossible and the absorption spectrum at pH 13 would be the same as in neutral solution.

The absorption spectrum of a 0·1 per cent solution of BSA was measured at pH 7·8 and pH 13. The spectrum at pH 13 was measured immediately after making up the solution and again two hr later. A sample of BSA was methylated by extensive treatment with diazomethane, a process known to cause methylation of the phenoxyl

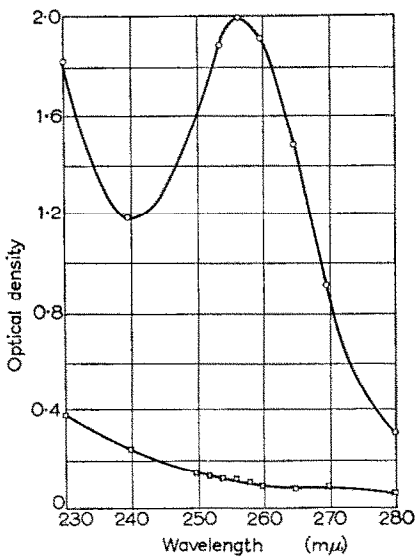


FIG. 3. Ultra-violet absorption spectrum of dialysate from a BSA solution after alkaline hydrolysis.
□ Control BSA.
○ BSA treated with *p*-$(ClCH_2CH_2)_2NC_6H_4(CH_2)_3COOH$.

groups. The absorption spectrum of the methylated sample was measured at pH 7·9 and at pH 13. There was no shift in the maximum of the peak showing that ionization of the tyrosine had become impossible.

Similar experiments were carried out on the samples of BSA that had been treated with the alkylating agents. The absorption at pH 13 was measured immediately and after two hr. The change in absorption with pH was exactly the same as with untreated protein and it was concluded that no reaction had taken place with the phenolic groups.

Reaction with imidazol group. The Pauly colour reaction using the modification of Weiss and Szabolew[18] was used on the intact protein, the absorption being measured at 3500Å. As tyrosine gives the same colour reaction, calibration curves were plotted for tyrosine and histidine, and then a similar curve was plotted for intact BSA. The colour developed with the intact protein agreed with the value obtained for a mixture of tyrosine and histidine at the concentrations at which they were present in the protein solution and it would appear from this that combination of the amino acids in the protein does not affect the amount of colour produced. A series of experiments was

carried out to compare the colour developed in the treated and untreated protein; solutions containg 6 mg of protein in 10 ml were used. From the change in colour the extent of reaction with the histidine was estimated.

Reaction with sulphydryl groups. The number of SH groups was determined by titrating a 2 per cent solution of crystalline egg albumin before and after alkylation with methyl mercuric nitrate.[19] In the native protein the —SH groups do not react with the reagent and it is necessary to denature it first by exposure to 4M guanidine hydrochloride.

Isolation of amino-acid derivatives. Alkylation of amino and imino groups should give products which are not decomposed under the conditions of protein hydrolysis. Such products were looked for by two-dimensional paper chromatography using the method described by Hardy, Holland and Nayler[20] on hydrolysates obtained with 0·2 per cent protein in 8N HCl for 24 hr at 110°C. in a sealed tube. HCl was removed by repeated drying *in vacuo* over pellets of NaOH.

RESULTS

The analytical results are summarised in Table 2.

TABLE 2. GROUPS OF BOVINE SERUM ALBUMIN ALKYLATED

Treatment	Reduction in carboxyl groups (10^5 g protein)	Reduction in primary amino groups (10^5 g protein)	Total no. of groups alkylated (10^5 g protein)	Per cent reduction in Pauly colour	Per cent change in electrophoretic mobility (pH 8·6)	Per cent change in electrophoretic mobility (pH 4·3)	pH of treated protein after dialysis
—	0	0	—	—			5·4
Di-(2: 3 epoxypropyl) ether	33	40	95	77	−18	+190	6·6
Propylene oxide	27	52	97	77	−18	+115	7·0
1:2-3:4-Diepoxy-butane	18	31	66	70	− 8	+ 67	6·0
Methane sulphonyloxy ethane	30	0	42	47	−30	+200	6·4
1:3-Dimethane sulphonyloxy propane	38	0	48	42	−30	+190	7·1
2:5-Dimethane sulphonyloxy hexane	21	6	30	12	− 6	− 33	6·6
Methyl di-2-chloroethylamine	Minimum of 12*	14	28–56	9	−76	+300	7·7
Diethyl-2-chloroethylamine		0	25	5	−60	+230	7·6
N′N″N‴ Triethy-leneiminophosphor amide	—	0	22–66	0	−12	+150	6·2
1-chloro-2-methane sulphohyloxyethane	12	3	15	1	− 6	− 33	6·9
NN-di-2-chloroethyl-*p*-aminophenyl butyric acid	30*	5	35	5	—	—	6·4

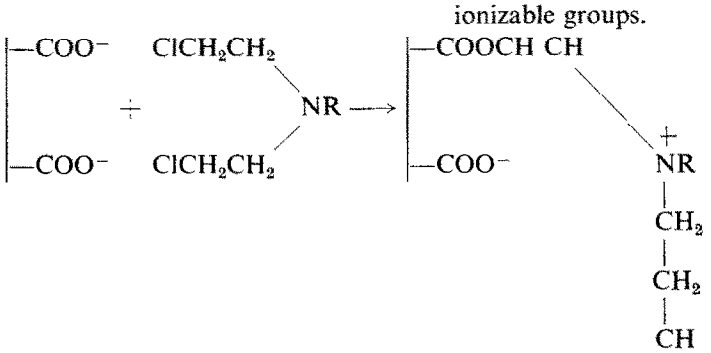
* From hydrolysis data.

Electrophoretic mobility. The proteins were run for two hr on horizontal strips of paper, using an instrument made by the Shandon Scientific Company. 0·025M buffers at pH 8·6 (barbiturate buffer) and 4·3 (acetate buffer), were used and at 40 V/cm a current of one mA/cm width of paper was passed. The papers were developed by the method of Flynn and DeMayo.[21]

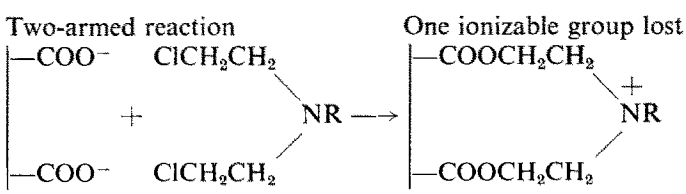
Esterification. The values obtained for the reduction in carboxyl groups from changes in the titration curve are unequivocal for the epoxides and methane sulphonates since reaction with any other groups would not affect the titration in the pH range from 2 to 5·5.

In the titration curves of the nitrogen mustard treated BSA there is an increase in the total number of ionizing groups per g of protein. This is possible after combination with the nitrogen mustard has taken place, since the nitrogen atom of the combined mustard is itself capable of ionizing (the p*Ka* of HN2 is 6·8). The introduction, by reaction, of another type of charged group into the protein makes interpretation of the titration curve difficult. If the nitrogen mustard has reacted with carboxyl groups, two effects are possible. Esterification of the carboxyl groups replaces one type of ionizable group by another, i.e. $—COO^-$ by $COO—CH_2CH_2—NRR'$ so that the net change will be zero. If both arms of the bifunctional mustards react with carboxyl groups, two charges will be destroyed for the introduction of one.

e.g. One-armed reaction — No change in total number of ionizable groups.



Two-armed reaction — One ionizable group lost



If the nitrogen mustard reacts with imidazole or amino groups, there will be an increase of one in the number of ionizing groups, for every mustard molecule introduced.

Inspection of the titration curves for the BSA treated with nitrogen mustards (Fig. 4), shows little change in the range pH 2 to 5·5, indicating that there has been no very extensive reaction with carboxyl groups. Although the titration range of the mustard will overlap this pH range slightly, the bulk of it will titrate between pH 6 to 8, and

indeed the increase in the number of groups titrating between these limits confirms this. From the increase in the number of ionizing groups titrated, about 40 moles of HN2 have combined with the BSA and about twelve of these can be assumed to have reacted with carboxyl groups. For the aromatic nitrogen mustard a more accurate

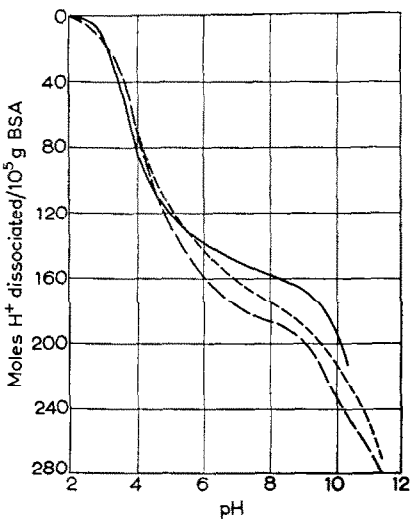


FIG. 4. Titration curve of bovine serum albumin treated with nitrogen mustards.
———— BSA untreated.
— — — — BSA treated with methyldi-2-chloroethylamine (HN2).
- - - - - - - BSA treated with diethyl-2-chloroethylamine.

1. Arginine.
2. Lysine.
3. Aspartic acid.
4. Glutamic acid.
5. Cystine.
6. Alanine.
7. Histidine.
8. Serine.
9. {Valine. / Methionine.
10. Phenylalamine.
11. Proline.
12. Threonine.
13. Tyrosine.
14. Glycine.
15. {Leucine. / iso Leucine.

estimate could be obtained by determining spectroscopically the amount of mustard released during alkaline hydrolysis.

Alkylation of amino and imino groups.—The van Slyke nitrogen analysis gives the reduction in free amino groups derived from the side chains of lysine and the terminal

groups of the polypeptide chain. With the bifunctional reagents and in particular the nitrogen mustards[22] bifunctional reaction giving rise to a cyclic tertiary amine (i.e. $—NH_2 \rightarrow —N<R$) probably occurs but cannot be distinguished by van Slyke amino acid analysis from a single reaction which gives a secondary amine. The substituted amines formed are, of course, stable compounds and will not be decomposed under the conditions of the analysis.

Of the four classes of compounds examined, only the epoxides react extensively with amino groups. The values in the table are reproducible to $\pm$ 2 groups/10^5 g of BSA, so that with the possible exception of HN2 reaction with all the other compounds is marginal. Under the conditions of the reaction between BSA and the alkylating agents, reaction with amino groups is not favoured since a high proportion of the groups will be in the unreactive ionized form. The extensive reaction indicated with epoxides is anomalous in this respect. It was thought that pH 8 might slightly favour the reaction and so BSA was treated with di (2:3-epoxypropyl) ether at pH 6 where even fewer of the amino groups will be unionized, but the results were exactly the same as for those at pH 8.

Since no reaction could be detected with the phenolic hydroxyl groups of tyrosine the change in the intensity of the Pauly colour reaction could be interpreted as reaction with imidazole side-chains, but it may not reveal the full extent of reaction since it is not known how substitution at the imino group effects colour development. The values obtained are, therefore, the minimum and the true values may be higher. Two dimensional paper chromatography clearly showed up the loss in histidine content by a diminution in spot size and approximate estimation using this method would indicate that the amount of reaction with histidine is greater than that deduced from the change in the reduction in the Pauly reaction. After treatment with the epoxides the spot due

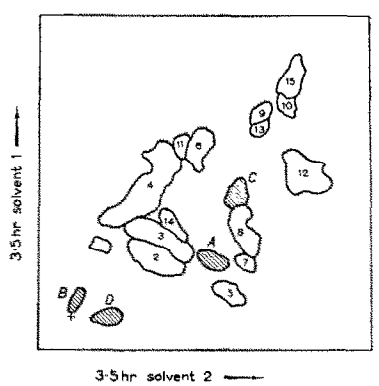


FIG. 5. Chromatogram of hydrolysate of bovine serum albumin showing the following new spots after treatment with:

A. $(CH_3)_2N{\cdot}CH_2{\cdot}CH_2Cl$.

B. $CH_3{\cdot}N(CH_2CH_2Cl)_2$.

C. $CH_2—CH—CH_3$ (O)

D. $(CH_2—CH{\cdot}CH_2)_2O$ (O)

to histidine had completely disappeared and there was an unambiguous reduction even with the nitrogen mustards.

Fig. 5 summarises the new spots formed in the chromatograms of treated proteins. The fact that a stationary spot appears only in BSA treated with bifunctional reagents suggests that a reaction had occurred in the protein, which after hydrolysis released two amino acids linked by the "alkyl bridge" of the bifunctional reagent. Unfortunately, it has not yet proved possible to identify the amino-acids involved, but in view of the reduction in the histidine spot it is probable that the stationary spot is formed either from two cross-linked histidine residues, or two lysine residues or a lysine–histidine cross-link. The two new spots which appeared on the chromatograms of BSA hydrolysates treated with the monofunctional agents, could also be explained as modified amino-acids but again, no definite information about their nature has been obtained.

Electrophoretic mobility. The results of the electrophoresis experiments show that, in general, the distance travelled by the protein at pH 8·6 has been reduced by the treatment, while at pH 4·3 it has been increased. Table 2 shows the change in the mobility of the treated samples expressed as a percentage of the mobility of untreated BSA. Included in the table are the pH values for the treated solutions after exhaustive dialysis, these values are an indication of the isoelectric point of the protein after treatment.

(*a*) *Epoxides.* The change in mass caused by the introduction of alkyl groups is quite small (it could not be detected by weighing), therefore the change in mobility can be attributed to a decrease in charge. The formation of intramolecular cross-links will not of itself alter the charge/mass ratio. This is borne out by the fact that the change in mobility is the same for both the mono-functional and bifunctional epoxides. At pH 8·6 the untreated BSA is negatively charged and a decrease in the mobility due to a decrease in the excess negative charge, could be caused by esterification of carboxyl groups. Reaction between the epoxides and the amino groups of the protein could also cause a change in the net charge, by altering the pK of the amino groups and therefore the number of groups in the ionized state at pH 8·6. The experiments carried out at pH 4·3, show that the reaction with epoxides causes an increase in the mobility of the protein. Excluding changes in mass, this must be due to an increase in the net positive charge since at pH 4·3, any changes in the pK values of the amino groups by reaction will not affect the total number of positive charges because almost all the cationic groups will be ionized at this pH. Therefore the increase in net positive charge must be due to removal of carboxylate ions by esterification.

(*b*) *Methane sulphonates.* The results of reaction with the methane sulphonates show a similar trend to those with the epoxides. Again the migration decreased at pH 8·6 and increased at pH 4·3, although 2:5-dimethane sulphonyloxy hexane did not affect the migration at all and the conclusion is that reaction has occurred with carboxyl groups.

(*c*) *Nitrogen mustards and triethyleneiminophosphoramide.* The nitrogen mustards affected the migration of BSA in the same way as the other classes of compounds, but to a much greater extent. The interpretation of these results is complicated by the introduction of the ionizable group in the mustard molecule. At pH 4·3 all the nitrogen atoms introduced by reaction with the mustard will be ionized and this probably

accounts for the fact that HN2 treated BSA has such a greatly increased migration at this pH. At the higher pH, the effect of the mustard groups should be smaller because not all the groups will be ionized. Therefore, it seems reasonable to assume that at least part of the increase in migration is due to esterification of carboxyl groups. The effect of the triethyleneiminophosphoramide is similar to that of the other compounds and again indicates reaction with carboxyl groups.

The general conclusion from the electrophoresis experiments is that reaction occurs between the carboxyl groups of BSA and each type of alkylating agent studied. The shift in the isoelectric point to more alkaline pH is also consistent with the reaction with carboxyl groups. These experiments do not give any information about reaction with amino-groups. Modification of the protein by alkylation causes a characteristic broadening of the bands and this shows most clearly on the electrophorograms obtained at pH 4·3, where part of the material has scarcely moved. It is well known that the albumin fraction of complete serum "tails" and this has been attributed to irreversible adsorption of the albumin on the paper. A similar explanation may hold in these experiments. Alternatively, it is possible that the extent of reaction has not been the same for all the protein molecules thus making the treated samples less homogeneous than the original BSA. No clear cut evidence for extensive cross-linking has been obtained from these experiments.

Reaction with sulphydryl groups. Since there is less than one SH group per molecule of bovine serum albumin, ovalbumin was used to determine the reactivity of these groups. The results are summarised in Table 3 That there is little or no reaction with

TABLE 3. REACTION OF ALKYLATING AGENTS WITH SH GROUPS IN EGG ALBUMIN

Alkylating agent	Per cent SH groups reacted	
	Native protein	Guanidine denatured protein
NN-di-2-chloroethyl-*v*-*p*-amino phenyl butyric acid	14	88
Methane sulphonyloxy ethane	0	55
Propylene oxide	0	100

the native protein is not surprising since these groups are not accessible even to specific sulphydryl reagents such as mercury compounds, except after denaturation. But once the groups have been revealed by treatment with 4 molar guanidine reaction is extensive.

DISCUSSION

All the different radiomimetic alkylating agents esterify the carboxyl groups and react with the imidazol side chains though to different extents. However, only the epoxides alkylated an appreciable proportion of the amino groups. This reaction is unexpected since the large majority of these groups will be present under the experimental conditions in the ionized and therefore non-reactive form. On the other hand, alkylation of the imidazol and carboxyl groups would be expected from nucleophilic reagents. The most reasonable explanation is that for epoxides the relative rate of

reaction with amino groups as compared with carboxyl ions is much greater than for the other alkylating agents.

In all cases, the reaction with BSA has only resulted in approximately 50 per cent of the available groups being alkylated. (The number of groups available for reaction with mustards and methane sulphonates is smaller than for the epoxides if the amino groups are excluded.) This occurs in spite of the fact that the alkylating agents were present in excess, but is in accord with earlier work done with sulphur mustard. Probably more extensive reaction is prevented by the non-availability of some groups for steric reasons.

The most reactive groups in the protein are the SH groups once they have been made sterically accessible although at pH 7·5 less than 1 in 1000 (p*Ka* 10·8) will be in the reactive —S^- form. Their very high reactivity which was defined in terms of a competition factor by Ogston[9] makes it possible for alkylation to occur relatively rapidly even at pH 7. However, there are many reasons for believing that the biological activity of the radiomimetic agents is not due to their reaction with SH groups.[3,4] Thus many reagents which combine readily with —SH groups both *in vivo* and *in vitro* are not radiomimetic. Recently, Roberts and Warwick[23] showed that ethyl methane sulphonate combined with low molecular weight SH groups *in vivo* and this reaction could be considered as a detoxification since the alkylating agent is thereby lost in a trivial reaction. Similarly, Hendry, Rose and Walpole[24] suggested that alkylation of amino groups was unlikely to be the significant reaction of the biologically active alkylating agents because many substances capable of reacting with amine under physiological conditions were not radiomimetic. This conclusion is supported by the present studies which show that a high reactivity for amine groups in protein is only shown by epoxides and not by other substances having the same biological properties.

The unique feature of the radiomimetic agents seems to be their ability to esterify anions under mild conditions but it seems more likely that the site of action is the phosphate group of the nucleic acids than the carboxyl group of proteins.[4,24]

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THE EFFECTS OF MYLERAN (1:4-DIMETHANESULPHONYLOXYBUTANE) AND HOMOLOGOUS COMPOUNDS ON THE BLOOD

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Abstract—The effects of Myleran (1:4-dimethanesulphonyloxybutane) and a series of homologous compounds on the chief types of blood cells in the rat have been investigated.

It is possible to give a dose of Myleran which will cause a considerable fall in neutrophils without appreciable effect on red cells or lymphocytes.

The effect on the blood of the normal rat appears to give a sufficient indication of the clinical behaviour of Myleran in myeloid leukemia to suggest that it may be a useful guide in the elaboration of new compounds for treatment of this disease.

The depressing effect on the neutrophils shown by Myleran and other members of the series may be greater in rats maintained on a low protein diet than in animals maintained on a normal or high protein diet.

The toxic effects of this series of components appear to be related almost entirely to the blood changes resulting from effects on the myeloid series. After a fatal dose the animals show a general haemorrhagic state with thrombocytopenia from about the 8th day and death occurs at about the 10th to 12th day as a result of anaemia following a massive haemorrhage usually in the stomach.

Myleran is the most active member of the series in causing a fall in blood neutrophils and also shows the greatest selectivity of action in producing a much greater relative depression of neutrophils than of lymphocytes.

MYLERAN,* the clinical use of which was first described by Galton,[1] has now become an established drug for the treatment of chronic myeloid leukaemia, and the results of Myleran therapy on this disease have recently been reviewed.[2]

In a brief account of the discovery and development of this drug[3] it was pointed out that the biological action of the series of dimethanesulphonyloxyalkanes $CH_3 \cdot SO_2 \cdot O—(CH_2)_n—O \cdot SO_2 \cdot CH_3$ (Myleran is the member in which $n = 4$) might resemble that of nitrogen mustard derivatives $R—N(CH_2 \cdot CH_2 \cdot Cl)_2$ since chemically both types of compounds can act as difunctional alkylating agents. The chemistry and chemical reactivity of the Myleran series of compounds in relation to their biological action is discussed by Hudson, Timmis and Marshall.[4]

Myleran was selected for clinical trial in cases of advanced malignant disease because of its inhibitory action on the growth of animal tumours particularly Walker rat-carcinoma 256, and because of its depressant action on the number of circulating neutrophils in the blood, clinical trials were concentrated on cases of chronic myeloid leukaemia. In therapeutic doses Myleran was found to depress myelopoiesis without seriously affecting other haemopoietic elements and the response of most patients was comparable with the best results of radiotherapy.[2]

Since many of the biological effects of the nitrogen mustards and other alkylating

*Approved name (British Pharmacopoeia Commission 1958) Busulphan.

agents resemble those induced by X-radiation these compounds have been described as "radiomimetic". In an investigation of the comparative physiological response to radiation, to Myleran, and to an aromatic nitrogen mustard derivative chlorambucil $COOH{\cdot}(CH_2)_3{\cdot}C_6H_4{\cdot}N(CH_2CH_2Cl)_2$ it has been found that whilst chlorambucil reproduces predominantly the lymphoid effects of radiation Myleran is rather more selective and produces mainly the myeloid and haemorrhagic effects. The complete effects of X-radiation can be imitated very closely by a combined treatment with Myleran and chlorambucil.[5,6,7]

Pharmacological and toxic properties of the series of dimethanesulphonyloxyalkanes in which *n* has been varied from 2 to 10 have now been investigated. These properties were found to be very closely related to their effects on the circulating blood and on the haemopoietic system.

EXPERIMENTAL

Methods

Animals. Wistar albino rats were used. They were kept in individual cages, and maintained on a constant diet, either "rat cake" of about 20 per cent protein content or a special diet of about 5 per cent protein content.

Preparations for injection. Since the compounds of this series are only very sparingly soluble in water, dispersions in arachis oil were used. These were prepared by thorough grinding of the compound with the oil. In order to obtain consistent results it is important that a fine dispersion should be used. In our preparations the size of the majority of the particles was about 10μ. The compounds were administered by intraperitoneal injection.

Preparations for oral administration. These were made by grinding the Myleran with Compound Powder of Tragacanth B.P.* with addition of water. A typical preparation consisted of Myleran 2 mg, Compound Powder Tragacanth 40 mg water 1 ml.

Blood counts. Blood was taken from a tail vein direct into the pipette in which it was diluted in the usual way. For differential counts the blood films were stained by Leishman's method.

Results

Blood counts. The average normal count for the three main types of blood cells in the rats of our colony (mean of 75 animals maintained on a 20 per cent protein diet) is erythrocytes 9,200,000/mm^3 (range 8,000,000 to 10,500,000); lymphocytes 15,000/mm^3 (range 10,000 to 26,000); neutrophils 3700/mm^3 (range 1500 to 5000). The lymphocyte/neutrophil ratio is about 75:25 which is the reverse of the human pattern.

Fig. 1 shows the blood response to Myleran in adult rats (over 200 g in weight) maintained on high and low protein diets. A 75 per cent fall in neutrophils was obtained with a dose of Myleran of 8 mg/kg in the 20 per cent protein diet rats and a 90 per cent fall in the 5 per cent protein diet group. In the low protein diet animals the neutrophils remained at a low level for a considerable time and thirty days after the injection had only returned to about half their normal value. There was also some effect on the red cells in these low protein diet animals but the lymphocytes showed

* Tragacanth 1·5 parts, acacia 2·0 parts, maize starch 2 parts, sucrose 4·5 parts.

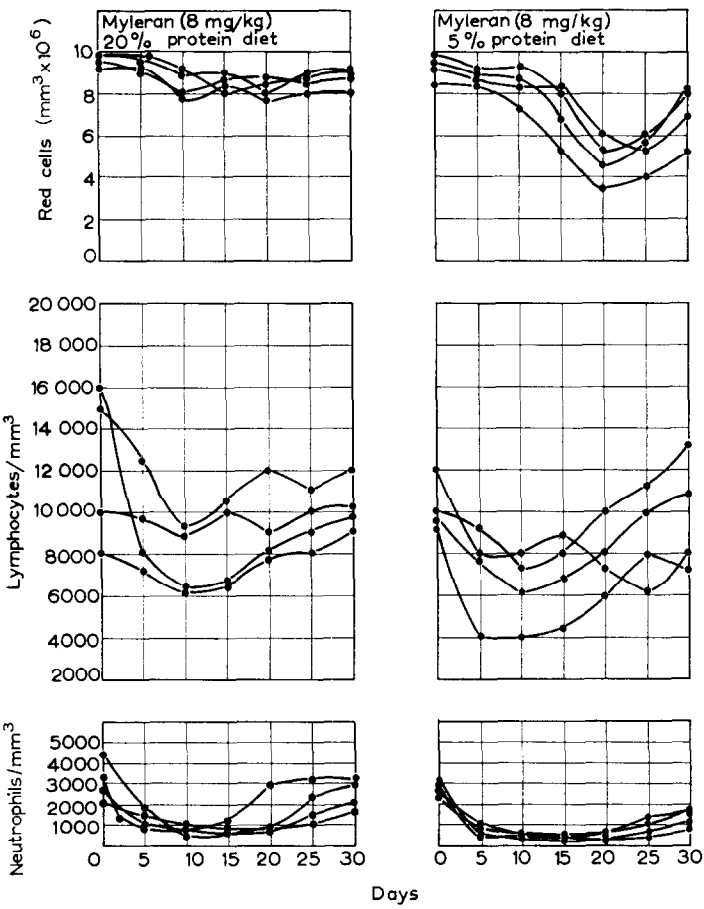

FIG. 1. Effects of Myleran on the blood in adult rats maintained on high and low protein diets.

little divergence from the values in normal rats and very little difference from the behaviour in the high protein diet animals. (In view of the large variations in lymphocyte count which occur in normal rats a fall of up to 40 per cent is barely significant).

This marked effect of low protein diet in increasing the response of neutrophils was

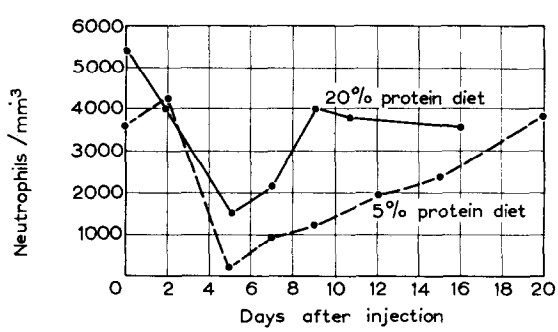

FIG. 2. Effects of high and low protein diets on the response of neutrophils to $CH_2{\cdot}SO_2{\cdot}O{\cdot}(CH_2)_8$ OSO_2CH_3. (Dose 80 mg/kg i.p.: adult rats). Mean values for groups of five animals.

also found with other members of the dimethanesulphonyloxyalkane series of compounds, the very marked effect in the case of the compound where $n = 8$ being shown in Fig. 2. Here again the neutrophils reach a lower absolute value in the low protein diet animals and return to normal is much slower.

In comparing the activities of a series of compounds therefore it is important that the weights of the animals should be fairly uniform and that they should all be maintained on the same diet. The response of adult animals maintained on a low protein diet may prove a more reliable guide for clinical applications of these compounds than the response of young rapidly growing animals.

In Table 1 are given the effects of the series of compounds ($n = 2$ to 10) upon the

TABLE 1. EFFECT OF SERIES $CH_3{\cdot}SO_2{\cdot}O{-}(CH_2)_n{-}O{\cdot}SO_2{\cdot}CH_3$ UPON CIRCULATING NEUTROPHILS IN THE RAT

n	No. of days after a single dose before neutrophils reach minimum numbers	5 per cent protein diet		$\frac{F}{d}$	20 per cent protein diet		$\frac{F}{d}$
		Dose (d) (mg/kg)	Per cent fall in neutrophils from normal (F)		Dose (d) (mg/kg)	Per cent fall in neutrophils from normal (F)	
2					200	0	
3	12	25	55	2·2	40	50	1·3
4 (Myleran)	12	8	95	12·0	8	70	8·0
5	13				12	85	7·1
6	8				40	60	1·5
7	6				60	40	0·7
8	5	80	90	1·1	80	50	0·6
9	6				100	40	0·4
10					300	0	

circulating neutrophils. The substances have been compared in rats maintained on our usual "rat cake" diet, containing about 20 per cent protein diet. There is no doubt of the outstanding activity of Myleran ($n = 4$) in depressing the circulating neutrophils in animals maintained on both diets. The next higher member of the series ($n = 5$) shows an activity approaching that of Myleran but all the other members are considerably less active.

The high activity of these two compounds ($n = 4$ and $n = 5$) compared to that of the other members of the series is reflected in their effect in inhibiting growth of the Walker rat carcinoma 256,[3] and in their induction of haemorrhagic state and fatal haemorrhage.

The relative selectivity of action of Myleran in depressing neutrophils and platelets more than lymphocytes is also observed with the other members of the dimethanesulphonyloxyalkanes. Although at doses near to or above the toxic dose, marked effects on lymphocytes and on lymphoid tissue can be obtained, at doses of the order of half the toxic dose or less all show preferential depressive action on the neutrophils. Table 2 shows the relative neutrophil–lymphocyte depressive action in the series of compounds from $n = 3$ to $n = 8$. The figures are approximate mean values usually from several experiments in each of which at least four animals were used. The most selective action on neutrophils (highest N/L ratio) is given by Myleran which even at

TABLE 2. RELATIVE EFFECTS OF SERIES $CH_3 \cdot SO_2 \cdot O—(CH_2)_n—O \cdot SO_2 \cdot CH_3$ UPON BLOOD LYMPHOCYTES AND NEUTROPHILS IN THE RAT

n	Dose (mg/kg)	Per cent fall in neutrophils from normal (*N*)	Per cent fall in lymphocytes from normal (*L*)	$\frac{N}{L}$
3	40	50	20	2·5
4	8	70	10	7·0
(Myleran)	12·5	80	15	5·3
	15	95	30	3·2
5	12	85	30	2·8
6	40	60	30	2·0
7	60	40	25	1·6
8	80	50	30	1·7

the highest doses still shows about three times as much effect on neutrophils as on lymphocytes.

Toxicity. The delayed toxic effects of Myleran and other members of this series of compounds can be related almost entirely to their effects on the blood. In rats given a lethal dose of Myleran (20 mg/kg i.p.) apart from some inhibition of growth, little other effect is seen for at least a week. The animals then begin to show signs of a general haemorrhagic state and begin to lose weight fairly rapidly until death occurs, usually between the tenth and twelfth day after injection of Myleran (Fig. 3). The immediate

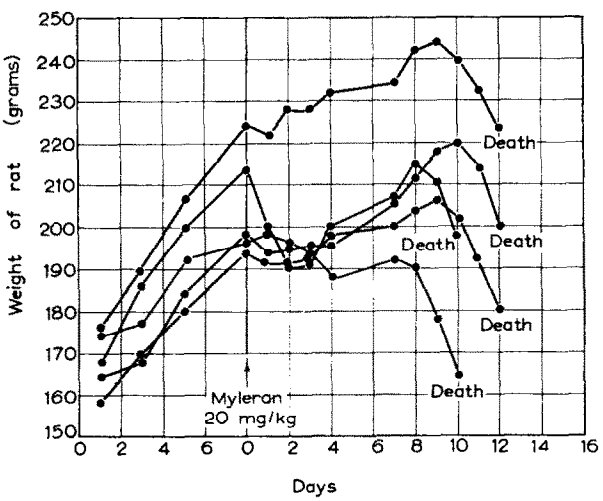

FIG. 3. Toxicity of Myleran in albino rats. Death occurs between the 10th and 12th day after a dose of 20 mg/kg (i.p.). All the animals in the group show a general haemorrhagic state from about the 8th day, and death is usually associated with a massive haemorrhage in the stomach.

cause of death is probably a massive haemorrhage which usually occurs in the stomach. The blood picture in a rat given a fatal dose of Myleran is illustrated in Fig. 4. The red cells show very little change until a major haemorrhage occurs when a sudden fall takes place, in this case about eight days after the injection of Myleran. There is some

fall in lymphocytes but this is considerably less than for the other blood cells. The main effect is on the neutrophils which drop steadily and practically disappear after about seven days. The platelets also disappear at about the same time coincident with the development of the haemorrhagic state.

At post-mortem examination multiple focal haemorrhages are seen in almost all organs, particularly skin, gastro-intestinal tract, lungs, brain, testes, etc. Lymph nodes

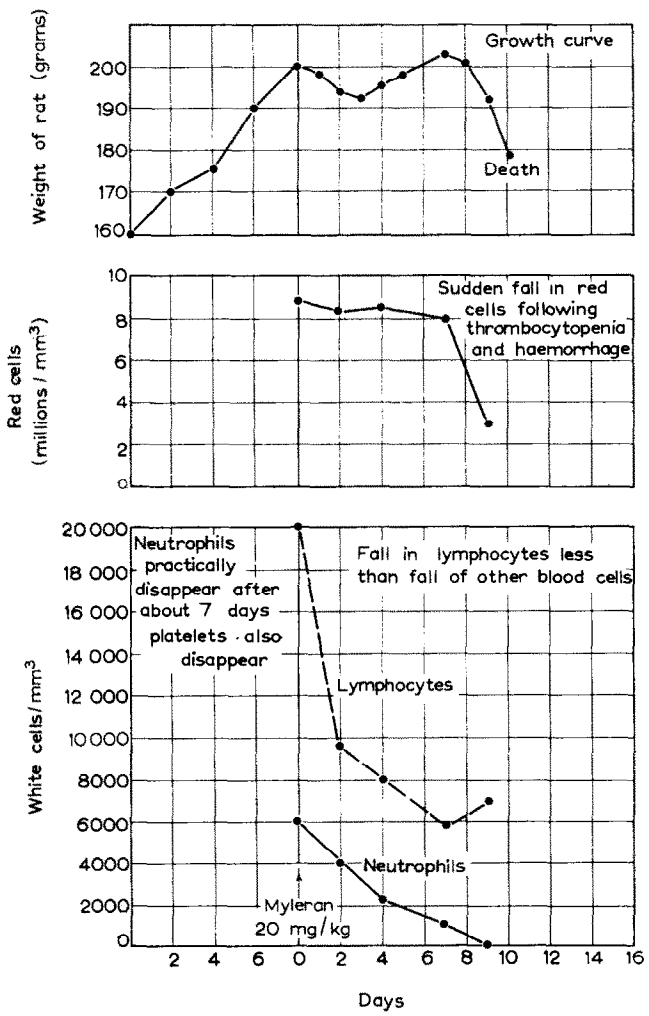

FIG. 4. Blood changes in a rat treated with a lethal dose of Myleran.

show some focal haemorrhage but the general appearances are more like the massive haemolymph changes seen in animals treated with carcinogenic substances.[8,9,10] The bone marrow is usually extremely pale with considerable destruction of haemopoietic tissue and is infiltrated with mast cells, lymphocytes and reticulum cells.

With sub-lethal doses of Myleran and related compounds after about eight to ten

days the haemorrhagic state can be easily demonstrated by plucking the fur from a small area on the chest of the animal, when a number of petichial haemorrhages appear almost immediately.

The toxicity of Myleran when given orally (by stomach tube) appears to be very nearly the same as when given by the intraperitoneal route. A single dose of 20 mg/kg caused death from haemorrhage of six out of eight rats at twelve to seventeen days after administration, and the surviving two rats suffered severe weight loss from about the 12th to 20th day, but subsequently recovered.

The whole series of compounds with values of n from 2 to 10 shows the same type of specific toxic action. Fig. 5 shows the variation in the toxic dose and in the dose

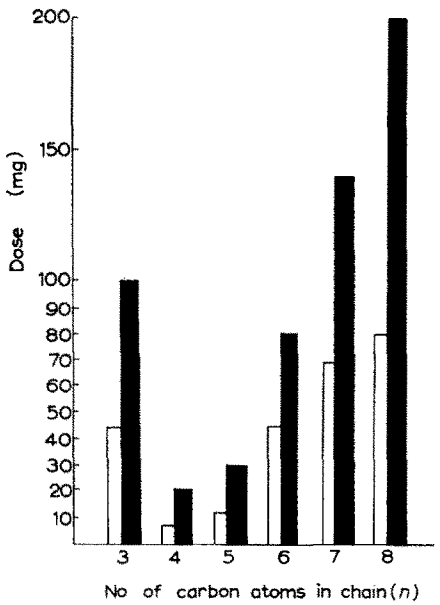

FIG. 5. Toxic dose (causing death in 80–100 per cent of the rats) ▮ in relation to dose causing 50–80 per cent fall in blood neutrophils □, in the series of compounds $CH_3SO_2{\cdot}O{\cdot}(CH_2)_n{\cdot}O{\cdot}SO_2CH_3$ as the value of n is increased from 3 to 8.

causing 50–80 per cent fall in blood neutrophils as the value of n is increased from 3 to 8, and provides some guide in selecting the most efficient neutrophil depressing compound for clinical trial. It must be remembered however that leukaemic cells, particularly immature neutrophils occuring in the blood in myeloid leukaemia are much more sensitive to the drug than the normal neutrophils so that it can be regarded as no more than a rough indication in selecting a compound with the most favourable therapeutic index. It is seen however that 1:4-dimethanesulphonyloxybutane ($n = 4$) (Myleran) as well as having the most selective action on the neutrophils (highest N/L ratio: Table 2) is also one of the best candidates in this respect.

The toxic dose, defined as the dose producing fatal haemorrhage in more than 80 per cent of the animals treated, decreases rapidly from the first member of the series

($n = 2$: not shown in Fig. 5), where a dose of more than 200 mg/kg is necessary to produce a fatal effect to Myleran ($n = 4$) where only 20 mg/kg is fatal. Thus Myleran is the most toxic member of the series and also the most active in producing neutrophil depression. As the number of carbon atoms n increases from 4 to 10 the toxic dose also increases following a practically linear relation to the last member investigated $n = 10$ which requires over 300 mg/kg for a fatal dose.

DISCUSSION

In spite of the difference in pattern of blood counts in the rat compared with the human pattern the effect of Myleran on the blood of the normal rat does appear to give a reasonable indication of its behaviour in clinical treatment of myeloid leukaemia.[1,2] The use of normal rats, especially of adult animals maintained on a low protein diet, should be of considerable assistance in evaluation of compounds for treatment of leukaemia.

Haddow and Timmis[3] discussing the chemical investigations which led to the discovery of Myleran pointed out that substances of this series might by reason of simplicity of structure be expected to show an advantage over the nitrogen mustards (e.g. $CH_3 \cdot N\ (CH_2 \cdot CH_2Cl)_2$ in presenting a less complex pharmacological picture. In accordance with this idea a particular feature of the Myleran series of compounds is their relatively selective action on the neutrophils. Thus it is possible to choose a dose of Myleran which will cause a considerable fall in neutrophils without any appreciable effect on red cells or lymphocytes. This cannot be achieved by whole body radiation or nitrogen mustards since these always show a greater effect on lymphocytes than on neutrophils.[11,12] This selective action of Myleran in particular is reflected in its rather specific toxicity. Its toxic action is associated closely with the blood changes resulting from effects on the myeloid series and is characterised by lack of other less specific toxic effects. This lack of undesirable side effects which often accompany treatment with nitrogen mustards etc.[13] is a very important clinical feature of Myleran.

Myleran is the most active member of the whole series of compounds described in the present investigation in depressing the number of circulating neutrophils. It also shows the greatest selectivity of action in that it has relatively much less effect on blood lymphocytes than on neutrophils. This peak of activity appears to be in some way associated with the length of the chain of $—CH_2—$ groups separating the two mesyl groups, and in a series of dimethyl myleran derivatives

$$(CH_3SO_2 \cdot O \cdot \underset{\displaystyle CH_3}{\underset{|}{CH}}—(CH_2)_n—\underset{\displaystyle CH_3}{\underset{|}{CH}}—O \cdot SO_2CH_3)$$

at present being investigated the highest activity is again found in the member with the 4 carbon chain separating the mesyl groups.[14] The possible relation of this activity to the physical chemical properties of the series, water and fat solubility etc. is discussed by Hudson, Timmis and Marshall.[4]

Acknowledgements—The work has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund, and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

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A PHYSICO-CHEMICAL INVESTIGATION INTO THE BIOLOGICAL ACTION OF MYLERAN AND RELATED SULPHONIC ACID ESTERS

R. F. HUDSON,* G. M. TIMMIS† and R. D. MARSHALL*

Abstract—The physical chemistry of Myleran (1:4-dimethanesulphonoxybutane) and related sulphonic acid esters has been investigated in an attempt to throw light on the mechanism of their biological action. Hypotheses which might explain the gradations of activity in the α, ω-dimethanesulphonoxyalkane series are examined. Differences between the reaction mechanism of these sulphonic acid esters and of nitrogen mustards, with nucleophilic centres related to those in nucleic acid and protein, are discussed. The synthesis of the compounds used is described.

INTRODUCTION

THE chemistry and biological action of the bifunctional alkylating agents has been well reviewed, see for example Ross[1] and there can be little doubt that the biological action of the mustards, epoxides, ethylene-imines and sulphonic acid esters is very closely related to their ability to alkylate at important sites in the body. There is, however, clinical evidence[2] that Myleran (Fig. 3, $n = 4$), an example of the sulphonic acid esters, differs from the mustards and the ethylene-imines in that at effective doses in the treatment of chronic myeloid leukaemia it is usually without side effects in contradistinction to the other two types when they are used in leukaemia, principally of the lymphocytic type. Also in experimental animals a marked difference was shown between Myleran and a clinically effective mustard, Chlorambucil (N,N-di-(2-chloroethyl)-*p*-aminophenylbutyric acid), especially in that the former depressed mainly the myelocytes and the latter chiefly the lymphocytes, in the rat peripheral blood.[3]

A detailed physico-chemical study has therefore been made of the mechanism of alkylation by Myleran and related compounds in an attempt to reveal differences that might exist between it and the mustards.

The sites of biological action now believed to be significant are in nucleic acid, namely singly ionized dialkyl phosphate groups and tertiary nitrogen atoms in the rings of purine and pyrimidine moieties. Similar types of alkylation site in certain co-factors may also be significant.

We found that the rates of reaction between singly charged phosphate ions and sulphonate esters are too low to be detected in water, and the velocity of the reaction with carboxylate ions could not be measured very accurately. For this reason we measured the rate of reaction of the esters with hydroxide ions, which are more electron releasing than acidic anions. This reaction was taken to be representative of the reaction of sulphonates with negatively charged groups. The rate of hydrolysis

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of the esters in initially neutral solution is taken to represent the reactivity of the esters towards uncharged electron donating reactants.

EXPERIMENTAL

Preparation of the esters

Two general methods were used, (A) the action of methanesulphonyl chloride on the appropriate sodium alkoxide and (B) the action of the alcohol on methanesulphonyl chloride in the presence of pyridine.

Method A. Powdered sodium (5 g) was covered with dry ether (100 ml) and the minimum quantity of absolute ethanol to give complete solution. This solution was added slowly to methanesulphonyl chloride (27·5 g), the sodium chloride filtered off, the ether removed, and the ethyl methanesulphonate distilled, b.p. 100°/24 mm. Methyl methanesulphonate, b.p. 84°/12 mm. was prepared similarly.

Method B. Freshly distilled methanesulphonyl chloride (0·2 mole) was added dropwise with stirring to a solution of *n*-butanol (0·2 mole) in carefully dried (KOH) redistilled pyridine (50 ml) at 0°. The temperature was not allowed to exceed 15° during the reaction. The mixture was acidified with ice-cold dilute HCl and the oil extracted with chloroform. After drying overnight (Na_2SO_4) the ester was purified by distillation. In the preparation of *iso*propyl and *n*-octyl methanesulphonates, the reaction was carried out in benzene solvent. The aqueous layer obtained after acidification was extracted twice with ether, and the combined benzene–ether solution was distilled after drying (Na_2SO_4). The following esters were prepared; *iso*propyl-, b.p. 96–97°/15 mm; *n*-butyl, b.p. 102°/10 mm; *n*-amyl, b.p. 112°/8 mm (Found: C, 43·27; H, 8·31; S, 19·1. $C_6H_{14}SO_3$ requires C, 43·36; H, 8·49; S, 19·3 per cent); *n*-octyl-, b.p. 98°/10^{-3} mm (Found: C, 51·70; H, 9·89; S, 15·95. $C_9H_{20}SO_3$ requires C, 51·89; H, 9·86; S, 15·4 per cent).

Dimethanesulphonates. All the esters were prepared by method B. On acidification, the esters normally precipitated as white solids, which were recrystallized from a chloroform–light petroleum mixture. Alternatively, an acetone–light petroleum mixture was used. The low melting pentamethylene ester precipitated as an oil. It was purified by dissolving in hot chloroform–light petroleum mixture, and freezing out in an ice–salt mixture. The ester was filtered off using a pre-cooled Hirsch funnel. The following melting points and analyses were obtained for the esters of general formula $CH_3SO_3{\cdot}(CH_2)_n{\cdot}CH_3SO_3$, Table 1.

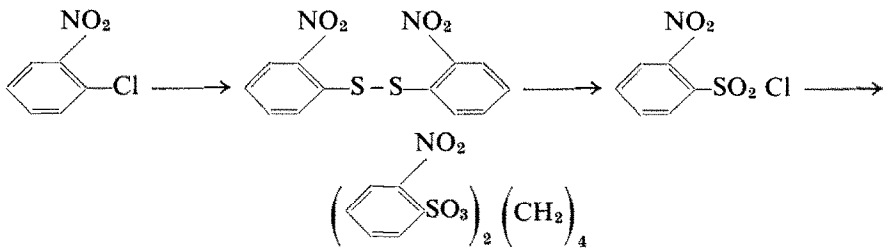
TABLE 1.

n	m.pt. (°C)	Required C	Required H	Required S	Found C	Found H	Found S
2	48	22·0	4·62	29·4	22·18	4·65	29·6
3	46·5	25·85	5·21	27·6	25·96	4·83	27·5
4	118–119	29·26	5·73	26·0	29·33	5·48	25·6
5	34–35	32·29	6·20	24·6	32·30	6·24	24·3
6	60	35·0	6·61	23·4	34·87	6·37	24·0
7	49	37·47	6·99	22·2	37·44	7·16	22·0
8	67·5	39·71	7·33	21·2	39·59	7·43	21·2
9	58	41·75	7·64	20·3	41·67	7·30	20·7
10	79–80	43·59	7·93	19·4	43·51	7·83	19·6

Tetramethylene p-*toluenesulphonate* was prepared by the dropwise addition of pyridine (0·4 mole) to a mixture of tetramethylene glycol (0·2 mole) and *p*-toluenesulphonyl chloride (0·2 mole). The ester was recrystallized from a benzene–light

petroleum ether mixture, m.p. 82·5° (Found: C, 54·37; H, 5·52; S, 16·0. $C_{18}H_{18}S_2O_6$ requires C, 54·26: H, 5·57; S, 16·1 per cent).

Tetramethylene di-o-nitrobenzenesulphonate was prepared by the following route,



o-Nitrochlorobenzene was converted to the disulphide by reaction with sodium disulphide in ethanol.[4] The di-*o*-nitrophenyl disulphide was oxidized with chlorine in aqua regia, and the sulphonyl chloride recrystallized from acetic acid.[5] The esterification was carried out in acetone, and the ester recrystallized from chloroform–light petroleum ether, m.p. 116° (Found: C, 41·40; H, 3·23; S, 13·5; N, 6·58. $C_{16}H_{16}O_{10}N_2S_2$ requires C, 41·75; H, 3·50; S, 13·9; N, 6·09 per cent).

Products of reaction. Tetramethylene dimethanesulphonate (5·6 g) was refluxed for seven days in a solution of 50 per cent aqueous acetone (500 ml). After neutralization, with potassium carbonate, the solvent was removed, and the residue distilled in a micro-distillation unit to give the glycol (1·9 g).

Rate measurements. The rate of reaction was measured by the conventional method of sealing 5 ml of the reaction mixture of known concentration of ester in the appropriate acetone–water mixture in ampoules which were placed in the thermostat. After pre-determined times, the tubes were rapidly cooled in acetone at —20°, and then broken in acetone. The liberated methanesulphonic acid was titrated against standard alkali using lacmoid as indicator. The rates of the alkaline reactions were followed in a similar way, the excess alkali being titrated against standard hydrochloric acid.

Olefin formation. In some cases the reaction tubes were analysed for olefin by reaking under a solution of bromine in dioxan of known concentration. The excess bromine was estimated iodimetrically. Blank experiments were performed in all cases.

TABLE 2.

Ester	Per cent H_2O	Temperature (°C)	Ester (mole/l.)	Per cent olefin After 24 hr
Ethyl	50	70·8	0·010	0·4
*iso*Propyl	100	69·8	0·010	0·7
*iso*Propyl	35	70·1	0·014	0·6
	50	61·0	0·013	0·6
Tetramethylene	50	61·0	0·0083	1·4

Solubility (a) Water. About 200 mg of the finely ground ester were placed in a sealed flask containing 25 ml of CO_2 free distilled water, and the mixture shaken

mechanically for a given time t. The acidity, a, was determined by alkali titration. The solubility s was then calculated from the equation,

$$s = a/k_1 t$$

(b) Ether. Approximately 100 mg of ester were washed with two 20 ml quantities of ether, and the ester dried. 20 ml of ether were then added, and the stoppered flask shaken mechanically for 20 minutes and rapidly filtered into a tared bottle and the volume determined. The ether was allowed to evaporate and the flask reweighed. Blank determinations led to weight increases of the order of 0·0001 g.

Calculation of rate constants

Monofunctional esters. The first order rate constants k_1 for the hydrolyses were calculated from the simple first order equation, and in all cases this law was closely followed. As the rate of reaction with hydroxide ions is of the same order as the rate of solvolysis the following method was used.

$$RX + H_2O \xrightarrow{k_1} ROH + HX \xrightarrow{NaOH} ROH + NaX + H_2O$$

$$RX + NaOH \xrightarrow{k_2} ROH + NaX$$

Let the initial concentration of ester RX be a mole/l and of sodium hydroxide, b mole/l. At time t let the concentration of ester be $a - x$ and of sodium hydroxide $b - x$. The rate of reaction is given by

$$dx/dt = k_1(a - x) + k_2(a - x)(b - x) \quad . \; . \; . \; (1)$$

$$\int_0^x \frac{dx}{a - x} = k_1 \int_0^t dt + k_2 \int_0^t (b - x)dt$$

$$\log_e \frac{a}{a - x} = k_1 t + k_2 \int_0^t (b - x)dt$$

so that

$$\log_{10}(a - x) + \frac{k_1 t}{2{\cdot}303} = \frac{-k_2}{2{\cdot}303} \int_0^t (b - x)dt + \log_{10} a \quad . \; . \; . \; (2)$$

By plotting $\log_{10}(a - x) + k_1 t/2{\cdot}303$ against $\int_0^t (b - x)dt$ the rate constant k_2 is obtained.

Bifunctional esters. The solvolysis of the bifunctional ester may be represented as follows,

$$AX_2 \xrightarrow{k_1} AX + X \xrightarrow{k_2} A + 2X$$

At time $t = 0$, let $[AX_2] = a_0$, and after time t, let $[AX_2] = a$, $[AX] = b$, and $[A] = c$. The appropriate differential equation gives on integration,[6]

$$c = a_0 \left[1 + \frac{1}{k_1 - k_2}\left(k_2 e^{-k_1 t} - k_1 e^{-k_2 t}\right)\right]$$

The rate constants may be evaluated by a general method suggested by Swain.[7] The concentration of acid F produced in time t is given by $F = b + 2c$

$$F = a_0\left[2 - \left(\frac{k_1 - 2k_2}{k_1 - k_2}\right)\exp(-k_1 t) - \frac{k_1}{k_1 - k_2}\exp(-k_2 t)\right]$$

Thus:

$$\frac{-\partial \cdot \log_{10}(2a_0 - F)}{\partial t} =$$

$$= \frac{1}{2{\cdot}303}\left[\frac{k_1\left(\frac{k_1 - 2k_2}{k_1 - k_2}\right)\exp(-k_1 t) + k_2\left(\frac{k_1}{k_1 - k_2}\right)\exp -k_2 t}{\left(\frac{k_1 - 2k_2}{k_1 - k_2}\right)\exp(-k_1 t) + \left(\frac{k_1}{k_1 - k_2}\right)\exp(-k_2 t)}\right] \quad . \quad . \quad . \quad (3)$$

Thus a graph of $\log_{10}(2a_0 - F)$ against t gives in the general case a curve of slope $-k_1/2 \times 2{\cdot}303$ at $t = 0$ and $-k_2/2{\cdot}303$ at $t = \infty$, when $k_2 < k_1$. In the special case when $k_1 = 2k_2$ the general case simplifies to

$$-\partial \log(2a_0 - F)/dt = k_1/2 \times 2{\cdot}303.$$

This is of the same form as a simple first order process.

Application of this equation to the rate measurements for the disulphonates showed that within experimental error $k_1 = 2k_2$ for the tetramethylene esters and the higher homologues. The following rate constants were obtained by graphical solution[8] of the general equation for the hydrolysis of the trimethylene 1:3-dimethanesulphonate; $k_1 = 0{\cdot}46\ \text{sec}^{-1}$; $k_2 = 1{\cdot}07\ \text{sec}^{-1}$.

Owing to the kinetic complexity of the mixed alkaline and neutral hydrolysis of the bifunctional compounds, the rate constants were obtained from the initial rate using the differential rate equation (1).

DISCUSSION

Before discussing the relationship between biological reactivity and chemical structure the mechanism of reaction of methanesulphonic acid esters will be discussed briefly, since this is highly dependent on the reaction conditions.

The nature of the reaction

Alkyl esters of sulphonic acids are approximately 50 times more reactive than alkyl bromides towards water, owing to the greater strength of the sulphonic acid group. This increases the ionization tendency,

(a) $$R{\cdot}CH_2{\cdot}O{\cdot}SO_2{\cdot}CH_3 \longrightarrow R{\cdot}CH_2^+ + CH_3SO_2{\cdot}O^-$$

This reaction is promoted by electron donating groups (nucleophiles) leading to a bimolecular displacement reaction,

(b) $$H_2O + R{\cdot}CH_2{\cdot}O{\cdot}SO_2CH_3 \longrightarrow \overset{+}{H_2O}{-}CH_2R + CH_3SO_3^- \quad \downarrow \quad R{\cdot}CH_2OH + H^+$$

The relative importance of the bond breaking process (a) and the bond forming process (b) is highly dependent on the substituents in R and on the reaction conditions.[9] Consequently the reactions of these esters in aqueous solution cannot be described by a common mechanism.

TABLE 3. RATE CONSTANTS k_1, ACTIVATION ENERGIES E_A, AND COLLISION FREQUENCIES PZ, FOR THE HYDROLYSIS OF ALKYL METHANESULPHONATES $ROSO_2Me$, IN 50 PER CENT AQUEOUS ACETONE AT 61 °C.

R	Me	Et	*n*-Bu	i-Pr
$10^5\,k_1$ sec^{-1}	4·82	1·59	0·813	37·0
E_A *k*.cal/mole	19·4*	20·6	20·5	23·0
$\log_{10}PZ$	7·63*	8·67	8·32	11·6

* In 25 per cent aqueous acetone.

Thus substitution by electron releasing (alkyl) groups successively decreases and increases the reactivity as shown by the rate sequence obtained for the monofunctional compounds (Table 3):—

$$CH_3{-} > CH_3{\cdot}CH_2{-} < (CH_3)_2{\cdot}CH{-}$$

The reaction mechanism of methyl methanesulphonate for example closely resembles that of primary alkyl halides, and the rate is highly sensitive to basic reagents, e.g. OH^- (Table 4).

TABLE 4. RATE CONSTANTS FOR THE NEUTRAL AND ALKALINE HYDROLYSIS OF MONOALKYL SULPHONATES IN ACETONE–WATER MIXTURES AT 61° (*45°).

R	Per cent H_2O	(OH^-) N	$10^5\,k_1$ (sec^{-1})	$10^5\,k_{OH}$ (l.mole^{-1} sec^{-1})	k_{OH}/k_{H_2O}	
Me	100	0·0106	28·4	8220	9·4	10^4
Et	25	0·0125	0·275	105	5·3	10^3
	50	0·0122	1·59	28·2	4·9	10^2
n-Bu	50	0·0104	0·813	44·3	1·51	10^3
i-Pr	25*	0·0125	0·53	9·01	2·41	10^2
	50*	0·0126	6·49	0	0	

On the other hand the substitution of α-alkyl groups increases the ionization tendency, so that in solvents of high water content, *iso*propyl methanesulphonate is unaffected by hydroxide ions (Table 4). Reactions of the kind (a) and (b) are strongly

promoted by polar solvents, whereas the rate of the competing reaction with ions (c) is slightly retarded.[10]

(c) $$OH^- + R{\cdot}CH_2OSO_2CH_3 \longrightarrow R{\cdot}CH_2OH + CH_3SO_3^-$$

Consequently as the water content, and hence the polarity of the reaction environment decreases, the ester becomes more reactive towards OH^- (Table 4) and other negatively charged groups. The reactivity of the electron donating groups available in proteins and nucleic acids at pH 7·4 is considerably less than that of the hydroxide ion. Thus the extent of reaction with these functional groups will change rapidly with the water content of their immediate environment. We have therefore investigated the change in the hydrolysis mechanism with change in solvent composition.

Although the exact nature of this change is not fully understood,[11] there is no doubt that the transition state (which characterizes the reaction mechanism and determines the reactivity) gradually becomes more ionic with solvent polarity. This is shown in Fig. 1 which gives the change in rate of hydrolysis with solvent composition. The slope

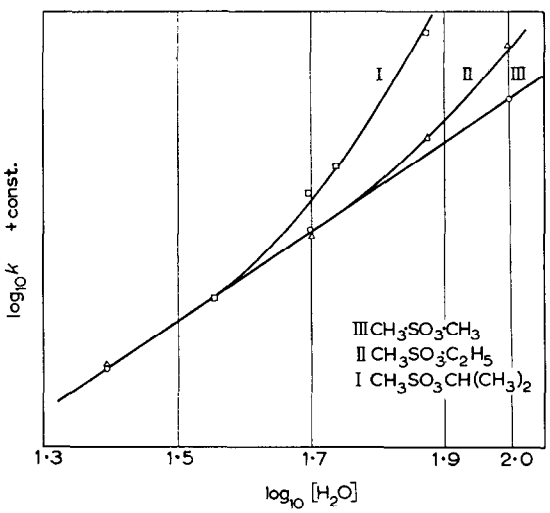


FIG. 1.

of the graph gives a measure of the polarity of the transition state relative to that of the reactant. It is observed that in solvents of low water content the three monofunctional esters react by essentially the same mechanism, whereas the slopes in Fig. 1. increase in the order Me < Et < i-Pr in the most aqueous solvents. The reaction may be described therefore in terms of a gradually changing mechanism from type (b) to type (a) as the water content increases, and in the most highly aqueous solvents (> 50 per cent water in acetone) the secondary esters react essentially by the limiting (S_N1) ionization,

(d) $$\text{i-Pr}{\cdot}OSO_2Me \longrightarrow \text{i-Pr}^+ + MeSO_2{\cdot}O^-$$
$$\text{i-Pr}^+ \xrightarrow{H_2O} \text{i-PrOH} + H^+$$

Now the exact nature of the intermediate cation is the subject of some controversy

which will not be discussed here.[12] It is important to note however, that although this reaction is formally analogous to the hydrolysis of β-chloroethylamines[13] (N- mustards):

(e) $$RR'N{\cdot}CH_2CH_2Cl \longrightarrow RR'N{\cdot}CH_2CH_2^+ + Cl^-$$

the stability of the cation (e) is considerably greater than that of cation (d). Consequently most electron donating groups react rapidly[13] with cation (e) leading to high competition factors. The secondary alkyl cation (d) is highly unstable leading to competition factors normally too small for experimental detection (Fig. 2). This

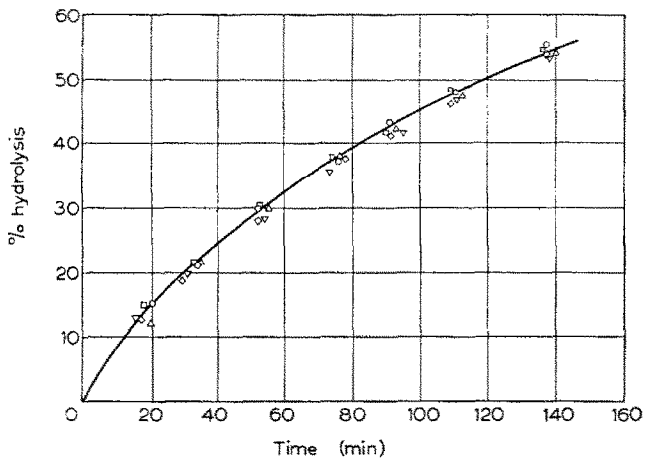


FIG. 2. Hydrolysis of *iso*propyl methane sulphonate in 70 per cent aqueous acetone at 35·0°C.
○ No added salt.
□ 0·0966 M KI.
△ 0·0483 M KI.
◇ 0·0973 M KCNS.
▽ 0·0487 M KCNS.

difference in the two kinds of alkylating agents may lead to milder and more selective action on the part of the sulphonate esters,[14] leading to the characteristic differences in biological action, e.g. on the various kinds of blood cells, and the vesicant action of the mustards and nitrogen mustards.

Polymethylene dimethanesulphonates

With the exception of the ethylene and trimethylene dimethanesulphonates the rate constants for the hydrolysis of the diesters are found to be approximately twice the values for the corresponding mono-esters (Table 5). This suggests that the ester groups in the mono- and diesters are equally reactive, and that the latter react independently to form the glycol. No tetrahydrofuran was detected in the hydrolysates of the tetramethylene diester, and ca. 90 per cent of the glycol was isolated.

The rate of hydrolysis of the diesters increases rapidly with chain length* to reach a constant value at C_4. This increase in chemical reactivity closely follows the increase

* A similar change in reactivity observed with the alkyl halides[15] is attributed to inductive changes in the carbon halogen bond energy (I) rather than to steric causes. As shown by the representation of the transition state for the present reaction, the second sulphonate group will probably exert little steric hindrance to the approach of the substituting group (N).

TABLE 5. COMPARISON OF RATE OF HYDROLYSIS k_1, SOLUBILITY IN WATER S_W AND IN ETHER S_E AND THE DEPRESSION OF NEUTROPHILS F/d FOR THE DIMETHANE SULPHONATES $(CH_2)_n(SO_3)_2$

n	$10^5 k_1$ (sec^{-1})		F/d†	S_W	S_E	S_W/S_E
	Mono-	Diester				
2	1·59	0·0645	0	—	—	—
3	—	0·486	1·3	284	139	2·0
4	0·813	1·79	8·8	23	4·15	5·7
5	0·789	1·66	7·1	250	211	1·2
6	—	1·69	1·5	50	95·2	0·51
7	—	1·86	0·7	9	187	0·045
8	0·813	1·71	0·6	1·2	61·8	0·02
9	—	1·70	0·4	0·4	147	0·003
10	—	1·65	0	0·07	29·2	0·002
4*	0·823	1·83	0	1·3	142	0·009

* *p*-toluenesulphonate. Solubilities are expressed as mol./1 × 10^4.
† Reference.[14]

in biological activity as measured by the growth inhibiting action on the Walker tumour and by the neutrophil depression[14] (Table 5).

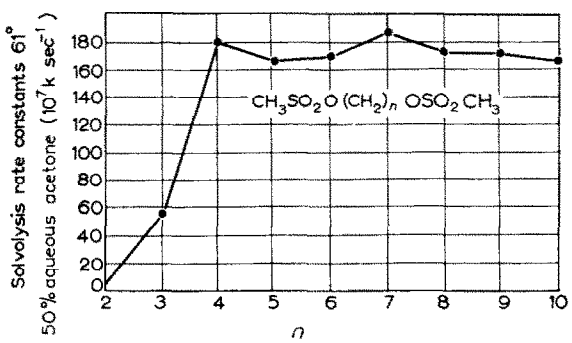
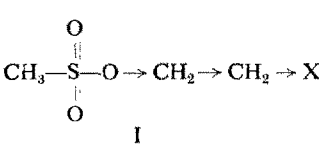


FIG. 3. Effect of chain length (n) on rate of hydrolysis (k_1).

The further decrease in biological activity along the homologous series for chain lengths greater than C_4 is therefore not due to changes in chemical reactivity and must be attributed to physical causes (see below).



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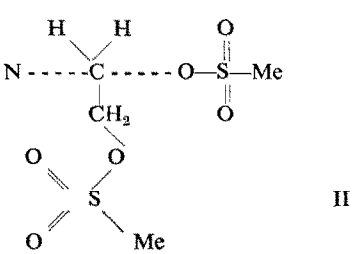


II

The influence of the acidic group

The results discussed so far have revealed a biological optimum at the tetramethylene

dimethanesulphonate, and have indicated that an increased inductive effect, which reduces the reactivity, also reduces the biological action. It follows that substitution which promotes the ionization (d) will increase the chemical activity, and it is of interest to observe the change in biological activity. The ionization reaction is promoted by electron attracting substituents in the released group, and to investigate this effect several diesters of aromatic sulphonic acids were prepared from tetramethylene 1:4 glycol. The rates of the hydrolysis and the reaction with hydroxide ions of the di-*p*-toluenesulphonate and the corresponding dimethanesulphonate were found to be almost equal (Table 6). The toluenesulphonate was however found to be biologically inactive. It was thought that a large increase in chemical reactivity might produce biological activity, but the di-*o*-nitrobenzenesulphonate, which is *ca.* 40 times more reactive towards water and ca. 10 times more reactive towards hydroxide ions than the corresponding methanesulphonate (Table 6) is also inactive towards the Walker tumour, but has so far not been tested as a neutrophil depressant.

Again we find esters of similar chemical reactivity and similar structure with very different biological actions. These differences may be attributed to one or both of the following physical factors, (a) increased steric hindrance of the esters of aromatic sulphonic acids towards the protein or nucleic acid chains, thus reducing sorption on the active sites. (b) the fat–water solubility ratio controlling the transport of the drug to the site of the reaction. These possibilities are examined further in the following section.

TABLE 6. RATE OF REACTION OF TETRAMETHYLENE DISULPHONATES, $(CH_2)_4(SO_3X)_2$ WITH WATER AND HYDROXIDE IONS IN 50 PER CENT ACETONE–WATER SOLUTION.

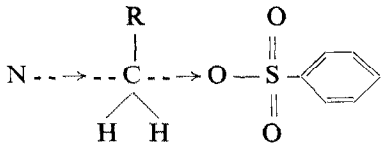
X	Temp. (°C)	(OH^-) N	$10^5 k_1$ (sec^{-1})	$10^5 k_{OH}$ (l.mole sec^{-1})	k_{OH}/k_{H_2O}
CH_3—	61	0·0212	1·79	339	$5{\cdot}26 \times 10^3$
p-$CH_3C_6H_4$	61	0·0127	1·83	252	$3{\cdot}8 \times 10^3$
o-NO_2·C_6H_4—	35	0·0197	6·42	298	$1{\cdot}3 \times 10^3$
	61	—	72·2	—	—

The influence of solubility

The results described in the two previous sections show that chemical reactivity alone does not determine the biological activity of these esters. Similar studies of the nitrogen mustards have led to a correlation between chemical and biological activity with different substituents in the aromatic ring[16], but in this case also increasing the chain length (but preserving the β-chloramine groups) leads to a decrease in activity. These changes have been explained by various workers in terms of the steric requirements of the bifunctional action.[17] Thus the maximum activity observed with the C_4 and C_5 compounds may seem, speculatively, to be related to the best separation distance in the crosslinking of polypeptide or nucleic acid chains,[18] or the cyclization on primary amino-groups[19] secondary phosphate[20] or neighbouring phosphate groups.[21] These speculations cannot be further substantiated until the sites of reaction on the protein or nucleoprotein have been identified.

The biological inactivity of diesters of aromatic sulphonic acids, which are as equally reactive chemically as the corresponding methanesulphonates, is difficult to

explain on steric grounds, as the substitution reaction occurs in the δ position to the reactive carbon atom.



Moreover, the displaced group attains a full negative charge which will therefore be directed towards the aqueous part of the gel and away from the polypeptide or nucleic acid chains. Although this substitution reaction would appear to be free from steric hindrance, the benzene ring may reduce absorption of the ester on the receptor in increasing the steric hindrance towards crosslinked polypeptide and nucleic acid chains.

As an alternative explanation of the large variations in biological activity of esters with similar chemical reactivities, the solubility characteristics of the esters were examined. The close relation between solubility and toxicity or drug action is well known, and in many cases quantitative correlations have been observed.[22] The transfer of the ester from the site of the injection (peritoneum) through the mainly aqueous medium will be facilitated by increased water solubility. The data in Table 5 shows that the water solubility reaches a maximum at the C_5 diester, and also that the solubility of the C_6 ester is approximately twice that of the C_4 ester whereas the neutrophil depressing action is approximately six times less. There is therefore no simple relation between biological activity and water solubility.

The solubilities of the esters in ether were also determined and taken as measures of their fat solubility. As shown by the data in Table 5 the ratio of the solubility in water to that in ether (S_W/S_E) closely follows the biological activity as measured by the extent of neutrophil depression (F/d), produced by the esters of varying chain length (Fig. 4). Moreover, the biologically inactive ester, tetramethylene 1:4-di-*p*-

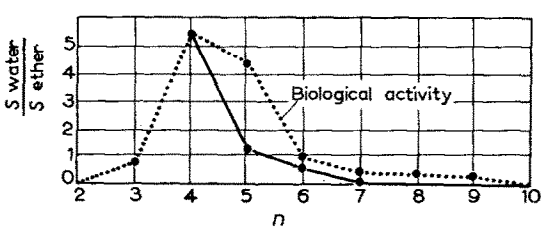


FIG. 4. Effect of chain length (*n*) on neutrophil depressing activity in the rats and on the water–ether solubility ratio.

toluenesulphonate has an extremely low water–ether partition coefficient, and the water solubility of the corresponding di-*o*-nitrobenzenesulphonate was too small to be estimated. It seems therefore that a water to oil partition coefficient may be used as a semi-quantitative measure of the biological activity of the series of esters considered here.

Frequently toxicity, and other biological effects e.g. narcotic action, are found to increase with oil–water partition coefficients.[22] This is usually explained by assuming

that sorption on specific sites is closely related to the oil–water distribution. The reverse trend found in the present case is taken to show that the efficiency of the transfer through aqueous media to these sites has a greater influence on the biological activity than sorption on the sites in a less polar environment. It is noted however, that the distribution coefficients of the most active esters are of the order of unity, so that these esters may pass equally readily through aqueous and organic media.

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STUDIES ON THE MODE OF ACTION OF TUMOUR-GROWTH-INHIBITING ALKYLATING AGENTS—I

THE FATE OF ETHYL METHANESULPHONATE ("HALF MYLERAN") IN THE RAT

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Abstract—The metabolic fate in the rat of ^{14}C-ethyl methanesulphonate (I), a growth-inhibitive (radiomimetic) alkylating agent, was studied.

Thirty-four per cent of the radioactivity is exhaled as carbon dioxide during the first 24 hr after intraperitoneal injection of the compound. Comparison of the rates of exhalation of carbon dioxide following injection of ethyl methanesulphonate and radioactive ethanol suggests that in the former case carbon dioxide is derived from ethanol formed by hydrolysis of the drug at a rate comparable to that which occurs in water at 37°.

Approximately 20 per cent of the injected radioactivity is excreted in the urine during three days after injection. While a number of radioactive metabolites are present in the urine, almost all the radioactivity is incorporated in N-acetyl-S-ethylcysteine and other conjugates or derivatives of S-ethylcysteine. This was demonstrated by comparing autoradiographs of chromatograms of urine obtained after injection of ^{14}C-ethyl methanesulphonate and ^{14}C-S-ethylcysteine. The nature of some of the metabolites is discussed. A small quantity of radioactive urea in the urine could have been derived from ethanol. A negligible quantity of radioactivity appeared in the faeces.

Methyl methanesulphonate and propyl methanesulphonate have also been shown to be excreted as derivatives of the corresponding alkylated cysteines.

S-ethylglutathione has been shown to be converted in the rat to derivatives of S-ethylcysteine, evidence to suggest that S-ethylglutathione and S-alkylated proteins may be intermediates in the formation of S-ethylcysteine conjugates from ethyl methanesulphonate.

The relevance of the findings in relation to the pharmacological properties of the alkylating agents in general is discussed.

INTRODUCTION

A WIDE variety of chemical agents such as the sulphur and nitrogen "mustards", dimesyloxy esters (cf. Myleran[1]), epoxides and ethyleneimines, possess a common ability to react *in vitro* with amino acids, proteins and nucleic acids.[2] They have been shown moreover to elicit diverse biological effects, notably vesication, the production of mutations and chromosome aberrations, growth inhibition, and the ability to act as carcinogens.[3] While the biochemical mechanisms underlying the effects of these so-called biological alkylating agents are not known, biological activity as measured by the inhibition of tumour-growth or the induction of chromosome abnormalities has been shown by *in vitro* studies to correlate both qualitatively and quantitatively with alkylating ability. It has furthermore been suggested that the cellular sites of reaction

may be ionized acid groups, either carboxyl groups of proteins[2] or phosphate groups of nucleic acid,[4] or alternatively purine molecules.[5,6] Various urinary metabolites have been identified following administration to animals of different types of alkylating agent[7] but as yet no direct evidence for *in vivo* alkylation as opposed to *in vivo* combination has been forthcoming.

The work described here, some aspects of which have been briefly reported,[8] concerns the metabolic fate of some alkyl methanesulphonates studied by Haddow and Ross.[9] These compounds have been shown to have some inhibiting effect on the growth of the

$$CH_3CH_2OSO_2CH_3$$

I

transplanted Walker rat carcinoma 256 and to cause mutations in *Drosophila melanogaster*.[10] The compound chosen initially for detailed investigation was ethyl methanesulphonate (I), (CB 1528), a compound closely related to the familiar series of dimesyloxy esters (II) of which Myleran (II; $n = 4$) is the most effective in the treatment of certain types of leukaemia; it is in fact a "half Myleran".

$$CH_3SO_2O(CH_2)_nOSO_2CH_3$$

II

The monofunctional alkylating agents generally manifest their biological effects, or more pertinently, their ability to inhibit tumour-growth at concentrations 50 to 100 times those of the bifunctional compounds; nevertheless it was considered that any *in vivo* alkylation reactions would be comparable to those undergone by their di- or polyfunctional analogues, and permit a more facile determination of the reaction products. It was hoped that a detailed investigation of the urinary metabolites following injection of ethyl methanesulphonate and related compounds might give some indication of the reactions undergone *in vivo*.

MATERIALS AND METHODS

Preparative methods

^{14}C-ethyl methanesulphonate labelled on the α-carbon atom, specific activity 1·6 μc/mg, was synthesised by Dr. V. C. E. Burnop of this Institute.

1-^{14}C-ethanol, specific activity 0·1 mc/mM, and 1-^{14}C-ethyl iodide, specific activity 0·1 mc/mM, were supplied by the Radio-chemical Centre, Amersham.

^{14}C-S-ethylcysteine.—Cysteine hydrochloride (217 mg) was dissolved in a mixture of 1 per cent caustic soda (1·1 ml) and water (1 ml) at 0°. ^{14}C-ethyl iodide (195 mg), containing 200 μc, in ethanol (3 ml) was added, and the stoppered mixture left at room temperature for 48 hr. The pH was adjusted to 7 by the addition of a few drops of dilute hydrochloric acid, a little acetone added, and the solid separated by filtration. It was washed with cold 50 per cent aqueous ethanol and ethanol, and dried in the air. The yield of colourless plates, specific activity 0·86 μc/mg was 111 mg. The compound was shown by paper chromatography and autoradiography to be pure S-ethylcysteine. Unlabelled S-alkylcysteines were prepared by the following general method.

Cysteine hydrochloride (0·003 M) was dissolved, with cooling, in a solution of

sodium hydroxide (0·006 M/10 ml water). To this was added with cooling and shaking, a solution of the appropriate alkyl bromide or iodide (0·003 M) in sufficient ethanol to keep the reaction mixture homogeneous. After standing overnight at 0°, the precipitate was collected by filtration, washed successively with cold 50 per cent aqueous ethanol, ethanol, and light petroleum (B.P. 60–80°), and recrystallized from aqueous ethanol. Yields were generally about 60 per cent.

Compounds made by this method included S-methylcysteine (M.P. = 245°; lit. M.P. = 248°[11]), S-ethylcysteine (M.P. = 255°; lit. M.P. = 260°[12]), S-propylcysteine (M.P. = 240°; lit. M.P. = 240–41°[13]).

N-Acetyl-S-ethylcysteine. S-ethylcysteine (10 g) was treated with acetic anhydride (50 ml) in a stoppered flask and the mixture vigorously shaken until solution was attained. After standing overnight at room temperature the mixture was diluted with an equal volume of water and the solvent removed by distillation from a water bath (40°) under reduced pressure. The material crystallized from chloroform as plates, M.P. = 122°.

Required for $C_7H_{12}O_3NS$: C, 44·0; H, 6·9; N, 7·3 per cent.

Found: C, 44·5; H, 7·0; N, 7·3 per cent.

S-ethylcysteine sulphone. S-ethylcysteine (6.0 g) was dissolved in 5N hydrochloric acid (10 ml) and water (60 ml). Ammonium molybdate (4 ml of 0·5 M) was added followed by 30 per cent hydrogen peroxide (12 ml), with cooling. After 2 hr at room temperature the yellow solution was treated with di*cyclo*hexylamine (sufficient to remove the hydrochloric acid as the insoluble hydrochloride). The filtrate was treated with acetone (1000 ml), and after standing overnight the sulphone was collected by filtration. Crystallization from hot aqueous ethanol afforded small colourless plates, M.P. = 169°, (3·9 g).

Required for $C_5H_{11}O_4NS$: C, 33·14; H, 6·12; N, 7·73; S, 17·7 per cent.

Found: C, 33·28; H, 6·17; N, 7·78; S, 17·5 per cent.

S-ethylcysteine sulphoxide. S-ethylcysteine (3 g) was dissolved in a mixture of concentrated hydrochloric acid (2·2 ml), water (15 ml), methanol (25 ml), and hydrogen peroxide (2·9 ml of 30 per cent) added. After standing for 1 hr di*cyclo*hexylamine (4·5 ml) was added, and the precipitated di*cyclo*hexylamine hydrochloride removed by filtration. The filtrate was neutralized by the dropwise addition of dilute hydrochloric acid, and after further filtration was treated with acetone (250 ml). The white precipitate of sulphoxide (1·6 g) formed microplates, M.P. 154–155°C, from aqueous ethanol.

Calc. for $C_5H_{11}O_3NS$: C, 36·35; H, 6·71; N, 8·68 per cent.

Found: C, 36·38; H, 6·51; N, 8·42 per cent.

Ethylmercaptopyruvic acid. Ethyl mercaptan (6·2 g) was dissolved in a solution of potassium hydroxide (5·6 g), in water (100 ml). Bromopyruvic acid[14] was added in portions with shaking and cooling to maintain the temperature at 0–5°. The pH was kept well on the alkaline side by the occasional addition of potassium hydroxide pellets. The mixture was allowed to stand at room temperature and then twice extracted with ether. The solution was made acid (pH 1–2) by the addition of hydrochloric acid, and the oil which separated was extracted into ether. After drying over anhydrous sodium sulphate the ether was distilled off, leaving a viscous syrup. Crystallization was induced by leaving the oil in a crystallizing dish in a desiccator over concentrated sulphuric acid for three to four weeks. The mass was dissolved in

hot benzene and on cooling, long colourless needles M.P. 123–125°, separated. Two further crystallizations raised the melting point to 130–131°. The yield of pure product was 1·8 g.

Required for $C_5H_8O_3S$: C, 40·5; H, 5·44; S, 21·6 per cent.

Found: C, 40·6; H, 5·38; S, 21·0 per cent.

When even slightly impure the compound appeared spontaneously to decompose in the course of several weeks, giving a deep brown syrup. Even the crystals from the purest preparation became slightly oily within several months, and evolved ethyl mercaptan.

S-Ethylglutathione. A mixture of reduced glutathione (sodium salt) (0·8 g), ethyl iodide (0·2 ml), normal sodium hydroxide solution (2 ml), water (2 ml), and ethanol (30 ml), was shaken vigorously for 3 hr. Addition of ethanol (50 ml) precipitated an oil which solidified on triturating with ethanol. Repeated crystallization from aqueous ethanol gave a hygroscopic white solid (200 mg), which was chromatographically homogeneous and liberated S-ethylcysteine when hydrolysed with boiling hydrochloric acid.

Found: N, 12·3 per cent.

$C_{12}H_{21}N_3O_6S$ requires: N, 12·54 per cent.

Animals and administration of compounds

Male Wistar rats weighing approximately 200 g and maintained on a 20 per cent protein diet were kept in metabolism cages which enabled separation of urine and faeces, and when required, collection of respired carbon dioxide. Both healthy and Walker-tumour-bearing rats were used. All compounds were administered by intraperitoneal injection.

Ethyl methanesulphonate exerts its inhibiting effect on the Walker rat carcinoma at a dose of 60 mg/200 g rat in arachis oil (2 ml) (A. Haddow, personal communication) —that used throughout these metabolic studies.

S-ethylcysteine (50 mg labelled (43 μc), 20 mg unlabelled) was administered suspended in arachis oil (2 ml).

Ethanol (60 mg unlabelled + 88 μc 1-^{14}C-ethanol (specific activity 0·1 mc/mM) was administered in water (2 ml).

Measurement of exhaled radioactive carbon dioxide. Carbon-dioxide-free air was drawn through the metabolism chamber at a rate of about 300 cm^3/min and the respired carbon dioxide was carried into a 5 per cent sodium hydroxide solution (80 ml). After dilution to 100 ml, 10 ml of this solution was treated with 25 per cent ammonium chloride (3 ml) and saturated barium hydroxide (15 ml). The precipitated barium carbonate was collected by centrifugation, washed successively with two portions of water, one each of ethanol and ether, dried at 100° to constant weight, plated to infinite thickness, and counted.

Estimation of radioactivity in faeces. Faeces were roughly dried, crushed into a homogeneous powder, dried to constant weight, and the radioactivity assayed at infinite thickness.

Investigation of urinary samples containing radioactivity

Estimation of radioactivity. Aliquots of urine were repeatedly applied to a 2 cm

plastic planchette and evaporated by infra-red heat until the layer was of infinite thickness and the weight constant. The radioactivity was assayed, using an end-window counter connected to an ECKO Scaler Type N 529 or Panex Equipment 100 C.

Preparation of samples for paper chromatography. Fig. 1 illustrates the manner in which the various fractions and hydrolysates were prepared for paper chromatography.

The cationic and anionic exchange resins, B.D.H. Amerbilte Resin IR 120 and B.D.H. Amberlite IRA 400 (OH) respectively, enabled separation of urinary constituents into neutral, acidic, basic, and amphoteric fractions and the removal of inorganic salts.

Acid hydrolysis of liquid samples was achieved by mixing an aliquot with an equal volume of concentrated hydrochloric acid and heating under reflux or in a sealed tube at 100° for 24 hr. Excess hydrochloric acid was removed by repeated evaporation with warm air. The hydrolysate was then chromatographed on paper and after desalting and separation into acidic, basic and amphoteric constituents by percolation through ion exchange columns (Fig. 1).

Alkaline hydrolysis was achieved in a similar manner using $2\bar{N}$ caustic soda solution.

Enzymic hydrolysis—an aliquot of urine was incubated with either Ketodase (Warner Chilcott Labs., New York, U.S.A.) or acid phosphatase (Worthington Biochemical Corpn., New Jersey, U.S.A.) at 37° in an equal volume of acetate buffer (pH 5·0).

Each fraction was analysed by ascending chromatography in at least two solvent systems.

Paper chromatography of urinary fractions.—A Shandon Multi-Sheet Frame Chromatank and Whatman No. 4 20 cm × 20 cm punched filter papers were found very satisfactory for the preliminary and rapid examination of mixtures. For the greater resolution necessary for the accurate determination of R_f values a Shandon 20-in. "Two-Way" Sheet Chromatank was used.

Solutions to be analysed were applied to chromatogram papers with an Agla microsyringe, and dried with warm air. For the examination of urinary samples never less than 10 μl was applied to the chromatograms.

The solvents used were: butanol–ethanol–propionic acid–water (20:10:10:4) (Solvent I); butanol–acetone–water–di*cyclo*hexylamine (20:20:10:4) (Solvent II); butanol saturated with 3 per cent ammonia (Solvent III); butanol–acetic acid–water (4:1:1) (Solvent IV) and phenol–water (NaCN + 0·3 per cent ammonia) (Solvent V). Paper chromatograms were exposed to Ilfex film for periods of up to four weeks and then developed with various spot reagents.

Spot-test reagents were prepared and used by the methods summarized by Block,[15] except the ninhydrin reagent which was made by dissolving ninhydrin (0·25 g) in a mixture of A.R. acetone (93 ml) and glacial acetic acid (7 ml). In the presence of di*cyclo*hexylamine (present in Solvent II) many of the amino acids gave characteristic colours when developed with ninhydrin, which proved a useful aid to identification; thus glycine appeared as a red-brown spot, aspartic acid as turquoise, phenylalanine and S-ethylcysteine as grey, and so on.[16]

The platinic iodide reagent was used frequently as a test for sulphur-containing compounds although its usefulness was somewhat restricted by its inability to give a positive reaction after papers had been in contact with di*cyclo*hexylamine, and the fact that oxygenated compounds failed to react.

RESULTS

The Metabolism of ^{14}C-Ethyl Methanesulphonate

Exhaled ^{14}C-carbon dioxide

In Table 1 are recorded the quantities of radioactive carbon dioxide exhaled at intervals during 24 hr after injection.

TABLE 1. RATE OF EXPIRATION OF ^{14}C-CO_2 FROM A RAT INJECTED WITH ^{14}C-ETHYL METHANESULPHONATE

	Hours after injection	Expired radioactivity (μc)	Per cent total injected radioactivity	Per cent total expired radioactivity
	0–2	3·1	3·2	9·2
	2–4	6·65	7·0	20·8
	4–6	6·35	6·7	19·5
	6–8	4·7	4·9	14·0
	8–10	3·9	4·1	12·0
	10–12	2·8	2·9	8·6
	12–14	2·1	2·2	6·4
	14–16	0·87	0·9	2·6
	16–18	0·88	0·9	2·6
	18–20	0·78	0·8	2·3
	20–22	0·66	0·7	2·0
	22–24	Negligible	—	—
Total		32·79	34·3	100·0

Radioactivity in faeces

The faeces contained never more than 1–2 per cent of the radioactivity of the injected drug and this possibly due to contamination with urine, since superficial washing with water removed the radioactivity.

Radioactivity in urine

Table 2 shows the rate of excretion of urinary radioactivity.

TABLE 2. URINARY RADIOACTIVITY FROM RATS INJECTED WITH ^{14}C-ETHYL METHANESULPHONATE

	Hours after injection	Per cent total administered radioactivity
	0–24	12–15 range for four experiments
	24–48	4
	48–72	1 approx.
	72–96	Negligible
Total		20 approx.

Nature of urinary metabolites. Urine was fractionated as outlined in Fig. 1, and the various fractions submitted to one- and two-dimensional paper chromatography using combinations of the solvent systems described.

The urinary excretion pattern was the same for normal and tumour-bearing rats.

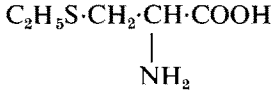
Autoradiography revealed the presence at various times of at least twelve areas containing radioactivity, while only two of these consistently constituted major areas

(see, for example, spots a and b, Figs. 2 and 3A). Treatment with ion exchange resins showed that they were both acids, while they were negative to the ninhydrin test and showed a positive sulphur test (platinic iodide reagent). Neither was associated with the protein fraction since they were still present in urine following deproteination with ethanol.

One of the compounds was stable to mild acid treatment (dilute hydrochloric acid overnight at room temperature) while the other altered in R_f in Solvent I (compare Figs. 3B and 3C), but retained the other properties listed.

After hydrolysis with boiling hydrochloric acid only one major area of radioactivity appeared on autoradiographs of paper chromatograms (Spot e, Figs. 3D and 4) and this could now be superimposed over a sulphur-containing ampholyte which gave a characteristic grey colour with ninhydrin in the presence of di*cyclo*hexylamine.

Urine which had been boiled with caustic soda solution still retained at least 50 per cent of its radioactivity. Acidification of this solution and evaporation prior to counting, however, led to a loss of all the radioactivity as did subjecting the alkaline hydrolysate to paper chromatography in acid solvents (no radioactive spots on the autoradiographed papers). It was evident that alkali had caused the conversion of one or more of the radioactive urinary constituents into the sodium derivative of a compound which was volatile at acidic pH. If the compound was a radioactive acyl compound formed from the drug, hydrolysis with alkali would give rise to sodium acetate which would give volatile radioactive acetic acid on acidification. The fact that solid counts on residues from radioactive urine which had been boiled with hydrochloric acid showed very little loss of the original radioactivity, proved that this was not the case, neither was the activity originally present as a carboxylic ester. It was therefore concluded that one or both of the original major radioactive metabolites was a sulphur-containing acid which was converted to a sulphur-containing ampholyte on acid hydrolysis without loss of radioactivity, but which decomposed with hot alkali to give the sodium salt of a volatile acid with loss of radioactivity on acidification. This data was consistent with the ampholyte present after acid hydrolysis being S-ethylcysteine (III), since this is stable to boiling acid, but decomposes giving the sodium salt of the volatile ethyl mercaptan on boiling with caustic soda.



III

Confirmation was achieved by comparison of the properties of S-ethylcysteine with those of the radioactive ampholyte present in acid hydrolysed urine. S-ethylcysteine had an identical R_f in the five solvent systems employed, behaved similarly on ion-exchange resin columns and on a column of deactivated charcoal[17] (S-ethylcysteine behaves like an aromatic amino acid in this respect) and on treatment with hot mineral acid, nitrous acid, and alkali. The alternative possibility that this S-ethyl amino acid could be ethionine (formed by ethylation of homocysteine) was rejected on the basis of its R_f in these solvents systems, it being easily distinguishable from S-ethylcysteine. The peptide S-ethylglutathione had been shown to be hydrolysed by the conditions used to hydrolyse the urine and to possess quite different chromatographic properties.

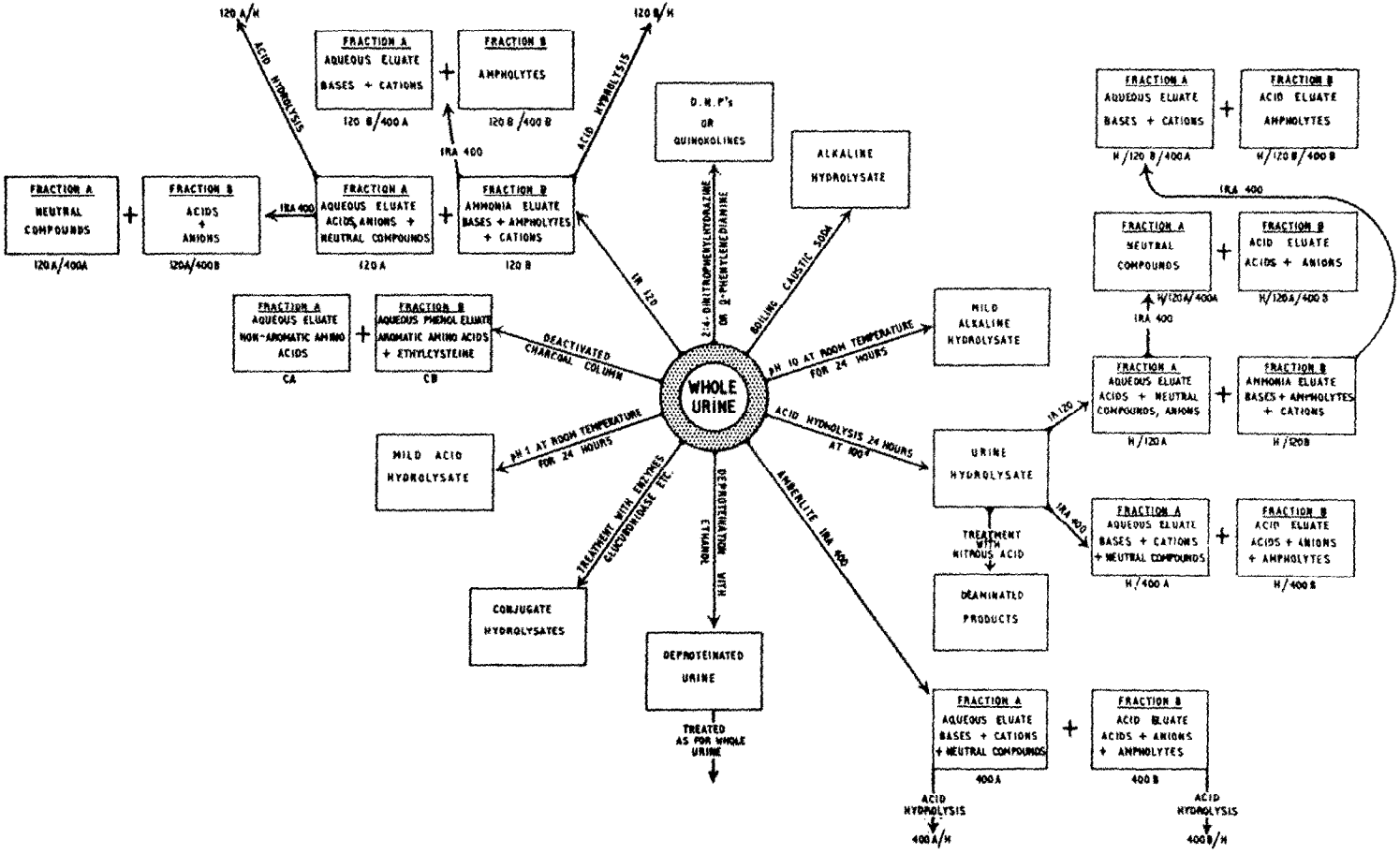


FIG. 1. Shows the methods used for fractionating urinary samples prior to paper chromatography or estimation of radioactivity.

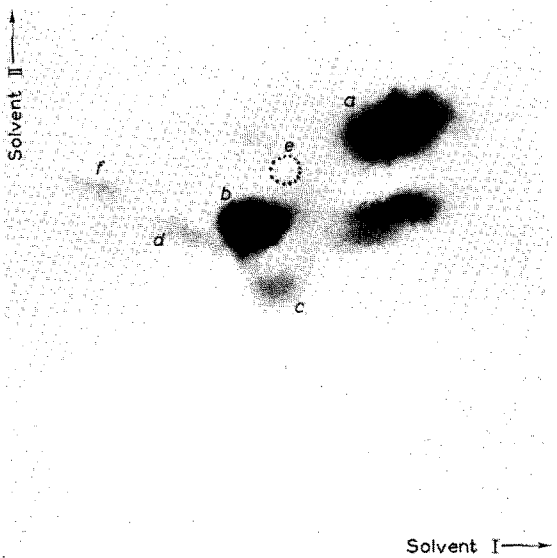
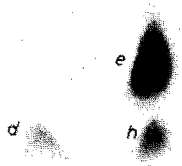


FIG. 2. Shows an autoradiograph of a two-dimensional chromatogram (Solvents I and II) of urine collected during the first 24 hr after injection of ethyl methanesulphate.

FIG. 3. Shows an autoradiograph of a unidimensional chromatogram (Solvent I) of urine obtained during the first 24 hr after injection of ethyl methanesulphonate, A, S-ethylcysteine, B, either A or B after treatment with cold dilute mineral acid, C, and either urine after heating with concentrated hydrochloric acid D.



Solvent I

FIG. 4. Shows an autoradiograph of a two-dimensional chromatogram (Solvent I and II) of urine collected during the first 24 hr after injection of either ethyl methanesulphonate or S-ethylcysteine which had been heated at 100 C for 24 hr with concentrated hydrochloric acid and then desalted.

Both major areas of radioactivity were hydrolysed on the paper chromatogram with hydrochloric acid (by heating papers sprayed with dilute acid between glass plates for 1 hr at 80°) and gave rise to ninhydrin-positive compounds containing radioactivity. Again, elution of the spots from the paper chromatogram, acid hydrolysis and re-chromatography (Solvents I and II), and autoradiography showed that each compound had been converted to S-ethylcysteine proving that each was originally a derivative of it. Since both major radioactive metabolites were ninhydrin-negative before acid hydrolysis, and gave rise to the same ninhydrin-positive ampholyte on acid treatment, it was concluded that they were conjugated, at least partly through the nitrogen atom. Since in most recorded instances S-substituted cysteines are excreted from the mammal as the N-acetyl derivative (mercapturic acid), N-acetyl-S-ethylcysteine was synthesized and shown to have an identical R_f to one of the radioactive components (spot a, Figs. 2 and 3A) in solvents I–V. The other major metabolite which altered in R_f on mild treatment with acid and gave rise to S-ethylcysteine on more vigorous treatment was concluded to be a more complicated conjugate of S-ethylcysteine.

Conclusive proof that almost all the radioactive compounds present in radioactive urine were derived from S-ethylcysteine was obtained by injection of ^{14}C-S-ethylcysteine, subjecting the urine to the detailed examination outlined in Fig. 1, and obtaining autoradiographs identical in almost all respects to those just discussed.

Only three areas of radioactivity were present on autoradiographs of paper chromatograms prepared from acid hydrolysed urine obtained from rats injected with ethyl methanesulphonate (Fig. 4). One of these, the major area, was clearly associated with S-ethylcysteine, while the other faint areas could be exactly superimposed over compounds which gave a brown colour with ninhydrin in the presence of di*cyclo*hexylamine. Since these compounds were also present on similar chromatograms prepared from urine of rats injected with S-ethylcysteine, it was obvious that they were derived from it. Both compounds were shown to be acids, and one (spot d, Fig. 4) definitely shown also to have basic properties. Before acid hydrolysis of urine these compounds were present as ninhydrin-negative acids (absence of radioactivity in 400 A or 120 B solutions, Fig. 1), one at least a conjugated amino acid. This compound was shown to be S-ethylcysteine sulphone, (the faint radioactive spot d, Fig. 2, is due to a trace of free sulphone in the original urine) since it had an identical R_f with an authentic specimen in Solvents I, II, IV and V, gave the same characteristic brown colour with ninhydrin in the presence of di*cyclo*hexylamine, was stable to boiling acid but unstable to boiling alkali, and was clearly derived from S-ethylcysteine *in vivo*. While the nature of the conjugation prior to acid hydrolysis is not known, it is relevant to mention that the homologue methionine sulphone is excreted by rats as a glutamyl derivative.[18] The sulphone, when injected into rats (180 mg/2 cm^3 water) was excreted in a conjugated form. Hydrolysis of the urine with acid liberated the free sulphone, but there was no evidence that reduction to S-ethylcysteine had occurred.

To determine whether the other ninhydrin-positive compound present in hydrolysed urine was S-ethylcysteine sulphoxide, this compound was synthesized but had a different R_f in Solvents I and II and did not give a brown colour with ninhydrin in the presence of di*cyclo*hexylamine. While the sulphoxide could not be detected on paper chromatograms of either unhydrolysed or acid-hydrolysed urine, its presence could not be precluded for, if formed *in vivo*, it would almost certainly be excreted in a conjugated ninhydrin-negative form, and model experiments have shown that it

would not survive acid hydrolysis. Injection of a rat with the authentic compound followed by ion exchange and paper chromatography, showed that none was excreted unchanged, and that no sulphone was detectable on chromatograms of acid-hydrolysed urine, which was evidence to suggest that the sulphoxide was not an intermediate in the oxidation of S-ethylcysteine to its sulphone.

When urine from rats injected with ^{14}C-ethyl methanesulphonate or ^{14}C-S-ethylcysteine, was treated with 2:4-dinitrophenylhydrazine,[15] subsequent paper chromatography in Solvent III and autoradiography, showed that a very small amount of radioactivity was associated with one of the dinitrophenylhydrazones. In view of the finding of Shen and Lewis[19] that S-benzylcysteine and related compounds undergo oxidative deamination *in vivo* to the corresponding keto acids, and the fact that S-ethylcysteine is readily deaminated by both d- and l-amino acid oxidase *in vitro*,[20] it was considered likely that this compound was the dinitrophenylhydrazone of β-ethylmercaptopyruvic acid (IV).

$$C_2H_5SCH_2COCOOH$$

IV

The dinitrophenylhydrazone of this keto acid had an entirely different R_f in Solvent III from that present in the urine. To determine whether the keto acid was excreted unchanged from the rat, a 24 hr urinary sample from a rat injected with it (15 mg/2 cm^3 water) was examined. No unchanged keto-acid was detectable on treatment with either 2:4-dinitrophenylhydrazine or *o*-phenylenediamine,[15] but instead it was found

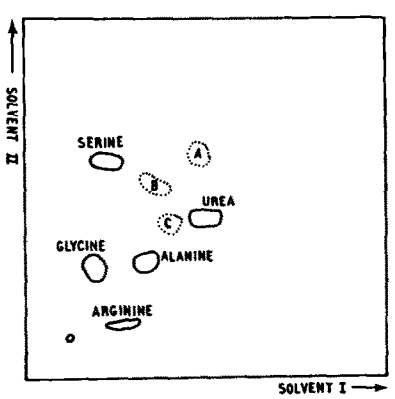


FIG. 5. Shows the ninhydrin-positive spots normally present on paper chromatograms (Solvent I and II) of urine from rats maintained on a 20 per cent protein diet (————), and those appearing in addition after injection of ethyl methanesulphonate (— — — —).

that a large proportion of the compound had been converted into derivatives of S-ethylcysteine. Hence while no conclusive evidence can be given for the formation of the keto acid from ethyl methanesulphonate or S-ethylcysteine *in vivo*, its participation as an intermediary metabolite may be considered.

Two-dimensional chromatograms of urine from rats maintained on a 20 per cent

protein diet showed fairly consistent amino acid excretion patterns (Fig. 5). Ninhydrin-positive areas corresponding in R_f to the amino acids serine, alanine, glycine and arginine were usually present. A white spot on the pale blue background was shown to be due to urea. Similarly prepared chromatograms of urine from animals injected with ethyl methanesulphonate showed on occasions, in addition to the normal excretion patterns, a faint brown spot due to unconjugated S-ethylcysteine sulphone (spot B, Fig. 5), a faint grey spot (more pronounced in the second day's urine) due to free S-ethylcysteine (spot A, Fig. 5), both areas containing radioactivity, and a bright yellow spot (spot C, Fig. 5). Urea was consistently found to contain an appreciable quantity of radioactivity, isolation of which was achieved by percolation of urine down columns of amberlite resin IR 120 and IRA 400. Evaporation of the final aqueous eluate (120 A/400 A solutions, Fig. 1), chromatograms of which revealed only one radioactive spot, that due to urea, gave a solid, M.P. 129°, undepressed on admixture with an authentic sample. The ninhydrin spot attributed to arginine invariably appeared associated with traces of radioactivity when papers were autoradiographed for a long time. Ninhydrin development of chromatograms of urine from rats injected with ^{14}C-S-ethylcysteine indicated the presence of traces of free S-ethylcysteine and S-ethylcysteine sulphone, but no radioactivity was found in urea. The yellow spot was also absent.

Absence of associated radioactivity proved that the compound giving a yellow colour with ninhydrin was not an ethylated product derived from the drug. The possibility of its being formed by interaction of methanesulphonic acid or ethanol, the hydrolysis products of ethyl methanesulphonate, with some cellular constituent, was eliminated following examination of the urine from rats injected with mixtures of these compounds in arachis oil. A considerable amount of work has failed to reveal the identity of this compound, but since it is not detected in urine from rats injected with other equally effective alkylating agents, it was not further investigated.

One small area of radioactivity on chromatograms of unhydrolysed urine from rats injected with ^{14}C-ethyl methanesulphonate (spot f, Figs. 2 and 3A) is not present on similarly prepared chromatograms from rats injected with ^{14}C-S-ethylcysteine. This compound which probably represents $< 0{\cdot}1$ per cent of the administered drug is almost certainly an alkylated product. It fluoresces intensely blue in ultra-violet light. No change in R_f of this compound was observed on treatment of the urine with cold alkali (pH 10 at room temperature for 24 hr) nor did the enzymes glucuronidase or acid phosphatase have any effect on it, while it is either decomposed or more probably undergoes a change in R_f on mild treatment with mineral acid.

Metabolism of 1-^{14}C-Ethyl Alcohol

Exhalation of ^{14}C-carbon dioxide

In Table 3 are recorded the quantities of radioactive carbon dioxide exhaled at intervals during 24 hr.

Nature of urinary metabolites

One per cent of the injected radioactivity was excreted in the urine. This radioactivity was associated with urea and to about the same extent as was found after injection of ^{14}C-ethyl methanesulphonate, indicating that only a few per cent of the urinary radioactivity derived from the drug is present in this form.

TABLE 3. TABLE SHOWING RATE OF EXHALATION OF ^{14}C-CO_2 AFTER INJECTION OF 1-^{14}C-ETHANOL

	Time after injection (hr)	Exhaled radioactivity (μc)	Per cent total injected radioactivity	Per cent total expired radioactivity
	0–0·5	9·0	10	13·5
	0·5–1·0	17·5	20	26·3
	1–1·5	12·9	14·6	19·5
	1·5–2·0	10·0	11·4	15·0
	2–3	9·3	10·1	13·9
	3–4	4·2	4·8	6·3
	4–5·5	1·8	2	2·7
	5·5–24	1·9	2·1	2·8
Total		66·7	75	100

Metabolism of ^{14}C-S-Ethylcysteine

Exhalation of ^{14}C-carbon dioxide

Only 4·6 per cent of the injected radioactivity is expired during 48 hr, 2·3 per cent of which is exhaled during the first 6 hr.

Radioactivity in urine

Table 4 shows the rate of excretion of urinary radioactivity. The nature of the urinary metabolites has been discussed earlier.

TABLE 4. TABLE SHOWING RATE OF EXCRETION OF URINARY RADIOACTIVITY AFTER INJECTION OF ^{14}C-S-ETHYLCYSTEINE

	Time after injection (hr)	Per cent total injected radioactivity
	0–28	27
	28–54	5·6
	54–120	8·6
Total		41·0

Radioactivity in faeces

Four per cent of injected radioactivity appeared in the faeces after two days.

Metabolism of Methyl and Propyl Methanesulphonates

The formation of mercapturic acids appears to be a general metabolic pathway for compounds of this type, since after injection of methyl methanesulphonate and propyl methanesulphonate (20 mg and 60 mg/200 g rat respectively), new sulphur-containing acids were detected in the urines, while S-methylcysteine and S-propylcysteine were identified after treatment with hot concentrated hydrochloric acid.

Metabolism of S-Ethylglutathione

Unidimensional chromatograms of urine obtained after injection of S-ethylglutathione (200 mg/2 cm^3 water) were essentially similar to those obtained

after injection of ethyl methanesulphonate or S-ethylcysteine in that two sulphur-containing acids were detected, one of which possessed an R_f comparable to N-acetyl-S-ethylcysteine. Heating the urine with hot concentrated hydrochloric acid liberated S-ethylcysteine.

DISCUSSION

^{14}C-ethyl methanesulphonate is metabolized by two major pathways, one involving hydrolysis to ethanol which is rapidly metabolized and excreted, and the other, reaction with the thiol group of cysteine or cysteine-containing compounds such as glutathione or protein. Evidence for the first route of metabolism is based on the exhalation of radioactive carbon dioxide and the excretion of radioactive urea, compounds which are also metabolites of ethanol. Furthermore since S-ethylcysteine gives rise to only a trace of ^{14}C-carbon dioxide, and since the exhalation of ^{14}C-carbon dioxide from injected ethanol is extremely rapid and almost quantitative, then the rate of exhalation of ^{14}C-carbon dioxide after injection of ^{14}C-ethyl methanesulphonate is a measure of the rate of decomposition *in vivo* of the drug *per se*, or of any intermediate ester formed from it. It is seen from Fig. 6 that 50 per cent of the total

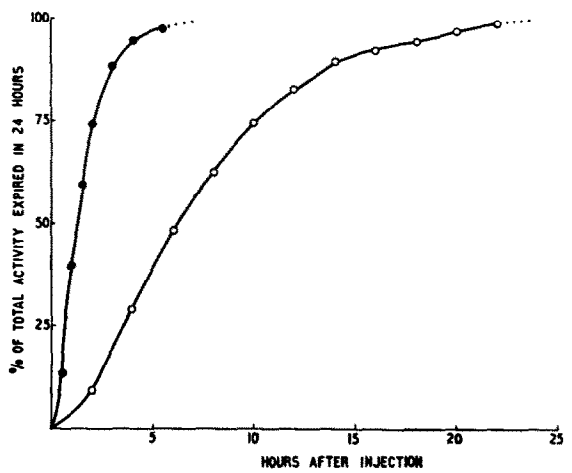


FIG. 6. Curves showing the rate of exhalation of C^{14}-CO_2 following injection of C^{14}-ethyl methanesulphonate (—○—○—) and 1-^{14}C-ethanol (—●—●—).

expired radioactive carbon dioxide is exhaled in 6·5 hr and if as seems reasonable this may be regarded as the half-life of the drug *in vivo*, it agrees well with the value of 7·5 hr obtained for its half-life in water at 37° (Dr. W. Davis, personal communication). The simplest explanation is that ethanol is derived directly from the drug rather than *via* an intermediate ester. As 75 per cent of the radioactivity from injected ^{14}C-ethanol, given in amount equivalent to that which would be formed if all the ethyl methanesulphonate was hydrolysed, is actually exhaled as carbon dioxide, then the amount of ethyl methanesulphonate which is converted to ethanol could well be as much as 40 per cent.

It has been shown by a comparison of the urinary excretion patterns obtained after injecting rats with ^{14}C-ethyl methanesulphonate and ^{14}C-S-ethylcysteine that almost all (probably more than 95 per cent) of the urinary radioactivity is associated with

derivatives of S-ethylcysteine. While only 20 per cent of the injected radioactivity from ^{14}C-ethyl methanesulphonate is excreted in this way, it would seem, again by comparing the quantities of radioactivity excreted after administration of S-ethylcysteine, that this could indicate that in fact twice this amount of reaction with cysteine had occurred, for only a total of 45 per cent of the radioactivity from ^{14}C-S-ethylcysteine is excreted in the urine, or exhaled as carbon dioxide during the five days after injection. Hence much of the S-ethylcysteine remains bound in the body, or more likely, is converted to a volatile product which is lost. In this connection it is relevant to mention the work of Quastel,[21] who has suggested (personal communication) that the antitubercular effect of S-ethylcysteine and similar compounds is related to the ease of fission of the drugs to volatile ethyl mercaptan. From these exterpolations it would seem that each of these two metabolic routes accounts for nearly 50 per cent of the administered ethyl methanesulphonate.

The origin of the cysteine involved in mercapturic acid synthesis was of considerable importance in assessing the relevance of the findings to the reactions of the drug *in vivo*. Many compounds including halogenated benzenes and certain cyclic hydrocarbons are excreted as mercapturic acids. While mercapturic acid synthesis from these compounds is probably a complex enzymic process, we are probably concerned in this study with a different reaction mechanism, namely direct alkylation of an ionized thiol group. Since it is generally believed that the level of free cysteine in the mammalian body is low, it seems unlikely that direct alkylation of cysteine by ethyl methanesulphonate has been the only route of mercapturic acid formation. Reaction between ethyl methanesulphonate and ionized cysteine is rapid *in vitro*, but so also is that with ionized glutathione, a compound which is present in large quantities in the reduced state in most mammalian viscera, connective tissue, and blood. Examination of the urine from rats injected with S-ethylglutathione has in fact revealed the presence of metabolites of S-ethylcysteine formed by hydrolysis of the peptide *in vivo*. This observation is not without precedent since a similar conversion of S-substituted glutathiones to the corresponding mercapturic acids has been observed.[22] For many years the view was held that protein thiol groups, that is those present in basic tissue and in enzymes, were not appreciably ionized at physiological pH and hence that their reactivity towards electrophilic reagents, such as the various classes of alkylating agents, would be low in the presence of other groups such as ionized carboxyl and ionized phosphate. The demonstrated ability of alkylating agents to inhibit numerous —SH enzyme systems, such as hexokinase[23] and papain,[24] and to inactive myosin in muscle,[25] and the more recent results of P. Alexander (personal communication) who has studied the action of ethyl methanesulphonate on denatured ovalbumin, would indicate that despite the apparent high p*Ka* of protein SH groups reaction does in fact occur. Very relevant is the work of H. P. Burchfield[26] who has elegantly drawn attention to the potentiation of reactivity of certain groups at the surface of large molecules due to the creation of microenvironments. It is clear from this work that the character of every SH group within a protein structure is unique, its properties being dependent on its particular environment within that structure, which will affect not only its p*Ka* but also its steric availability, factors which will affect its reactivity towards different reagents. It is clear therefore that reactions between alkylating agents and protein thiol groups could conceivably produce diverse pharmacological effects depending on the particular group attacked (see later). The salient

factor to be mentioned here is that such alkylated proteins would almost certainly be metabolized to smaller fragments and give rise in this case to the derivatives of S-ethylcysteine found in the urine.

The demonstrated reaction between methanesulphonates and thiol groups *in vivo* provides an explanation for the remarkable properties of the related chloroethyl methanesulphonate (CB 1506) which is a powerful inhibitor of the growth of the transplanted Walker rat carcinoma, and atypical of the mesyloxy compounds in its mutagenic effects on *Drosophila melanogaster*.[27] An earlier communication[8] suggested that reaction with cysteine in an analogous manner to that found in the case of ethyl methanesulphonate produces *in situ* S-chloroethylcysteine, or a related compound such as S-chloroethylglutathione which would function as a monofunctional sulphur mustard. The finding by Fahmy and Fahmy[28] that S-chloroethylcysteine (synthesized by Dr. W. C. J. Ross) has the same characteristic high mutagenic effects on the early germ cells of *Drosophila melanogaster* as chloroethyl methanesulphonate has led them to support this view.

The conversion of a compound *in vivo* to one which is able to exert more potent effects on the host is not without precedent. It could be envisaged therefore that the tumour-growth-inhibiting effects of ethyl methanesulphonate might be due to one of its metabolites. Accordingly S-ethylcysteine, S-ethylcysteine sulphone, S-ethylcysteine sulphoxide and ethylmercaptopyruvic acid were tested for activity against the transplanted Walker rat carcinoma by Professor A. Haddow. Each of these compounds except the keto-acid had a low toxicity and none had any inhibitory effect on tumour-growth. The keto-acid although converted to harmless metabolites was unexpectedly toxic. Since the products formed *in vivo* from ethyl methanesulphonate do not appear to be responsible for the biological effects of the drug, one is left with a number of alternative possibilities. The drug may be acting on functional groups other than the thiol group, as has been postulated by a number of other workers, or as is more likely, on a whole variety of functional groups throughout the body.

It should be stressed that in our present state of knowledge one cannot refer to particular reactions on the components of tumour cells different from those on normal cells which result in specific tumour-growth-inhibiting effects by the alkylating agents; they do however exert a unique effect on dividing cells. While certain phenomena such as the production of gene mutations and chromosome breaks could be the result of reactions at particular loci, the effect on tumours, like the systemic toxicity usually associated with it, could be the result of diverse and distant reactions. Considering only alkylation of the thiol group it is not difficult to envisage the multiple pharmacological effects which could result. It has been shown that compounds reacting with cysteine, sufficient to cause its depletion, can inhibit body growth. Inhibition of enzymes or reaction with co-factors such as coenzyme A, requiring intact —SH groups for their activity, could lead to an interference with many energy-yielding biosynthetic systems which could prevent cell division. The importance of thiol groups in the process of cell division has been frequently emphasized;[29] for not only has it been shown that their concentration varies throughout the mitotic cycle but compounds which can react with thiol groups inhibit cell division.[30]

In addition to their possible usefulness in the treatment of neoplastic disease, the alkylating agents have aroused considerable interest in cancer research by their ability to produce mutations and under certain conditions to initiate tumour-growth. In this

connection it is interesting to recall the work of Crabtree[31] which implicates reaction with thiol groups as a possible step in carcinogenesis.

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THE ENZYMIC OXIDATION OF CHLORAL HYDRATE TO TRICHLOROACETIC ACID*

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Abstract—A DPN-dependent enzyme system which catalyses the oxidation of chloral hydrate to trichloroacetic acid has been isolated from liver and partially purified. No naturally occurring substrate for this enzyme has been found.

THAT the major metabolic pathway of chloral hydrate metabolism involves its reduction to trichloroethanol, while its oxidation to trichloroacetic acid is a minor metabolic alteration, has been known for some time.[1,2] In addition, it has been reported that liver slices cause the dehalogenation of chloral hydrate.[3] However, in no instance has an attempt been made to determine the specific nature of the enzymes catalysing these reactions.

In this paper are described the isolation and partial purification of an enzyme which catalyses the oxidation of chloral hydrate to trichloroacetic acid and for which, so far, no physiological substrate has been found.

MATERIALS AND METHODS

Chloral hydrate and trichloroacetic acid were obtained commercially and assayed according to methods described in the U.S. Pharmacopoeia (Revision XV). Trichloroethanol was obtained from E. R. Squibb and Sons through the courtesy of W. A. Lott. DPN, DPNH, TPN, FMN and FAD† were purchased from the Sigma Chemical Company. SKF525A, generously supplied by Glenn Ullyot of the Smith, Kline and French Company, was hydrolysed to diphenylpropylacetic acid. Tetraethylthiuram disulphide ("Antabuse") was a gift of the Ayerst Laboratories. Bromal, DEAE-cellulose resin and 2-benzyl-2-thiopseudourea were purchased from Distillation Products Industries, Division of Eastman Kodak Company. Liver and yeast alcohol dehydrogenase were obtained from Worthington Biochemical Corporation.

Bromal hydrate, prepared by heating bromal in water and recrystallizing the product from hot water, melted at 53°C. Monoamine oxidase was prepared according to the procedure of Weissbach *et al.*[4] Liver aldehyde dehydrogenase was obtained from beef liver following the method of Racker.[6] Protein was determined by the method of Warburg and Christian.[7]

The enzyme mediating the oxidation of chloral hydrate to trichloroacetic acid was prepared as follows. An acetone powder of rabbit liver, prepared in the usual manner, was extracted at room temperature three times, each time for 10 min and with three volumes of phosphate buffer (0·001 M, pH 7·4). Centrifugations and all subsequent

* Taken from a Thesis to be submitted by Paul J. Friedman for the degree of Doctor of Medicine.
† DPN and DPNH, oxidised and reduced diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide.

operations were performed at 0—3°. To the pooled extracts was added slowly (dropwise with stirring), 1·22 volumes of a saturated solution of ammonium sulphate (pH 8·0), to yield a mixture 55 per cent saturated with respect to ammonium sulphate. The mixture was then centrifuged at 20,000 × *g* for 15 min. To the supernate was added 0·50 volume of the saturated solution of ammonium sulphate to bring the saturation to 70 per cent. The preparation was again centrifuged for 15 min at 20,000 × *g*. The supernate was discarded and the precipitate (55–70 per cent ammonium sulphate fraction) was taken up in a small volume of water and dialysed against 6 l. of water for 16 hr with continuous stirring.

The dialysed preparation (but not more than one gramme of protein) was put on a DEAE-cellulose column (2 × 35 cm) previously prepared by washing with a solution of sodium hydroxide (1 N) and then with water. The column to which the enzyme preparation was added was washed with water (500 ml) and phosphate buffer (0·005 M, pH 7·4, 250 ml), and the enzyme was then eluted with a stronger phosphate buffer (0·02 M, pH 7·4). Fractions of 10 ml each were collected and assayed. The contents of the tubes containing the enzyme activity were pooled and lyophilized. Since the purified enzyme is most stable in this condition, it was maintained as a lyophilized powder in a vacuum desiccator at 0–3°. When needed, a portion of the powder was taken up in a minimal amount of water and dialysed against water for 3 hr to remove the phosphate buffer. After dialysis, the preparation was centrifuged at 20,000 × *g*. for 10 min and the precipitate discarded. This preparation was completely free of alcohol dehydrogenase, lactic dehydrogenase, aldehyde dehydrogenase and DPNH oxidase. Whether the enzyme solution was kept frozen or was repeatedly frozen and thawed, the activity slowly declined at about the same rate, i.e., about 50 per cent in a week. The addition of serum albumin (3 mg/ml) reduced the rate of inactivation of the enzyme. A summary of the method of purification is given in Table 1.

TABLE 1. PURIFICATION PROCEDURE

Preparation	Total units	Specific activity*
Acetone powder extract	252,000	40
Dialysed ammonium sulphate fraction	65,800	130
Column eluate	19,500	1,240

* Units (× 1000) per milligram of protein.

Two procedures were employed in assaying enzyme activity. The first one, outlined below, was based on the appearance of an absorption band at 340 mμ when DPN is reduced. With chloral hydrate in excess, the rate of reduction of DPN was proportional to enzyme concentration.

Reagents: glycine-pyrophosphate buffer (0·1 M, pH 9·5)
DPN (10 mg/ml)
chloral hydrate (0·1 M)
enzyme (7–50 units)

To a micro quartz cell with a 1 cm light-path were added buffer (0·5 ml), DPN (0·1 ml), chloral hydrate (0·05 ml), the enzyme sample to be assayed, and water to give a final volume of 1 ml; the reaction was started by the addition of enzyme.

Density readings were taken in a Beckman spectrophotometer at 340 mμ at one-minute intervals.

A unit of enzyme is defined as that amount which caused an increase in optical density of 0·001 unit/min under the conditions described.

In some crude preparations, in which DPN oxidase activity precluded an accurate assay of enzyme activity by the above procedure, another method was adopted which involved the direct determination of the trichloroacetic acid produced in the reaction. This procedure was also used in experiments in which it was necessary to determine the concentration of trichloroacetic acid. This method is as follows: concentrations and compositions of reagents were the same as was described above.

To buffer (0·5 ml) in a test tube were added DPN (0·5 ml), chloral hydrate (0·2 ml), enzyme and water to give a final volume of 3 ml. The tube was placed in a water bath at 37° for 30 min, following which a solution of sulphosalicylic acid (50 per cent, 0·3 ml) was added. The contents of the tube were mixed and centrifuged at 2000 × *g* for 10 min. An aliquot (0·5 ml) was taken for measurement, according to the procedure of Friedman and Cooper[8] of the amount of trichloroacetic acid formed in the reaction. Appropriate standards were run simultaneously to correct for any effect of DPN or the enzyme preparation on the values obtained.

Unless otherwise specified, all the data in the "Results" section of the paper are expressed in terms of the first assay procedure outlined above, i.e. the direct spectrophotometric assay based on the rate of reduction of DPN.

RESULTS

Coenzyme requirement

A thoroughly dialysed acetone powder extract or fresh homogenate of liver showed no activity unless supplemented with DPN. TPN could not substitute for DPN and, in fact, inhibited the activity of DPN when the two pyridine nucleotides were added to the purified preparation (Table 2). Fig. 1 is a Lineweaver-Burke plot[9] of the data

TABLE 2. INHIBITION OF DPN BY TPN

Coenzyme added	△ O.D./min × 1000
DPN (1 mg)	78
TPN (1 mg)	8
DPN (1 mg) and TPN (0·5 mg)	8
DPN (1 mg) and TPN (1 mg)	8
DPN (1 mg) and TPN (2 mg)	6

Procedure as described under *Methods*.

obtained by following the increase in the rate of the reaction with increasing concentrations of DPN. The K_M for DPN was calculated to be $1{\cdot}7 \times 10^{-3}$ M. Although the optimal rate of enzyme activity was obtained at a level of about 2 mg DPN/ml, half that amount was used in all experiments, unless otherwise noted, since this extraordinarily high requirement for DPN made this procedure somewhat costly.

No stimulation of activity was observed when glutathione, FAD, FMN, ADP or CoA were added to an aged preparation of the enzyme which had lost about one-half of its original activity.

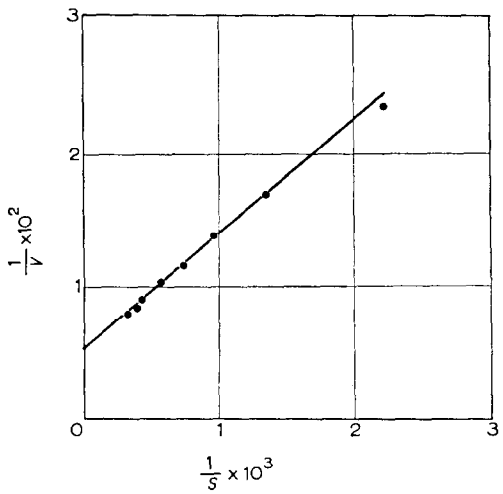

FIG. 1. Lineweaver-Burke plot of reaction velocity as a function of DPN concentration.

Optimal pH

The optimal pH for the enzymic oxidation of chloral hydrate, under the conditions of the assay, was 9·4–9·6. Using either tris or glycine-sodium hydroxide buffer, the reaction was stimulated by the addition of either phosphate or pyrophosphate (Table 3). The stimulatory activity of these anions could not be replaced by ethylenediamine

TABLE 3. STIMULATION BY PHOSPHATE AND PYROPHOSPHATE

Phosphate or pyrophosphate (μmoles)	$\triangle$ O.D./min $\times$ 1000
0	34
2	54
6	68
12	80

Procedure described under *Methods* section, except that glycine-NaOH buffer was used.

tetraacetate. At pH 8·0 and pH 10·5, the activity was decreased to about one-third that observed at pH 9·4–9·6; below pH 8·0 a negligible rate of enzymic activity was obtained.

Localization of enzyme activity

(*a*) *Species.* The oxidation of chloral hydrate to trichloroacetic acid has been observed in the liver of beef, rabbit and guinea pig.

(*b*) *Tissue.* In the rabbit, enzyme activity was found in the liver and kidney. No activity was observed in heart, brain or muscle, in confirmation of the experiments of Butler[5] using slices of canine tissues.

(*c*) *Intracellular.* In a freshly prepared liver homogenate centrifuged at 105,000 $\times$ *g* for 30 min, the enzyme catalysing the transformation of chloral hydrate to trichloroacetic acid was completely recoverable in the soluble fraction of the cell.

Inhibitors

The effect of various compounds, including several inhibitors of aldehyde dehydrogenase, on the reaction was examined; these included "Antabuse" (10^{-3} M), *p*-chloromercuribenzoate (10^{-4} M), and arsenite (10^{-3} M), each of which was without effect. Similarly, diphenylpropylacetic acid (SKF-acid) which inhibits a variety of metabolic transformations of many drugs,[10] caused no inhibition of the enzyme system when tested at a final concentration of 10^{-3} M. On the other hand, some substrates for liver aldehyde dehydrogenase, e.g. formaldehyde, acetaldehyde, and benzaldehyde, markedly inhibited the oxidation of chloral hydrate by the enzyme system under study.

Balance studies

The correspondence between trichloroacetic acid produced and DPNH formed when chloral hydrate is incubated with DPN and the enzyme preparation is shown in Table 4. No reversal of the reaction could be shown when trichloroacetic acid was incubated

TABLE 4. BALANCE STUDY

	DPN reduced (μmoles)	TCA formed* (μmoles)
Experiment I	0·100	0·096
Experiment II	0·101	0·104
Experiment III	0·144	0·130

* Determined as described in text under *Methods*.

with DPNH at pH 7·4, even when liver alcohol dehydrogenase was added to pull the reaction and, in addition, to increase the sensitivity two-fold.

Identification of trichloroacetic acid

An acetone powder extract of rabbit liver (40 ml), together with chloral hydrate (500 mg), DPN (20 mg), and glycine-pyrophosphate buffer (0·1 M, pH 9·5) was incubated for 2 hr at 37° in air. Subsequently, 4 volumes of 95 per cent ethanol were added; the mixture was chilled, filtered, and the filtrate concentrated to 15 ml in a flash evaporator at 32°. The residue in the flask was then extracted twice with 10 volumes of ether, the ether extracts were pooled and evaporated to 20 ml under a stream of air. A saturated solution of 2-benzyl-2-thiopseudourea hydrochloride (5 ml) was added and the white crystals which formed were collected by filtration, washed with water and recrystallized from hot water, *iso*amyl alcohol, and finally ethanol (yield = 72 mg). The melting point of the derivative was 150°C.* No depression of the melting point was observed when the sample was mixed with an authentic sample of thiocarbamide benzyl trichloroacetate.[11]

Substrate specificity

Table 5 lists the compounds tested as substrates for the enzyme preparation. Glyceraldehyde, glycerol and 5-hydroxyindoleacetaldehyde also were without activity

* When determined simultaneously, both the apparent derivative and the authentic product melted at the same temperature. However, since the product melts with decomposition, melting points could be obtained from 139 to 150° depending on the rate of heating. The 150° melting point was arbitrarily used in the text since the discoverer of the reaction lists this value.

TABLE 5. COMPOUNDS INACTIVE AS SUBSTRATES

Compound	Compound
Formaldehyde	Glycerol
Acetaldehyde	3-*o*-Toloxy-1:2-propanediol
Butyraldehyde	Trichloroethanol
Benzaldehyde	α-Hydroxy-β-dimethyl-ν-butyrolactone
Chlorobenzaldehyde	
5-Hydroxyindole acetaldehyde*	Pyridoxal
Theophylline	Pyridoxal phosphate
Theobromine	
Caffeine	Formic acid
	L-cysteine
D-Glucose	
Uridine diphosphoglucose	
α-Methylglucoside	
β-Methylglucoside	
Alloxan	
DL-Glyceraldehyde	

* Prepared by incubation of 5-hydroxytryptamine with monoamine oxidase. That 5-hydroxyindole acetaldehyde was formed was shown by running a simultaneous control with liver aldehyde dehydrogenase replacing the "chloral hydrate dehydrogenase". A rapid reduction of DPN occurred in this control cuvette.

at pH 8·0. The only substrates, other than chloral hydrate, which caused a reduction of DPN in the presence of the enzyme, were bromal hydrate and monochloroacetaldehyde; the rate of oxidation of each was about one-half that of chloral hydrate. In an attempt to find a natural substrate for the enzyme, "kochsafts" were made of guinea-pig liver, heart, brain and kidney and incubated with DPN and the purified preparation. No reduction of DPN was observed with any kochsaft.

DISCUSSION

The most remarkable feature of this enzyme system lies in its unusual substrate specificity. Although it may be assumed that the enzyme has a function in the body other than to catalyse the oxidation of chloral hydrate to trichloroacetic acid, no naturally occurring substrate has so far been found.

In studies *in vivo* on bromal hydrate metabolism in dogs, Butler[11] obtained no evidence for either reduction to tribromethanol or oxidation to tribromoacetic acid. However, bromal hydrate, in the rabbit liver preparation, is oxidised, and like chloral hydrate, is reduced in the presence of liver alcohol dehydrogenase.[13] With respect to chloral hydrate reduction, it is not known whether alcohol dehydrogenase is the only enzyme in the body which is capable of causing a reduction of the compound to trichloroethanol.

In addition to the substrate specificity, two other curious phenomena exhibited by the enzyme system are the stimulation by phosphate and pyrophosphate and the usually high DPN requirement for saturation. No explanation for these observations can be offered at the present time.

Returning to the question of the natural substrate of this enzyme, it is conceivable that this enzyme has never before been described. This investigation may then point out a hitherto lightly regarded facet of drug metabolism studies, namely, the use of a drug as a substrate to uncover new enzyme systems.

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DER EINFLUSS MÖGLICHER ADRENALINVORSTUFEN AUF DIE AUSSCHEIDUNG VON ADRENALIN IM HARN VON RATTEN

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Abstract—Using a fluorometric determination the urinary content of epinephrine in normal and 1-DOPA resp. HOT treated rats was investigated.

The following results could be obtained:

(1) In normal rats the 24 hr urinary output was of the order of 1 μg.

(2) Daily administration of 100 μg Methyldihydroxyphenylserine (MDOPS), or of 10 mg 1-DOPA and HOT respectively did not cause any change in the 24 hr urinary epinephrine output.

In a second series of experiments the urinary excretion of subcutaneously administered epinephrine was investigated in rats. It was found that following the injection of 50 μg to 400 μg epinephrine/animal, 4–5 per cent of the injected epinephrine was recovered in the urine. The percentage of the urinary excretion of epinephrine was independant of the dosage and was not altered by repeated administration of epinephrine (every 6th day) to the same animal group throughout a period of fifty days.

Daily treatment with 10 mg 1-DOPA per animal did not lead to a significant change of the urinary excretion of subcutaneously administered epinephrine. However the daily administration of 10 mg HOT per animal produced a slight decrease in the urinary recoveries of injected epinephrine.

Über den Abbau des Adrenalins im tierischen Organismus herrscht bis heute noch keine endgültige Klarheit. Ein Abbau über Indolderivate,[1] wie er *in vitro* vorliegt, konnte *in vivo* nicht mit Sicherheit nachgewiesen werden.[2,3,4,5] Der von Blaschko[5] angenommene Abbau durch oxydative Desaminierung wurde durch den Nachweis einer vermehrten Ausscheidung von 3-Methoxy-4-Hydroxymandelsäure nach Verabreichung von Noradrenalin durch Armstrong und McMillan[6] gestützt. Auch die Zufuhr von Dihydroxyphenylalanin (DOPA) und Hydroxytyramin (HOT) führte zu einer vermehrten Ausscheidung von Catecholsäuren bzw. zu den in 3-Stellung methylierten Derivaten (Dihydroxyphenylessigsäure, Homovanillinsäure).[7,8,9] Allerdings verläuft der Abbau in quantitativer Hinsicht bei diesen Verbindungen nicht gleichwertig, da beim Adrenalin nach i.v. oder s.c. Zufuhr in der Regel nur etwa 1–4 prozent der zugeführten Menge in unveränderter Form durch die Nieren ausgeschieden werden[10,11,12,13], während von DOPA bis zu 16 prozent im Harn unverändert wiedergefunden wurden.[9]

In der vorliegenden Arbeit wurde nun der Einfluß eines gleichzeitigen Abbaues von 1-DOPA bzw. HOT auf die quantitativen Verhältnisse des Adrenalinabbaues im Gesamtorganismus an Hand der Veränderungen der Harn-Adrenalinausscheidung bie gleichzeitiger Zufuhr der beiden Vorstufen an Ratten untersucht. Hiebei war es aber zunächst notwendig, den Einfluß einer massiven DOPA bzw. HOT-Verabreichung auf die Adrenalin-Leerausscheidung im Harn der Tiere festzustellen, damit eine

eventuelle Neusynthese von Adrenalin aus diesen Verbindungen nicht einen veränderten Abbau vortäuschen könnte. Bei diesen Untersuchungen wurde auch noch N-Methyl-Dihydroxyphenylserin (MDOPS)* als ebenfalls mögliche Vorstufe mituntersucht.

METHODIK

Der Nachweis von Adrenalin im Harn

Der Nachweis von Adrenalin im Harn wurde zunächst nach der Methode von Euler und Floding[14] durchgeführt. Die Versuche scheiterten jedoch daran, daß uns zu diesem Zeitpunkt keine geeigneten Aluminiumoxydpräparate zur Verfügung standen. Wir waren daher gezwungen, auf eine alte Vorschrift von Euler[15] zurückzugreifen und die Adsorption des Adrenalins an frisch gefälltem Aluminiumhydroxyd durchzuführen. Bei der Elution wichen wir dann insoferne von der Euler'schen Vorschrift ab, als wir Phosphorsäure verwendeten, um die Entfernung des Aluminiumions durch Alkohol-Azeton-Fällung möglichst quantitativ zu gestalten. Im Einzelnen wurde folgendermaßen verfahren:

Je 50 ml Harn wurden auf ein pH von 2,0 (elektrotitrimetrisch) gebracht und 5 Min. aufgekocht. Nach Abkühlung und Filtration wurden 2,5 ml 25% ige Aluminiumsulfatlösung zugesetzt und hernach unter kräftigem Umrühren (magnetisches Rührwerk) durch tropfenweisen Zusatz einer 2 n NaOH elektrometrisch auf ein pH von 8,5 eingestellt. Das ausgefällte Aluminiumhydroxyd wurde zweimal (einmal Nachwaschen mit Wasser) abzentrifugiert und anschließend mit 0,7 ml konzentrierter Phosphorsäure in ca 20 ml Gesamtvolumen gelöst. Nach vollständiger Lösung (pH 2,5–2,7) wurde das Aluminiumphosphat unter kräftigem Umschütteln durch Zusatz von 80 ml Alkohol-Azetongemisch (1:1) ausgefällt und abfiltriert. Nach Nachwaschen mit Alkohol-Azeton wurde das Filtrat am Wasserbad im Vakuum von den organischen Lösungsmitteln befreit und auf etwa 10 bis 15 ml eingeengt. Hierbei muß vermieden werden, daß das Filtrat völlig zur Trockenen gebracht wird, da dadurch eine teilweise Zerstörung des Adrenalins (vermutlich unter Einwirkung konzentrierter Phosphorsäure) bewirkt wird. Der wässrige Rückstand wurde auf ein pH von 3,5 eingestellt und auf das ursprüngliche Harnvolumen (50 ml) aufgefüllt. Je 1 ml dieser Endverdünnung wurde mit 1 ml 2 n Acetatpuffer pH 3,5 versetzt und nach der Vorschrift von Euler und Floding[14] durch Zusatz von 0,1 ml 0,25% Kaliumferricyanid sowie 0,1 ml 0,5% Zinksulfat zu Adrenochrom oxydiert. Nach kräftigem Umschütteln wurde 3 Min. später die Umlagerung zu Adrenolutin durch weiteren Zusatz von 1 ml Ascorbinsäure-Natronlauge-Mischung (0,2% Ascorbinsäure, 20% NaOH) durchgeführt. Nach Auffüllung mit dest. Wasser auf 10 ml wurde die Fluoreszenzintensität dieser Lösung in einem Beckman-Fluorometer unter Verwendung eines Chininstandardes (0,25 μg/ml Chininsulfat in 0,1 n H_2SO_4) sowie der Filter Schott BG 12 (Eingangsfilter) und Schott OG 4 (Ausgangsfilter) gemessen. Die Bestimmungen wurden 1.) in der oben angeführten Weise mit der Probe allein sowie 2.) nach Zusatz von 0,2 μg Adrenalin und .3) in der oben angeführten Weise, jedoch ohne Zusatz von Ascorbinsäure zur Ermittlung des Fluoreszenzleerwertes der einzelnen Harneluate durchgeführt.—Berechnet wurden A) die Differenz der Fluoreszenzintensitäten von 1.) und 3.) (die durch den Adrenalingehalt im Harn verursachte Fluoreszenz) und B) die Differenz der Intensitäten zwischen 1.) and 2.). Aus diesen Differenzwerten konnte

* Wir danken den Chemikern der Fa. Philips, Weesp, Holland für die Synthese und Bereitstellung der für diese Versuche benötigten Substanzmengen von MDOPS.

durch Vergleich der Adrenalingehalt der Probe bzw. nach Multiplikation mit dem Verdünnungsfaktor im Harn berechnet werden.

Zunächst wurde die Brauchbarkeit der Nachweismethodik an Hand einer Reihe von Leerversuchen geprüft. Nach Adsorption von Adrenalin aus Wasser und Bestimmung in den Eluaten wurden im Mittel 95,5 ± 5,0% der zugesetzten Adrenalinmenge (10 Werte zwischen 10–20 μg) wiedergefunden.

Eine Wiederholung dieser Versuche mit Adrenalinzusätzen zu Rattenharn ergab eine Wiedergewinnungsquote von durchschnittlich 70%. Hierbei konnte jedoch festgestellt werden, daß auch die Fluoreszenzintensität der Kontrollzusätze (0,2 μg Adrenalin) in den Harnextrakten bis zu 50% abgeschwächt war. Eine Hemmung der Adrenalinfluoreszenz in unverdünnten Eluaten von Harnproben wurde auch von Pitkänen[16] beobachtet, wobei dieser Effekt durch entsprechende Verdünnung zum Verschwinden gebracht werden konnte[17]. Aus diesem Grunde führten wir unsere Untersuchungen in der Folge zusätzlich zum unverdünnten Eluat mit 2 Verdünnungen des Eluates (1:2 und 1:4) durch. Es zeigte sich, daß dem Harn zugesetztes Adrenalin (10 Versuche mit 5–20 μg) bei Nichtverdünnung mit 71,1 ± 12,2%, bei Verdünnung 1:2 mit 80,4 ± 9,2% und bei Verdünnung 1:4 mit 95,9 ± 7,1% wiedergefunden werden konnte.

Aus diesen Ergebnissen geht hervor, daß in unseren Versuchen erst beiein er Eluatverdünnung auf das Vierfache des ursprünglichen Harnvolumens eine weitgehende verlustfreie Bestimmung des Adrenalins möglich war. In der Folge wurde bei allen untersuchten Harnproben die Adrenalinbestimmung in den Eluaten nach Verdünnung auf das Doppelte sowie auf das Vierfache des ursprünglichen Harnvolumens durchge führt. Um gleichzeitig auch einen Anhaltspunkt dafür zu haben, ob der Verdünnungsgrad genügend war, wurde fallweise auch eine Verdünnung 1:8 untersucht. Die untere Grenze des Nachweises von Adrenalin aus Menschen-oder Rattenharn lag bei der hier mitgeteilten Methode bei 0,5 μg/100 ml.

Versucheüber den Einfluß von MDOPS, l-DOPA und HOT auf die Adrenalinbestimmung im Harn

In den in den Abschnitten II und IV beschriebenen Versuchen erhielten die einzelnen Tiergruppen während der Periode der Harnsammlung maximal 300 μg MDOPS, sowie 30 mg l-DOPA bzw. HOT. Bei roher Annahme einer Ausscheidungsquote von rund 10% im Harn mußte mit einem maximalen Gehalt dieser Substanzen von ca 30 μg MDOPS, 3 mg l-DOPA bzw. HOT in den untersuchten 24-Stunden-Harnmengen bzw. in den verdünnten Elutionsansätzen der fluorometrischen Bestimmung mit einem Gehalt von rund 0,3 μg MDOPS bzw. 30 μg l-DOPA oder HOT gerechnet werden. Bei der Ausführung der Oxydation bzw. der fluorometrischen Bestimmung nach der von Euler und Floding[14] angegebenen Vorschrift bei einem pH von 3,5 konnten wir feststellen, daß nur l-DOPA in den oben angeführten Konzentrationen eine nennenswerte Fluoreszenz bzw. Störung der Adrenalinbestimmung verursacht. Diesbezüglich durchgeführte Leerversuche mit der von uns verwendeten Nachweismethode ergaben, daß l-DOPA zu rund 50% der im Harn zugesetzten Menge in den Eluaten aufscheint und Störungen des Adrenalinnachweises ab Harn-DOPA-Konzentrationen von 1 mg/50 ml in Erscheinung treten. Bei Annahme einer 10% igen DOPA-Ausscheidung in unseren Versuchen würde eine solche Störung größenordnungsmäßig zu einer 50%igen Vermehrung der Adrenalinleerausschüttung

führen. Tatsächlich konnte in den unter II beschriebenen Versuchen in keinem Falle ein Anstieg der Adrenalinleerausscheidung im Harn unter 1-DOPA festgestellt werden. Dies scheint dafür zu sprechen, daß 1-DOPA in unseren Versuchen nahezu vollständig abgebaut wurde. Dies ist ohne weiteres möglich, da Pellerin und D'Iorio[9] bei ihren Versuchen mit 15% Ausscheidung von DOPA im Harn das Racemat verwendet haben und d+DOPA bekanntlich fermentativ langsamer abgebaut wird.

Durchführung der Rattenversuche

Die Versuche wurden an Albinoratten im Gewicht von 150–200 g durchgeführt. Je 3 Tiere wurden dabei in einen Diuresekäfig zusammengesetzt und der 24-Stunden-Harn der Gruppe unter Vorlage von 1,0 ml konz. HCl gesammelt. Alle 24 Stunden wurden die Tiere 1 Stunde lang in einem Käfig mit Rattenkeks (Zusammensetzung siehe Lembeck[18]) gefüttert. Während der Harnabnahme erhielten die Tiere Wasser ad libitum und geschnittene Feldrüben. Unter diesen Bedingungen konnten wir die Tiere bis zu 4 Wochen in den Diuresekäfigen ohne allzu großen Gewichtsverlust halten. Alle untersuchten Substanzen wurden s.c. verabreicht.

VERSUCHSERGEBNISSE

I. *Leerausscheidung von Adrenalin im Harn von Ratten*

Zu Beginn jeder Versuchsreihe wurden an 5 hintereinanderfolgenden Tagen die pro Gruppe (jeweils 3 Tiere) innerhalb von 24 Stunden ausgeschiedenen Adrenalinmengen bestimmt. An insgesamt 6 Gruppen konnten dabei die nachfolgenden Durchschnittswerte aus den einzelnen 5 Tage-Perioden ermittelt werden:

3,34; 2,86; 3,42; 2,46; 3,30; und 4,04 μg/24 St.

Diese Werte stimmen ausgezeichnet mit den Ergebnissen von Pitkänen[16] überein, die ebenfalls mit einer fluorometrischen Nachweismethode an Wistar-Ratten eine Adrenalinleerausschüttung von rund 0,2 μg/5 Stunden/Tier, d.s. annähernd 1.0 μg/24 Stunden, als tägliche Ausscheidung eines Tieres fand.

An 2 Gruppen wurde ferner die Reaktionsfähigkeit des Nebennierenmarkes an Hand der zusätzlichen Adrenalinausscheidung durch die Nieren nach vorheriger

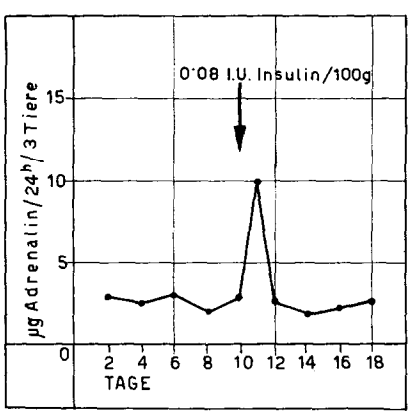

ABB. 1. Adrenalingehalt des 24-Stunden-Harnes von Ratten unbehandelt und nach Verabreichung von 0,08 I.U. Insulin/100 g.

Verabreichung von Insulin untersucht. Abb. 1 gibt einen derartigen Versuch mit einer der Gruppen wieder. (Abb. 1).

In insgesamt 4 Versuchen wurde nach Verabreichung von 0,08 I.E. Altinsulin NOVO/100 g Tiergewicht s.c. ein Ansteigen der 24-Stunden-Harn-auscheidung von Adrenalin auf Werte von 10,2 μg; 11,0 μg; 9,5 μg u. 9,8 μg pro Gruppe beobachtet. Nach Abzug des Wertes der Leerausscheidung ergibt sich somit aus unseren Versuchen eine durch die Insulinverabreichung bedingte Mehrausscheidung von etwa 7,0 μg pro Gruppe bzw. 2,3 μg pro Tier. Dieser Wert stimmt ebenfalls ausgezeichnet mit der von Pitkänen[16] (Fig. 17) für dieselbe Insulindosierung beobachteten Adrenalinausscheidung überein.

II *Leerausscheidung von Adrenalin im Harn nach Verabreichung von* 1-*DOPA, HOT und MDOPS*

Die Versuche wurden in der Weise durchgeführt, daß an Ratten einheitlichen Geschlechtes in Gruppen zu je 3 Tieren zunächst 8 Tage lang in Zwei-Tagesperioden 4 mal die Leerausschüttung von Adrenalin im Harn bestimmt wurde. Ab dem 8. Tag erhielt eine Gruppe 100 μg/Tag/Tier MDOPS, je eine Gruppe 1 mg/Tag/Tier 1-DOPA bzw. HOT und je 2 Gruppen 10 mg /Tag/Tier/1-DOPA bzw. HOT subcutan durch 14 Tage hindurch zugeführt. Während dieser 14-tägigen Untersuchungsperiode wurde bei

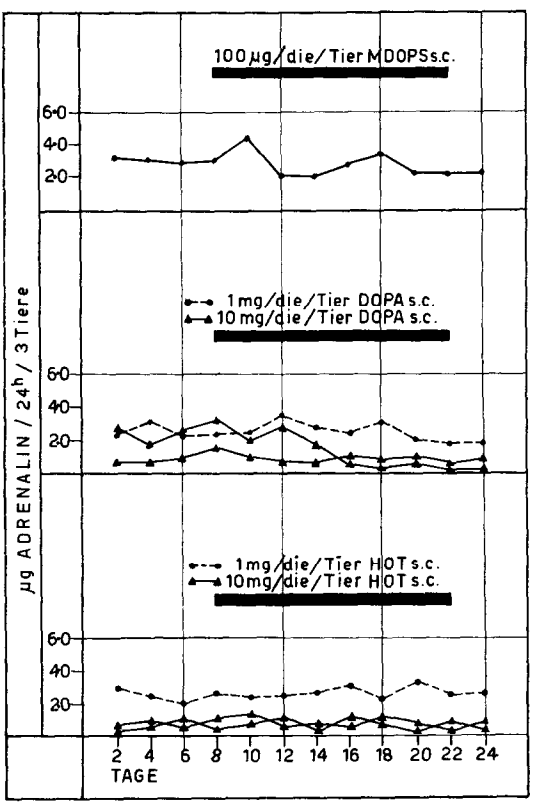

ABB. 2. Adrenalinleerausscheidung im Harn von Ratten mit und ohne gleichzeitige Verabreichung von MDOPS (oberes Drittel), 1-DOPA (Mitte) und HOT (unteres Drittel). Die schwarzen Balken geben die Zeitperiode der zusätzlichen MDOPS, 1-DOPA und HOT-Verabreichung an.

allen Gruppen die Adrenalinausscheidung im Harn in 48-Stundenperioden laufend durchgeführt. Die Ergebnisse dieser Untersuchungen sind in Abbildung 2 wiedergegeben. (Abb. 2).

Aus der Abbildung kann entnommen werden, daß bei keiner Gruppe eine Erhöhung der Adrenalinausschüttung durch die Behandlung mit den angeführten Brenzkatechinderivaten zu beobachten war. Aehuliche negative Ergebnisse wurden nach einmaliger Verabreichung von MDOPS an Kaninchen von Schmitterlöw[19] mittels biologischer Nachweismethodik und von Pellerin und D'Iorio[9] für 1-DOPA nach einmaliger Verabreichung an Ratten mittels radioaktiver Messmethode erhoben. Unsere Ergebnisse zeigen, daß auch nach 14 tägiger Verabreichung höchster Dosen dieser als Vorstufen für Adrenalin angesprochenen Verbindungen keine zusätzliche Ausscheidung von Adrenalin im Harn von Ratten in Erscheinung tritt.

III *Ausscheidung von Adrenalin im Harn nach s.c. Zufuhr von Adrenalin an Ratten*

(A) *Abhängigkeit des Bruchteils des im Harn wiedergefundenen Adrenalins von der subcutanen Adrenalindosis.*—In diesen Versuchen wurde der Bruchteil des im Harn nach subcutaner Verabreichung wiedergefundenen Adrenalins in der Weise bestimmt, daß wieder an Gruppen zu je 3 Tieren zunächst in 2 aufeinander folgenden 48-Stunden-Perioden die Adrenalin-Leerausscheidung bestimmt wurde. Anschließend an diese Vorperiode wurde Adrenalin-bitartrat in 0,5–1% Lösung in Dosen von 25 μg bis 400 μg/Tier subcutan verabreicht und der Adrenalingehalt des darauffolgenden 24-Stunden-Harnes bestimmt. Von dem Adrenalinwert der auf die Injektion folgenden 24-Stunden-Harnperiode wurde der aus den Vorperioden ermittelte durchschnittliche Wert der Adrenalin-Leerausschüttung abgezogen und die so erhaltenen Werte in Abb. 3 als Ordinate gegenüber der verabreichten Adrenalindosis als Abszisse aufgetragen (Abb. 3).

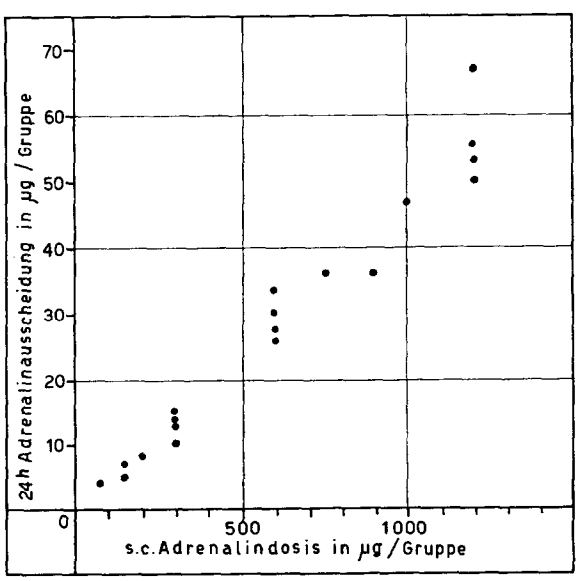

ABB. 3. Abhängigkeit der Adrenalin-24-Stundenausscheidung im Harn von der subcutanen Zufuhr verschiedener Adrenalinmengen.

Aus den Werten der Abb. 3 ist ersichtlich, daß zwischen der verabreichten Dosis und der im Harn wiedergefundenen Adrenalinmenge eine lineare Abhängigkeit besteht. Die Berechnung der Regressionsgeraden aus den einzelnen Werten ergab die folgende Gleichung: $y = -0{,}96 + 0{,}0477x$ (y: μg Adrenalin im Harn, x: μg Adrenalin s.c.). Der entsprechende Korrelationskoefizient beträgt + 0,986 ($P < 0{,}001$). Der Wert des Regressionskoefizienten von 0,0477 zeigt an, daß in sämtlichen Versuchen unabhängig von der verabreichten Dosis durchschnittlich 4,77% als unverändertes Adrenalin im Harn wiedergefunden werden konnte.

(B) *Verhalten der Adrenalinausscheidung im Harn bei wiederholter s.c. Verabreichung von Adrenalin.*—Für das Studium des Einflusses der verschiedenen Brenzkatechinderivate auf die Ausscheidung von zugeführtem Adrenalin im Harn war es zunächst notwendig, das Verhalten der Adrenalinausscheidung durch die Nieren bei wiederholter subcutaner Verabreichung zu untersuchen. Zu diesem Zwecke wurde an einer Gruppe von 3 Ratten über einen Zeitraum von 6 Wochen jeden 6.Tag 600 μg Adrenalin (200 μg je Tier) injiziert und die Adrenalin-Harnausscheidung sowohl in den Leerperioden wie auch in den unmittelbar auf die Injektionen folgenden 24-Stunden-Harnen untersucht. Das Ergebnis dieser Versuchsreihe ist in Abb. 4 wiedergegeben und

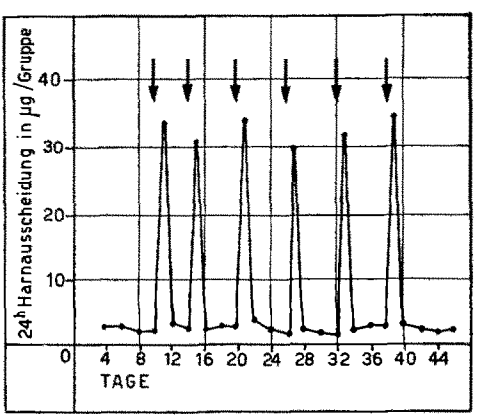

Abb. 4. Adrenalinausscheidung im Harn (24-Stundenwert) einer Gruppe von 3 Ratten unter intermittierender Zufuhr von je 200 μg Adrenalin pro Tier. Die Pfeile am oberen Rand der Abbildung geben den Zeitpunkt der einzelnen Adrenalinverabreichungen an.

zeigt, daß auch bei 6 maliger Wiederholung der Adrenalinbelastung keinerlei Aenderung der Größe des Bruchteils des im Harn wiedergefundenen Adrenalins in Erscheinung trat. Als unangenehme toxische Nebenerscheinungen traten gegen Ende dieser Versuchsreihe bei den Tieren an den Injektionsstellen großflächige Fellnekrosen auf, die jedoch eine sehr gute Heilungstendenz aufwiesen. Bei einer zusammenfassenden Betrachtung der hier mitgeteilten Versuche über die Harnausscheidung von subcutan zugeführtem Adrenalin muß vor allem die Konstanz-und Dosisabhängigkeit der Adrenalinausscheidungsquote hervorgehoben werden. Diese Konstanz des ausgeschiedenen Bruchteils erfolgte auch unabhängig von der jeweils produzierten Harnmenge, da die bekannte diuretische Wirkung des Adrenalins[20,21] auch in unseren Versuchen mit einer deutlichen Dosisabhängigkeit in Erscheinung trat, wobei vor allem bei Dosen über 100 μg/Tier ein Ansteigen der 24-Stunden-Harnmenge von etwa

durchschnittlich 11 ml in den Leerperioden auf 30 bis 50 ml zu beobachten war. Aus diesen Ergebnissen erscheinen die folgenden Schlußfolgerungen berechtigt, daß 1.) von der jeweils subcutan zugeführten Adrenalinmenge nach Resorption ein bestimmter gleichbleibender Bruchteil in den Nierenglomeruli abfiltriert wird. Dieser Anteil scheint unbeeinflußt von der gleichzeitigen Wirkung des Adrenalins auf die tubuläre Rückresorption von Wasser und Chlorid zur Ausscheidung zu gelangen; 2.) daß der Anteil der Nierenclearance an der Totalclearance des Adrenalins ein sehr geringer ist und sich dieses Verhältnis in einem Dosenbereich von 25–400 μg/Tier (125 μg–2,0 mg/kg) als konstant erweist. Diese letztere Feststellung beinhaltet jedoch, daß die Kapazität der extrarenalen Eliminationsmechanismen des Adrenalins selbst bei toxischen Dosen ausreicht, um den Anteil der extrarenalen Elimination unvermindert aufrecht zu erhalten.

IV. *Einfluß von 1-DOPA und HOT auf die Harnausscheidung von subcutan zugeführtem Adrenalin*

Nachdem in den beiden voranstehendue mitgeteilten Untersuchungsreihen die Dosisunabhängigkeit sowie die zeitliche Konstanz der Ausscheidungsquote des Adrenalins bei wiederholter Zufuhr festgestellt werden konnte, wurde nunmehr in weiteren Versuchsreihen die Wirkung einer Belastung der chemischen Adrenalinabbaumechanismen durch tägliche massive Zufuhr von 1-DOPA und HOT auf die renale Elimination von Adrenalin studiert. Die Versuche wurden in der Weise durchgeführt, daß an je 4 Gruppen zu je 3 Ratten zunächst die Ausscheidungsquote des Adrenalins nach verschiedenen subcutanen Dosen ausgetestet wurde. Anschließend an diesen Vorversuch wurden durch 21 Tage hindurch an 4 Gruppen täglich 10 mg/Tier 1-DOPA und an 4 weiteren Gruppen 10 mg/Tier HOT subcutan verabreicht.

Innerhalb dieser 3 wöchigen Verabreichungsperiode wurde am 6., 12., u. 18. Tag das renale Adrenalinausscheidungsvermögen bei Verabreichung 4 verschiedener subcutaner Adrenalindosen (50, 100, 200 u. 400 μg/Tier) untersucht. Die s.c. Adrenalininjektion wurde dabei immer 1/2 Stunde nach der an diesem Tage fälligen 1-DOPA oder HOT Injektion verabreicht und von da ab die 24-Stunden-Harnmenge gesammelt, in der dann der Adrenalingehalt bestimmt wurde. Die bei beiden Versuchsreihen erhaltenen Ergebnisse sind in Abb. 5 für 1-DOPA und HOT getrennt graphisch dargestellt (Abb. 5). In der Abb. 5 wurde der Prozentsatz des nach subcutaner Verabreichung wiedergefundenen Adrenalins als Ordinate gegenüber der Dauer der 1-DOPA-bzw. HOT—Behandlung aufgetragen. Es ist daraus ohne weiteres ersichtlich, daß unter der 21 tägigen 1-DOPA Behandlung keine Aenderung des Anteiles der renalen Adrenalinausscheidung an der Gesamtelimination in Erscheinung tritt. Hingegen ist bei den HOT-Versuchen eine deutliche Tendez der Verringerung der renalen Ausscheidungsquote zu erkennen. Zusammenfassend kann aus dem Verlauf dieser Versuche festgestellt werden, daß durch die gleichzeitige Belastung des Organismus mit 1-DOPA und HOT in keinem Falle eine Erhöhung des Anteiles der renalen Adrenalinausscheidung an der Gesamtelimination beobachtet werden konnte. Dies läßt darauf schließen, daß der oxydative Abbau des Adrenalins im Organismus durch Zufuhr hoher Dosen von 1-DOPA und HOT in seiner Wirkung nicht beeinträchtigt wird, obwohl 1-DOPA und HOT größtenteils im Organismus zu Derivaten der Dihydroxyphenylessigsäure sicher in ähnlicher Weise wie Adrenalin oxydativ abgebaut werden.[6,7,9] Was das Absinken der renalen Ausscheidungsquote von Adrenalin unter

der chronischen Zufuhr von HOT anbetrifft, so muß berücksichtigt werden, daß die verwendete Dosierung (10 mg/Tier entsprechend etwa 50 mg/kg) bereits zu ziemlich

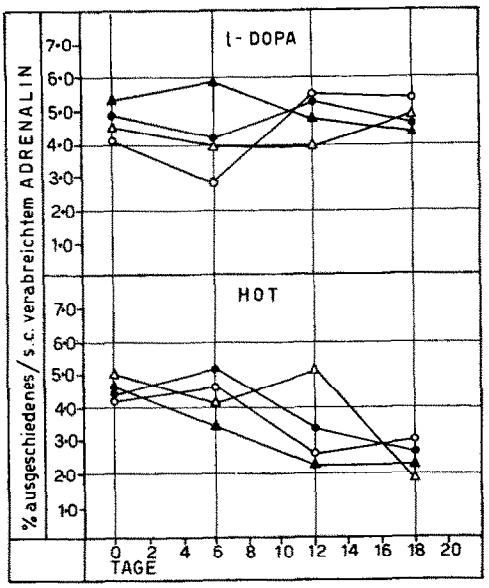

ABB. 5. Einfluß von l-DOPA bzw. HOT (in täglichen s.c.-Dosen von etwa 50 mg/kg) auf die renale Ausscheidung von subcutan zugeführtem Adrenalin an Ratten. Ausgefüllte Dreiecke: 50 μg, volle Kreise: 100 μg, leere Dreiecke: 200 μg und leere Kreise: 400 μg Adrenalin pro Tier s.c..

starken toxischen Erscheinungen führte. Es kann daher nicht ganz ausgeschlossen werden, daß das beobachtete Absinken der renalen Ausscheidung von Adrenalin in ursächlichem Zusammenhang mit dem toxischen Zustand der Tiere steht.

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SOME SULPHUR CONTAINING AMINO-ACIDS OF BIOLOGICAL INTEREST

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Abstract—α-Methylmethionine, α-methylethionine, α-amino-α-methyl-γ-propylthiobutyric acid, α-methylcysteine, and S-2-chloroethylcysteine hydrochloride have been prepared. Preliminary results indicate that α- and β-methylmethionine have tumour growth promoting properties towards the Fujinami fowl sarcoma in egg culture whilst α-methylcysteine and α-methylethionine have similar properties towards the transplanted Walker rat carcinoma. S-2-chloroethylcysteine produces a similar mutagenic effect on the male germ line in *Drosophila* to that produced by 2-chloroethyl methanesulphonate (CB 1506).

AMINO-ACID antagonists merit investigation as possible chemotherapeutic agents because the synthesis of proteins must be an essential factor in the continued growth of tumours.[1] Work[2] has recently suggested that mixed anhydrides of nucleotides and amino-acids are intermediates in the synthesis of both proteins and nucleic acids. The implied possibility that amino-acid antagonists might also inhibit nucleic acid synthesis receives support from the investigations of Pardee and Prestidge.[3] It has been known for some time that certain methylated amino-acids are antagonists of the corresponding naturally occurring amino-acid.[4] Another point of interest is that α-methyl amino-acids are more strongly concentrated intracellularly, the structure of the methylated amino-acid favouring chelation and hence transport across the cell membrane.[5] The preparation of three new α-amino-acids is now reported.

Ethionine is a well-established antagonist of methionine and its α-methyl derivative has been prepared as a compound of similar properties which would more readily concentrate within cells. Ethanethiol condensed with methyl vinyl ketone giving 1-ethylthiobutanone-3 (I, R = Et) which was converted successively into the hydantoin (II, R = Et) and α-methylethionine (III, R = Et) essentially by the method of Reisner.[4b] α-Amino-α-methyl-γpropylthiobutyric acid (III, R = C_3H_7) was similarly prepared by way of the ketone (I, R = C_3H_7) and the hydantoin (II, R = C_3H_7)

(I) $RSCH_2CH_2COMe$ (II) $RSCH_2CH_2C(Me)(CONH)(NH{-}{-}{-}{-}CO)$

(III) $RSCH_2CH_2C(Me)(COOH)(NH_2)$

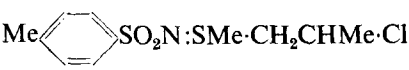
The work of Weisberger[6] has indicated that cysteine may play a unique role in the metabolism of leucocytes and he has shown that at least one cysteine antagonist, selenocysteine, exerts an inhibitory effect on experimental and clinical leukaemias. Although αα′-dimethylcystine has been referred to in the literature[7] its preparation

and that of α-methylcysteine do not appear to have been published yet.* 1-Benzylthiopropane-2 (IV), formed by condensing bromoacetone with benzylmercaptan, may be converted into the hydantoin and S-benzyl-α-methylcysteine (V, R = $PhCH_2$) in the usual manner. The benzyl derivative affords α-methylcysteine (V, R = H), isolated as the hydrochloride, on reduction with sodium in liquid ammonia (cf. [8]).

(IV) $PhCH_2SCH_2COMe$ (V) $RSCH_2\underset{\underset{NH_2}{|}}{C}\cdot Me\cdot COOH$

These α-methylamino-acids cannot be titrated by the normal formol method; very indefinite end-points are obtained. This is probably due to the effect of the methyl group on the dissociation of the carboxyl group. Weak or negative ninhydrin colours are given in aqueous solution but a strong positive reaction can be obtained by heating on paper as in the development of a paper chromatogram. This is consistent with the report[9] that amino-acids carrying an α-methyl substituent are less readily decarboxylated for such decarboxylation is presumed to precede colour formation in the ninhydrin test.

α- and γ-methylmethionine have previously been examined as methionine antagonists[4b] and the β-methyl derivative has now been prepared for test. Propylene oxide condensed with methanethiol to give 1-methylthiopropan-2-ol (VI, X = OH) which on heating with hydrochloric acid yielded 2-chloro-1-methylthiopropane (VI, X = Cl); this was characterised by the preparation of the sulphilimine (VII) by the method of Mann and Pope.[10]

(VI) $MeCHX\cdot CH_2SMe$ (VII) 

(VIII) $MeSCH_2CHMe\cdot\underset{\underset{NHAc}{|}}{C}:(COOEt)_2$ (IX) $MeSCH_2CHMe\cdot\underset{\underset{NH_2}{|}}{C}H\cdot COOH$

Condensation of the chloride (VI, X = Cl) with diethyl acetylaminomalonate afforded the diethyl ester of acetylamino-(2-methylthio*iso*propyl)malonic acid (VIII) and this on acid hydrolysis gave β-methylmethionine (IX). The chloride (VI, X = Cl) was previously prepared by Hunt and Marvel[11] by the action of thionyl chloride on the product obtained by condensing 1-bromopropan-2-ol with methylmercaptan in alkaline solution: the method described in the present paper is more convenient.

2-Chloroethyl methanesulphonate (CB 1506) has been shown to inhibit the growth of transplanted animal tumours[12] and to have unusual mutagenic properties.[13] Roberts and Warwick[14] have shown that following administration of ethyl methanesulphonate to rats, S-ethylcysteine appears in the urine and it seems reasonable to assume that S-2-chloroethylcysteine would be formed *in vivo* following the administration of the chloroethyl methanesulphonate. This cysteine derivative has now been prepared for examination as a cytotoxic agent. Cysteine was hydroxyethylated by treatment with ethylene oxide in buffered solution and the resultant compound (X, X = OH) was converted into the hydrochloride of S-2-chloroethylcysteine (X,

* Since this paper was submitted Arnstein (*Biochem. J.* **68**, 333 (1958) has described the preparation of αα′-dimethylcysteine.

X = Cl) by heating with concentrated hydrochloric acid. An attempt to prepare a sulphilimine from S-2-chloroethylcysteine was unsuccessful.

(X) $XCH_2CH_2SCH_2\underset{\underset{NH_2}{|}}{C}H\cdot COOH$

EXPERIMENTAL

All melting points, which are corrected, were taken in sealed capillaries.

1-Ethylthiobutan-3-one

A mixture of methyl vinyl ketone (35 g) and ethylmercaptan (41 g) containing cupric acetate (300 mg) and hydroquinone (300 mg) was stirred at room temperature for $2\frac{1}{2}$ hr. After heating at 70° for $\frac{1}{2}$ hr the mixture was allowed to stand overnight and then after diluting with ether (200 ml) it was washed with lead acetate solution (to remove unreacted mercaptan), then with water and finally dried over calcium chloride. Distillation of the product gave 1-ethylthiobutan-3-one, b.p. 100–104°/25 mm, 2:4-dinitrophenylhydrazone, m.p. 89–92°. Cardwell[16] gives b.p. 84°/19 mm, 2:4-dinitrophenylhydrazone, m.p. 90–92°, for the ketone obtained by condensing ethyl mercaptan with 2-chlorethyl methyl ketone.

4[2'-(Ethylthio)ethyl]-4-methylhydantoin

Ammonium carbonate (187 g) was added to a well-stirred mixture of 1-ethylthiobutan-3-one (52 g), sodium cyanide (31 g), and 30 per cent aqueous ethanol (410 ml) and the whole was maintained at 58–62° for 18 hr. The solution was then evaporated to low bulk, acidified to Congo red with concentrated hydrochloric acid, and finally evaporated to dryness. A 98 per cent aqueous ethanol extract of the residue was evaporated to dryness and then extracted with ether. The addition of light petroleum (b.p. 40–60°) to the ethereal solution caused the precipitation of solid, m.p. 116–119° (yield: 45·8 g). After recrystallization from ether–light petroleum the *hydantoin* formed plates, m.p. 117–118° (Found: N, 13·6; S, 15·1. $C_8H_{14}O_2N_2S$ requires N, 13·8; S, 15·8 per cent).

α-Methylethionine

The above hydantoin (15 g), barium hydroxide octahydrate (50 g), and water (240 ml) were heated in a pressure bottle at 155–160° for $\frac{1}{2}$ hr. The precipitated barium carbonate was filtered off and ammonium carbonate (7·4 g) was added to the filtrate. The clear filtrate from this mixture was evaporated to dryness under reduced pressure and the residue was ground under acetone and then collected by filtration. The yield was 8·5 g of material, m.p. 280–282°. This *α-methylethionine* was crystallized by dissolving in water (10 parts) and carefully adding hot acetone (40 parts) followed by slow cooling. In this way fine matted needles, m.p. 280–282° (after drying at 90°) were obtained (Found: C, 47·6; H, 8·7; S, 16·9; N, 7·8. $C_7H_{15}O_2NS$ requires C, 47·4; H, 8·5; S, 18·0; N, 7·9 per cent).

α-Amino-α-methyl-γ-propylthiobutyric acid

1-Propylthiobutan-3-one, b.p. 104–105°/21 mm, prepared from *n*-propylmercaptan

by the above method (Rothrock[17] gives b.p. 107–111°/20 mm) was characterized by the preparation of its 2:4-*dinitrophenylhydrazone*, orange needles, m.p. 62°, from ether-*cyclo*hexane (Found: C, 47·5; H, 6·0; N, 17·1. $C_{13}H_{18}O_4N_4S$ requires C, 47·8; H, 5·6; N, 17·2 per cent). 4-[2′-(*Propylthio*)*ethyl*]-4-*methylhydantoin*, m.p. 98–100°, fine needles from *iso*propyl ether (Found: C, 49·4; H, 7·4; N, 12·8. $C_9H_{16}O_2N_2S$ requires C, 50·0; H, 7·5; N, 13·0 per cent), obtained in the usual manner from the thiobutanone, on hydrolysis with barium hydroxide gave *α-amino-α-methyl-γ-propylthiobutyric acid*, m.p. 279°, fine needles from aqueous acetone (Found: C, 50·5; H, 8·8; N, 7·2; S, 16·2. $C_8H_{17}O_2NS$ requires C, 50·2; H, 9·0; N, 7·3; S, 16·8 per cent).

1-Benzylthiopropan-2-one

To a solution of sodium (4·6 g) in absolute ethanol (200 ml) was added benzylmercaptan (23·5 ml) and then bromoacetone (18·4 ml) was run into the vigorously stirred mixture during 15 min at room temperature. After heating at 45° for 50 min the alcohol was removed under reduced pressure and the residue was extracted with ether. The washed ether extract was stirred with a saturated solution of sodium bisulphite when a precipitate slowly formed. Next day this was collected by filtration and washed with ethanol and ether. The finely ground bisulphite complex (57·5 g) was shaken with hydrochloric acid (200 ml, 2 N) and ether (500 ml) until all the solid had dissolved. The ethereal solution was then dried over calcium chloride and concentrated under reduced pressure. The light yellow oil (19 g), which tended to decompose on distillation, formed a semicarbazone, m.p. 121–122°; Wahl[18] gives m.p. 123° for this derivative of the ketone obtained by condensing chloroacetone with benzylmercaptan.

S-Benzyl-α-methylcysteine

The above ketone (38.5 g) was submitted to a modified Strecker reaction as already described and the resulting 4-(*benzylthiomethyl*)-4-*methylhydantoin* (22·8 g), m.p. 120–122°, formed fine needles from ether-light petroleum (b.p. 60–80°) (Found: C, 57·5; H, 5·6; N, 11·7; S, 12·2. $C_{12}H_{14}O_2N_2S$ requires C, 57·6; H, 5·6; N, 11·3; S, 12·8 per cent). Hydrolysis of the hydantoin (16·2 g) at 160° with aqueous barium hydroxide afforded *S-benzyl-α-methylcysteine* (13·8 g) which on recrystallization from water formed rosettes, m.p. 237–238° (Found: C, 58·4; H, 6·9; N, 6·6; S, 13·7. $C_{11}H_{15}O_2NS$ requires C, 58·7; H, 6·7; N, 6·2; S, 14·2 per cent).

α-Methylcysteine hydrochloride

The S-benzyl derivative (5 g) was dissolved in liquid ammonia (100 ml) and to the well-stirred solution sodium shavings were added until the blue colour in the solution persisted for 15 min. Ammonium chloride was added carefully until the blue colour was discharged and then two equivalents of the salt (2·4 g) were introduced. The ammonia was allowed to evaporate and the residue was extracted with dry ether to remove toluene and then dissolved in water (20 ml). The addition of warm mercuric chloride solution (350 ml, 10 per cent) gave a white precipitate which was collected by centrifuging and washed with fresh mercuric chloride solution. An excess of hydrogen sulphide was passed through an aqueous suspension of the precipitate and the mixture again centrifuged. The clear colourless supernatant liquid was evaporated to dryness in an atmosphere of nitrogen under reduced pressure and the residue extracted with

methanol (20 ml). On being allowed to evaporate in a vacuum desicator over calcium chloride this solution gave large prismatic crystals of *α-methylcysteine hydrochloride* (2·0 g), m.p. 190–195° (Found: C, 28·1, H, 6·0; N, 8·1; Cl, 20·8; S, 18·4. $C_4H_9O_2NS$. HCl requires C, 28·2; H, 5·8; N, 8·2; Cl 20·7; S, 18·6 per cent).

1-Methylthio-2-chloropropane

Methylmercaptan was generated by the action of alkali on S-methyl*iso*thiourea sulphate (206 g, see Organic Syntheses[19]) and passed into an ice-cooled aqueous solution of sodium hydroxide (200 ml, 5 N) and the resulting solution was 3·33N with respect to thiol (iodine titration). A slight excess of propylene oxide (35 ml) was then added to the cooled solution and the mixture was allowed to reach room temperature overnight. An ethereal extract of the product was dried over sodium sulphate and distilled; the fraction (34 g) of boiling point 74–75°/26 mm, n_D^{23} 1·4802 being collected. Hunt and Marvel[11] give b.p. 67°/20 mm, n_D^{20} 1·4869. This 1-methylthiopropan-2-ol was heated with concentrated hydrochloric acid (120 ml) for one hour when an oily layer formed. The chloro-compound was extracted with ether and washed with saturated sodium chloride solution and then with water and finally dried over calcium chloride. 1-Methylthio-2-chloropropane (yield: 26 g), b.p. 55°/24 mm, n_D^{24} 1·4820 (Hunt and Marvel give b.p. 67°/37 mm, n_D^{20} 1·4905) was characterized by the preparation of the *sulphilimine derivative* (VII), m.p. 149°, needles from ethanol (Found: C, 45·1; H, 5·5; N, 4·8; Cl, 11·9; S, 22·9. $C_{11}H_{16}O_2NS_2Cl$ requires C, 45·0; H, 5·5; N, 4·8; Cl, 12·1; S, 21·9 per cent).

Diethyl acetylamino-(2-methylthioisopropyl)-malonate

Diethyl acetylaminomalonate (40·6 g) was added to a solution of sodium (4·6 g) in absolute ethanol (500 ml). After the further addition of 1-methylthio-2-chloropropane (30 ml) ethanol was removed by slow distillation through a Widmer column. This took 2 hr. and the mixture was subsequently heated for a further 6 hr at 160° in a glycerine bath. The cooled product was extracted with ether when a light yellow oil was obtained. This solidified on standing and proved to be a mixture of the required ester with unchanged acetylaminomalonate: it was resolved by a preliminary chromatographic separation (using benzene as eluant on an activated alumina column) followed by fractional crystallization from *iso*propyl ether. The *diethyl thioester* (12 g), m.p. 85–87°, formed needles from *iso*propyl ether (Found: C, 51·0; H, 7·6; N, 4·6; S, 10·1. $C_{13}H_{23}O_5NS$ requires C, 51·1; H, 7·6; N, 4·6; S, 10·5 per cent).

β-Methylmethionine

The diethyl thioester (6·8 g) was heated under reflux with dilute hydrochloric acid (70 ml, 5N) for 1½ hr and then the solution was evaporated to dryness under reduced pressure. The residue was dissolved in hot water (10 ml) and pyridine (7 ml). Hot ethanol (50 ml) was then added and after cooling the solution to −20° *β-methylmethionine* (2·2 g) slowly separated. It was purified by crystallization from ethanol containing a little water. The amino-acid was obtained as a microcrystalline powder, m.p. 237–239° (Found: C, 44·0; H, 8·4; N, 8·5; S, 19·5. $C_6H_{13}O_2NS$ requires C, 44·2; H, 8·0; N, 8·6; S, 19·6 per cent). In contrast to the α-methyl amino-acids β-methylmethionine could be assayed by a formol titration when the equivalent was found to

be 164 as compared with the calculated value of 163. The R_F value for β-methylmethionine in ethanol: ammonia (dens. 0·88): water (95:5:5) is 0·32; under similar conditions the R_F values for methionine and α-methylmethionine are respectively 0·41 and 0·45. β-Methylmethionine gives a strong positive ninhydrin coloration in aqueous solution.

S-2-Chloroethylcysteine

Ethylene oxide (6 ml) was added to a solution of cysteine hydrochloride (15·8 g) and sodium bicarbonate (18·5 g) in water (100 ml) and the mixture was heated on a steam bath for 25 min. After the further addition of ethylene oxide (2 ml) the mixture was heated for another 45 min. An iodine titration of the solution then showed that over 95 per cent of the sulphydryl groups had reacted. The residue obtained on evaporating the solution under reduced pressure was heated with concentrated hydrochloric acid (120 ml) for ½ hr. Concentration of the acid solution under reduced pressure led to the separation of a crystalline solid which was collected by filtration. Recrystallization from *iso*propanol afforded *S-2-chloroethylcysteine hydrochloride* as plates (5.5 g), m.p. 186–188° (Found: C, 27·5; H, 5·1; N, 6·3; Cl, 31·3; 2, 14·4. $C_5H_{10}O_2NClS$ requires C, 27·3; H, 5·1; N, 6·4; Cl, 32·2; S, 14·5 per cent). Formol titration of an ice-cold solution of the chloroethylcysteine hydrochloride indicated an equivalent weight of 109 (theoretical value, 110); on heating the solution a further equivalent of acid was formed due to the hydrolysis of the chloroethyl group. In buffered aqueous solution at pH 7·5 and 37° the half-life of chloroethylcysteine hydrochloride (with respect to the hydrolysis of the chloroethyl group) is 7 min (personal communication from Dr. W. Davis). When warmed with one equivalent of cysteine in an excess of aqueous sodium bicarbonate chloroethylcysteine hydrochloride yielded homodjenkolic acid, m.p. and mixed m.p. 265–270°.

RESULTS

Dr. R. J. C. Harris has studied the effect of α- and β-methylmethionine on the Fujinami fowl sarcoma implanted into eggs. In a typical experiment the tumour was implanted into the chorioallantoic membrane on the ninth day and 5 mg of α-methylmethionine in 0·2 ml of water was injected into the yolk sac on the twelfth day. The eggs were opened and the embroyos and tumours weighed on the sixteenth day. In the control series the average weight of the embryos was 11·98 ± 1·25 g and of the tumours 1·47 ± 0·53 (average of eight eggs) whilst in the test series the average weight of embryos was 12·55 ± 1·27 g and of the tumours was 2·25 ± 1·15 g (average of six eggs); a treated/control tumour weight ratio of T/C 1·53. In another experiment β-methylmethionine at the same dose level gave a treated/control tumour weight ratio of 1·55/0·95 or 1·63.

Professor A. Haddow has examined the action of four methylated amino-acids on the Walker rat carcinoma. In one experiment twelve rats of about 200 g weight were implanted with the tumour and six of these were given twelve daily intraperitoneal injections of 100 mg of α-methylcysteine (dissolved in 2 ml of water) starting on the day following implantation. At the end of the experiment the average weight of the tumours in the control animals was 21 g and in the treated animals, 58 g (T/C ratio 2·76). Similarly α-methylethionine gave a T/C ratio of 2·4. α- and β-methylmethionine had relatively little effect on the tumour under these conditions.

Following the observation that the sulphilimine formed from di-2-chloroethyl sulphide and chloramine-T will inhibit the growth of the transplanted Walker rat carcinoma when given as a single intraperitoneal injection of 10 mg in arachis oil[15] the the monofunctional chloroalkyl derivative (VII) was also tested but found to be inactive at 20 mg per rat.

Dr. and Mrs. O. G. Fahmy report that the mutagenicity of S-2-chloroethylcysteine on the male germ line of *Drosophila* is comparable with that of 2-chloroethyl methanesulphonate but with higher selectivity on the early germ cells. An injected dose of $0{\cdot}5 \times 10^{-9}$ mole per male of chloroethylcysteine induces a sex-linked recessive lethal rate of 15 per cent in the most sensitive spermatogonia, as compared with an average of only 0·7 per cent in the sperm and spermatid stages. Roughly the same mutation rate (i.e. 15 per cent) in spermatogonia is induced by a dose of $2{\cdot}0 \times 10^{-9}$ mole per male of 2-chlorethyl methanesulphonate, but this induces an average mutation rate of 1·7 per cent in sperm and spermatids.

DISCUSSION

These very preliminary biological results suggest that the methylated amino-acids now described have no useful tumour inhibiting properties under the conditions so far employed, that is, using animals maintained on normal diets. It would seem desirable to repeat the rat experiments using diets low in the natural amino-acid if an effect is to be produced by a competitive inhibitor. N-methylation of amino-acids produces analogues some of which cannot be utilized by the organism[20] and others which are used.[21] The ability of an organism to demethylate the compound could well be the deciding factor. Although C-methylated amino-acids are inhibitors of the growth of certain micro-organisms the behaviour of these acids in normal rats does not appear to have been examined. It is reasonably certain that C-demethylation will not occur *in vivo* and if the amino-acid is utilized this must be a property of the molecule as a whole.

The observation of Ghadially and Wiseman[22] that an excess of methionine inhibits the growth of normal itssue but increases the growth rate of the RD3 carcoma is reminiscent of our own results and does indicate that further investigation of the more readily transported methylated methionines should be made.

As already indicated α-methylcysteine was prepared as a cysteine antagonist for examination of its effect against experimental leukaemias. This investigation will be carried out when the behaviour of animals on suitably low cysteine diets has been established.

The results obtained with S-2-chloroethylcysteine in the rat suggest that because of the high reactivity of the compound (the half-life is only 7 min towards hydrolysis under physiological conditions) very little may survive to reach the target site. Using more localized treatment (cf. [13]) in *Drosophila* very definite effects are observed and it is clear that the mutability of spermatogonia under the influence of 2-chloroethyl methanesulphonate could be due entirely to the *in vivo* formation of S-2-chloroethylcysteine as had been postulated earlier.

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SHORT COMMUNICATIONS

Enzymic oxidation of mescaline by mammalian plasma

It is now known that amine oxidases, distinct from the intracellular amine oxidase, occur in the blood plasma of a number of mammalian species. Two enzymes of this kind have been studied:

(a) the enzyme spermine oxidase, first described in the sheep[1] but present in all ruminants[2];
(b) the enzyme benzylamine oxidase, first studied in the horse[3] but present also in certain other species.

We wish to draw attention to the fact that mescaline is a substrate of these two plasma oxidases. For bovine plasma this has already been noted by Tabor, Tabor and Rosenthal.[4] In the pig, mescaline is the amine most rapidly oxidized, but the plasma enzymes in the llama and the dog also oxidize mescaline at a relatively high rate.

The plasma amine oxidases differ from the intracellular "mono" amine oxidase in their substrate and inhibitor specificities. However, there is good reason to believe that tissue enzymes exist which are related to the plasma oxidases: Bernheim and Bernheim[5] first noted that the enzymic oxidation of mescaline by rabbit liver preparations differed from that of other amines, and it is now generally believed that in this organ mescaline is oxidized by an enzyme which is distinct from the amine oxidase present[6,7,8]; the inhibitor specificity of the rabbit liver enzyme for mescaline is reminiscent of that of the plasma oxidases.

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Note upon the block produced in the tricarboxylic acid cycle by fluorocitric acid.

The lethal synthesis of fluoroacetate to fluorocitrate blocks the citric acid metabolism *in vitro* at the stage in the tricarboxylic acid cycle of aconitase. So far there is no evidence that fluorocitric acid blocks any other stage.[1] In particular Morrison and Peters[2] found no block in the *iso*citric dehydrogenase. However there is some suggestion from the experiments of Braunstein and Azarkh[3] and from our own unpublished work that fluorocitrate might block the formation of α-ketoglutarate and combine with the Mn needed for this enzyme. It seemed possible therefore that in particle preparations fluorocitrate might be blocking α-ketoglutarate formation by combining with Mn, and that this effect might have been missed. The matter has been tested by studying the effect of fluorocitrate on the citric acid metabolism in kidney particles with and without the addition of extra Mn. To avoid re-cycling, malonate was added, as it blocks the cycle at the stage succinate — fumarate. Table 1 contains one of two similar experiments in which a slight reduction found was almost within the experimental error. It can be concluded that the main action of fluorocitric acid is on the aconitase, as has hitherto been believed.

TABLE 1.

Kidney mitochondrial particles were prepared from the guinea pig using a homogenizer in 1 per cent KCl solution at 2°C. After centrifuging once in a Servall angle centrifuge at 5000 *g* for 25 min, the supernatant was discarded and the particles stirred into cold 1 per cent KCl to which was added $\frac{1}{5}$ volume of M/2 phosphate buffer (pH 7·2). One kidney is made up to approx. 26 ml of the enzyme mixture of particles. 50 ml Erlenmeyer flasks were prepared containing enzyme mixture 1·9 ml, $MgCl_2$ 0·1 ml (4·0 μmoles), ATP 0·1 ml (1·2 μmoles), Na citrate 0·4 ml (10 μmoles), Na malonate 0·1 ml (0·016 M), fluorocitrate 0·1 ml (5·79y synthetic acid as the Na salt), $MnCl_2$ 0·1 or 0·05 ml (124y or 62y), KCl 1 per cent added to a total volume of 3·0 ml.

The flasks were shaken for 30 min at 37°C.

Additions	Citric acid left after 30 min	
	μMoles	Diff.
None	0·18	
Fluorocitrate	2·92	2·74
Mn 124y	0·16	
Same + F cit	2·92	2·76
Mn 62y	0·21	
Same + F cit	2·76	2·55

The citric acid was estimated by the method of Taylor[4] after deproteinisation with trichloracetic acid.

Acknowledgement—I am indebted to Dr. Rivett for the sample of synthetic barium fluorocitrate used and to Miss R. Shawdon for valuable technical assistance.

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Following this fundamental work the sessions became progressively more practical and took the form of, Conversion of Collagen into Gelatin, Chemical and Physical properties of Gelatin and Glue, and finally, Relation of Properties to Use. Each section had a chairman's introduction given by an authority in his particular field.

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In such a treatise, it may be that more work might have been included on the protein/polysaccharide relationships in collagens and allied materials. Presumably this has been reduced as the organisers of the conference were themselves most interested in the gelatin complexes, and much discussion of the practical application of the modern theories of gelatin structure is an essential part of the work of the organising body. Even in the last session on 'Relation of Properties to Uses' it is clearly obvious how the scientific method is now invading current industrial thought and practice in this industry.

Finally, I think the editor and his collaborators are to be congratulated on the speed with which they have turned the conference proceedings into an authorative publication.

F. HAPPEY

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The volume shows clearly that the study of nervous tissue and in particular, of its metabolism, cannot be pursued except in reference to the properties of living cells in general. Superimposed upon this basis of common properties there are the problems arising out of differentiation. These two aspects, common properties of all cells and specific differences, such as those peculiar to some neurones or to some fibres, determine the pattern of the general picture. To select only one example: the unique properties of the fatty material of the myelin sheath is seen to arise as a special and complex development of a membrane similar to that found in many locations other than medullated nerve.

Again, specificty of localization is apparent in the uneven distribution of the pharmacologically active substances and the enzymes taking part in their metabolism. But also these substances are found and metabolized in non-nervous tissue.

All the main aspects of the metabolism of nervous tissue are fully covered, but there are, in addition, reports which cover a field much wider than the nervous system; this is true particularly for the articles on enzymic group activation and transfer, on transmethylation and on fatty acid metabolism, articles which will be read with great interest and profit by all biochemists.

The final sections of the book deal with acetylcholine and other pharmacologically active substances. The work of the past few years has taught us much about the distribution of these compounds, their disappearance and accumulation under the influence of drugs. These data will be important for the understanding of substances like noradrenaline and 5-hydroxytryptamine in the central nervous system, but the book also makes it clear that this is a goal yet to be attained.

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RESPIRATORY INHIBITION AND GLYCOLYTIC STIMULATION IN NORMAL AND NEOPLASTIC TISSUES BY THE ANTI-TUMOUR AGENT 4-(4′-DIMETHYLAMINOSTYRYL) QUINOLINE

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EXTENSIVE biological tests performed in a number of laboratories in the U.S. and England* have shown that certain styrylquinoline derivatives exhibit marked toxic effects on normal and neoplastic tissues. Nothing is known, however, about the mode of action of these compounds at the enzymic level. In the course of biochemical investigations carried out in this laboratory some interesting effects of the styrylquinolines on the respiratory, phosphorylative and glycolytic processes of normal liver mitochondria and ascites tumour cells were noted. The compound which was chosen for a more detailed study into the nature of these effects was 4-(4′-dimethylaminostyryl) quinoline†, henceforward abbreviated as 4-DSQ‡. It is the object of this communication to report briefly on the mode of action of 4-DSQ as an inhibitor of respiration and an activator of glycolysis. In the accompanying paper[1] a survey of the activities of a number of closely related compounds together with the available biological data will be given.

In the experiments carried out according to the standard Warburg technique 0·02 ml of an ethanolic solution of 4-DSQ ($1{\cdot}2 \times 10^{-2}$ M) was added to the media giving a final concentration of $1{\cdot}5 \times 10^{-4}$ M in 1·6 ml. Approximately 20 per cent of the added dose was in real solution at 37°C in the absence of the biological material. 0·02 ml ethanol was always added to the control flasks.

It appeared to be a general property of 4-DSQ to interfere with DPN-linked oxidations of isolated mouse liver mitochondria as shown by its marked inhibitory effect on the oxidation of glutamate (90–100 per cent inhibition with mitochondria from 200–250 mg fresh weight of liver; controls consumed 7–8 μatoms oxygen during the 20 min incubation period), pyruvate, malate and β-hydroxybutyrate. The extent of the inhibition was influenced by the amount of mitochondria being present, but practically not by the addition of the coenzyme DPN (10^{-3} M) nor by EDTA (10^{-3} and 10^{-2} M)

* Compare reference 1 in next paper.

† 4-(4′-Dimethylaminostyryl) quinoline was a generous gift from Dr. Carl T. Bahner of Carson-Newman College, to whom our sincere thanks are due.

‡ In addition, the following abbreviations will be used: diphosphopyridine nucleotide, DPN; ethylenediamine tetraacetate, EDTA; ratio of μmole inorganic phosphate esterified to μatom oxygen utilized, P/O; 2 : 4-dinitrophenol, 2 : 4-DNP; inorganic *ortho*phosphate, P_i; adenosine di- – triphosphate, ADP and ATP.

which is known to promote mitochondrial integrity.[2] It had been found in earlier experiments[3] that DPN or EDTA completely reversed the inhibitions of mitochondrial glutamate oxidation brought about by hepatic carcinogens of the *p*-aminoazobenzene type. In this connection it may be of some interest to note that the hepatic carcinogen 4-(4′-dimethylaminophenyl)azoquinoline*, which is isoteric with 4-DSQ through the replacement of the ethylene by an azo bridge, was found to inhibit glutamate oxidation for about 40–50 per cent, but to be markedly less inactive in the presence of added DPN. By contrast, 4-DSQ might have been active either by inhibiting the dehydrogenases non-competitively in respect to DPN or by blocking any of the electron or phosphate transferring steps of the respiratory chain and associated phosphorylations. However, neither the oxidation of succinate nor that of ascorbate plus excess cytochromec was found to be depressed in the presence of 4-DSQ, while the inhibition of the glutamate oxidation was the same whether the uncoupling agent 2 : 4-DNP was present or not. The rate of reduction of DPN ($4{\cdot}7 \times 10^{-4}$ M) by a crystalline beef liver glutamic dehydrogenase preparation (15 μg in a total volume of 2 ml buffered with phosphate, pH 7·4) was inhibited in the presence of $1{\cdot}3 \times 10^{-5}$ M 4-DSQ for about 55 per cent as measured at 340 mμ in the Unicam spectrophotometer. The inhibition was neither of the competitive nor of the non-competitive type. No inhibition was apparent in the presence of 0·1 per cent albumine.

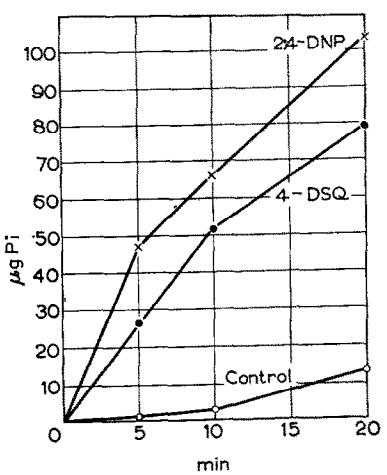

FIG. 1. Activation of the latent ATPase of mouse liver mitochondria by 4-DSQ.
Each flask contained 1·7 ml buffer: KCl (0·1 M), Tris (0·05 M) and $MgCl_2$ (0·005 M), 0·1 ml Na_4ATP (0·1 M) and 0·1 ml of a mitochondrial suspension corresponding with 25 mg wet weight of liver; pH 7·2. Incubation at 27° C. Reaction terminated by adding trichloroacetic acid. Inorganic phosphate determined according to Fiske-Subbarow.

When conditions were so arranged, by using mitochondria prepared from 300–350 mg wet weight of mouse liver, that the oxidation of β-hydroxybutyrate was inhibited by 4-DSQ approximately 50–60 per cent, it appeared that the phosphorylations were partly uncoupled from the remaining oxidations. The P/O ratio fell from 2·2 to 1·2

* 4-(4′-Dimethylaminophenyl)azoquinoline was a gift from Dr. E. V. Brown of Seton Hall University to Dr. C. T. Bahner.

(average of 7 experiments, conditions as stated previously[3]). Marked uncoupling was noted (P/O: 0·6, controls: 2·3) when in addition 10^{-2} M EDTA was present in media containing either 5 or $7{\cdot}5 \times 10^{-3}$ M Mg^{++}. However, 4-DSQ was hardly active in the presence of 10^{-3} M EDTA (P/O: 1·9, controls: 2·2). The uncoupling effect of 4-DSQ led to the discovery that the latent mitochondrial ATPase was markedly activated by 4-DSQ (Fig. 1). It was further observed that 4-DSQ caused an extensive swelling of the fresh liver particles (50 per cent decrease in optical density at 520 mμ) provided that Mg^{++} was present. The latter finding is somewhat puzzling, since Mg^{++} is known to preserve the mitochondrial morphology[4] and to counteract the swelling induced by other biologically-active compounds such as thyroxine.[5] This paradox may, however, provide a lead for further study into the mode of action of 4-DSQ and related compounds.

The respiration of intact mammary carcinoma ascites cells (S3A, from Dr. G. Klein) suspended in Krebs-Ringer phosphate buffer either in the absence (QO_2 = 7–10) or in the presence of glucose (QO_2 = 4–5), was found to be inhibited by 4-DSQ for at least 50 per cent. On the contrary, the anaerobic glycolysis of the cells, suspended in Krebs-Ringer bicarbonate buffer containing glucose or fructose, was enhanced two-fold or more in the presence of 4-DSQ. This stimulation was consistently found in numerous experiments by measurement of the carbon dioxide evolution and the lactic acid production[6] after 45, 60 or 120 minutes of incubation at 37°C. (Table 1) The manometric data showed that the rate of glycolysis was enhanced from the very beginning of the experiments and that it rose instantaneously when 4-DSQ was tipped in from the side arm of the respirometers after some time during the incubation. 2:4-DNP (5×10^{-5} M) which also activates the latent mitochondrial ATPase, produced a similar effect on the anaerobic glycolysis while the lactate production was stimulated even from 3 to 4·5-fold in the presence of 10^{-4} M 2 : 4-DNP.

In the course of these experiments it was found in many cases that the aerobic lactate production of the ascites cells (i.e. the "controls" in Krebs-Ringer bicarbonate) was not markedly below the anaerobic level, but sometimes even higher. As shown in Table 1, 4-DSQ or 2 : 4-DNP stimulated the aerobic glycolysis to the same extent as the anaerobic glycolysis.* However, when incubation was carried out in Krebs-Ringer phosphate buffer a marked Pasteur effect was noted ($Q_L{}^{O_2} = \frac{1}{2} Q_L{}^{N_2}$) which was completely abolished in the presence of 4-DSQ or 2 : 4-DNP (Table 1). The absence of a marked stimulatory effect on the anaerobic glycolysis in the latter

* Similar experiments have recently been carried out with lymphosarcoma ascites cells (T86157 from Dr. W. M. de Bruyn) suspended in Krebs-Ringer bicarbonate buffer. The anaerobic lactate production in the presence of 4-DSQ was 233 per cent of that of the controls, whereas the lactate production was 35 per cent less under aerobic than under anaerobic conditions. The aerobic lactate production in the presence of 4-DSQ was 382 per cent of that found in the absence of 4-DSQ. 2 : 4-DNP was still more active. Experiments with Ehrlich ascites carcinoma yielded comparable results. The same experiments were also carried out with two transplanted solid tumours of the mouse (hepatoma T 28012 and sarcoma UV 256). 4-DSQ had no effect on the anaerobic glycolysis of the tumour slices suspended in Krebs-Ringer bicarbonate buffer, while 2:4-DNP had only a very weak effect (10 per cent stimulation at the most). The aerobic lactate production of the slices was more than 50 per cent below the aerobic level (marked Pasteur effect). Both 4-DSQ and 2:4-DNP counteracted the Pasteur effect by causing a two-fold rise in the aerobic lactate production. The difference between the solid and ascites tumours in their glycolytic response towards 4-DSQ and 2:4-DNP under anaerobic conditions is thus most likely due to a difference in glycolytic capacity, which in turn may be conditioned by such factors as enzyme concentration, available cellular phosphate and phosphate acceptors, and the rate of entrance of glucose. As far as we are aware, the enormous stimulation of the anaerobic glycolysis of the ascites cells by 2:4-DNP found in the present experiments, has not been reported earlier and apparently has no parallel in any other tissue studied as yet.

TABLE 1. THE STIMULATORY EFFECT OF 4-DSQ AND 2 : 4-DNP ON THE GLYCOLYSIS OF ASCITES TUMOUR CELLS SUSPENDED IN KREBS-RINGER BICARBONATE AND PHOSPHATE BUFFER

Freshly harvested mammary carcinoma ascites cells, grown in $F_1(BxC_3H)$ mice for 7–9 days, were suspended in physiological salt solution and freed from erythrocytes by low speed centrifugation in the cold. The cells were incubated in amounts corresponding to 4·5–6·0 mg dry weight in the various experiments during 45 min at 37°C in the presence of glucose (0·03 M). Total volume 1·6 ml; buffer as indicated; 4-DSQ: $1·5 \times 10^{-4}$M and 2 : 4-DNP: 10^{-4}M, both present during the equilibration period. In each series two experiments are reported.

Medium	Addition	Lactate production (μ-mole)			
		Anaerobic		Aerobic	
Krebs-Ringer Bicarbonate		(95% N_2, 5% CO_2)		(95% O_2, 5% CO_2)	
	none (control)	6·0	7·2	6·5	7·1
	4-DSQ	12·0	17·7	12·3	16·5
	none (control)	5·8	5·0	5·5	5·7
	2 : 4-DNP	22·7	23·3	20·0	—
Krebs-Ringer Phosphate		(100% N_2)		(air)	
	none (control)	8·9	9·3*	4·9	5·9
	4-DSQ	11·4	12·5	12·9	12·0
	none (control)	7·7**	10·1	4·7	4·7
	2 : 4-DNP	10·8	11·6	11·2	11·9

* These cells (5·9 mg dry) produced 6·8 μmole lactate in the K.R. bicarbonate buffer in the absence and 18·9 μmole lactate in the presence of 4-DSQ.

** This was the only one of the listed experiments which was carried out for 30 min. The cells (4·5 mg dry) produced 5·0 μmole lactate in the K.R. bicarbonate buffer in the absence and 16·9 μmole lactate in the presence of 2 : 4-DNP.

experiments might have been due to the limited buffer capacity of the phosphate medium which did not allow glycolysis to proceed much further.

The anaerobic glycolysis of homogenates of the ascites cells which were prepared by osmotic shock followed by mild homogenization and incubated with the medium according to LePage[7] except for the omission of fructose diphosphate, was stimulated about 1·5-fold in the presence of 4-DSQ or 2 : 4-DNP. This stimulation was apparent both in the presence and the absence of the ATPase-inhibitor fluoride and especially so in the latter case (Table 2, exp. 1 and 2). The lactate production of the controls was always markedly less in the presence of fluoride (Table 2: System A, enolase block circumvented by adding pyruvate) than in its absence (System B). When fluoride plus 4-DSQ or 2 : 4-DNP were present, approximately the same amount of lactate was produced as in the absence of any added compound. These phenomena were also found to be true for whole ascites cells. The enhanced lactic acid production by the homogenates in the presence of 4-DSQ or 2 : 4-DNP was accompanied by a decrease in the uptake of P_i from the medium. It was further shown that the rate of glycolysis of the controls was indeed limited by the concentration of P_i and ADP, in that the addition of ADP (9·6 μmole) and extra P_i (3·8-7·2 μmole) to the homogenate system led to an enhanced glycolytic rate. The stimulatory effect of 4-DSQ on the anaerobic glycolysis was progressively less or absent under the latter conditions

with increasing concentrations of P_i added (Table 2, exp. 3 and 4). Similar results were obtained with 2 : 4-DNP.

The anaerobic lactate production of the homogenates incubated in the presence of fructose diphosphate, instead of glucose, was also stimulated by 4-DSQ. This was not the case when 3-phosphoglycerate served as the substrate in the presence of alcohol dehydrogenase to generate reduced DPN. The acetaldehyde and the lactate produced in the latter experiments were measured separately.

TABLE 2. THE STIMULATORY EFFECT OF 4-DSQ AND 2 : 4-DNP ON THE ANAEROBIC GLYCOLYSIS OF HOMOGENATES PREPARED FROM ASCITES TUMOUR CELLS

Ascites cells, freed from erythrocytes, were held in bidistilled water at 0°C during 15 min and homogenized for 10 sec at moderate speed. Very few cells remained intact. 0·3 ml of the homogenate was pipetted into the respirometers, which contained the medium according to LePage[7] with omission of fructose diphosphate. System *A* contained KF (0·01 M) and pyruvate (0·006 M), system *AA idem* plus 9·6 μmole ADP; system *B* no fluoride added and pyruvate (0·0006 M) present, system *BB idem* plus 9·6 μmole ADP. Inorganic phosphate was present as indicated. Incubation during 60 min at 37°C; 9, 6, 7·5 and 9·2 mg dry weight of homogenates were present respectively in the four experiments. All quantities are in μmoles.

Exp. No.	Conditions	P_i present at time 0	Lactate production	Extra lactate produced	P_i uptake (−) release (+)
1	*A* (control)	4·4	7·7	4·9	−4·2
	A, 4-DSQ	4·4	12·6		−3·5
	B (control)	4·4	14·4	6·2	−2·3
	B, 4-DSQ	4·4	20·6		−1·4
2	*A* (control)	3·9	4·4	2·2	−2·9
	A, 2 : 4-DNP	3·9	6·6		−2·2
	B (control)	3·9	6·6	4·2	− ·5
	B 2 : 4-DNP	3·9	10·8		+0·3
3	*A* (control)	3·9	5·4	2·8	−3·8
	A, 4-DSQ	3·9	8·2		−3·7
	AA	7·7	15·8	0·6	−6·0
	AA, 4-DSQ	7·7	16·4		−5·3
	AA	11·1	18·3	−0·3	−8·0
	AA, 4-DSQ	11·1	18·0		−6·2
4	*B* (control)	3·8	8·6	5·2	−1·8
	B, 4-DSQ	3·8	13·8		+0·1
	BB	7·6	13·0	2·3	−2·9
	BB, 4-DSQ	7·6	15·3		−1·6
	BB	11·0	15·0	1·7	−3·5
	BB, 4-DSQ	11·0	16·7		−1·9

When the homogenate was incubated anaerobically in the medium of LePage (system A) from which glucose and fructose diphosphate were omitted, approximately 1 μmole of lactate was produced and 0·5 μmole P_i taken up. However, after incubation in the presence of 4-DSQ 5·6 μmole P_i were found in the medium instead of the 3·6 μmole P_i initially present.

The unfortified homogenate incubated during 20 minutes in Tris buffer in the presence of ATP and 4-DSQ released from 2- to 2·5-fold as much P_i as the controls to which no 4-DSQ was added. The insignificant phosphate release from glucose-6-phosphate and fructose-1 : 6-diphosphate was not enhanced in the presence of 4-DSQ.

The results obtained thus far are compatible with the view that 4-DSQ activates the ATPases of the ascites tumour cells and homogenates under anaerobic conditions. The subsequent liberation of P_i and ADP from the ATP generated during glycolysis allows the latter process to proceed at a higher rate by continuously supplying the triose phosphate dehydrogenase system with a higher concentration of the necessary components P_i and ADP. The result is, however, a decreased net output of glycolytic ATP (Table 2).

In conclusion the following comparison between the modes of action of 4-DSQ and 2 : 4-DNP may be made: (i) In the concentrations tested 2 : 4-DNP does not inhibit the respiration of liver mitochondria or ascites tumour cells. On the contrary, the uncoupling of the oxidative phosphorylation brought about by this nitrophenol stimulates the oxygen consumption of the mitochondria. The inhibition of respiration following the addition of glucose to ascites cells (reverse Pasteur effect) is counteracted by 2 : 4-DNP (10^{-4} M). However, high concentrations of 2 : 4-DNP ($>10^{-4}$ M) are known to inhibit oxidation. By contrast, 4-DSQ inhibits mitochondrial oxidations (DPN-linked) under all conditions. (ii) When its limited solubility is taken into account, the styrylquinoline behaves under anaerobic conditions as an ATPase activator of similar potency as 2 : 4-DNP The latter aspect of the mode of action of the two compounds is especially pronounced in actively glycolysing ascites tumour cells in which the rate of glucose dissimilation appears to be severely restricted by the unfavourable balance between free and esterified phosphate even in the absence of oxygen utilizing oxidative processes. (iii) The Pasteur effect is readily abolished by 4-DSQ and by 2 : 4-DNP, the latter being active by the uncoupling of the oxidative phosphorylations in the respiratory chain,[8] whereas in the case of 4-DSQ inhibition of the oxidation might also be involved.

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INVESTIGATIONS ON GROWTH-INHIBITORY STYRYLQUINOLINE COMPOUNDS AND ANALOGUES—II

THE RELATIVE ACTIVITY IN A SERIES OF STYRYLQUINOLINE AND ANALOGOUS COMPOUNDS TO INTERFERE WITH INTERMEDIARY METABOLISM AND TUMOUR GROWTH

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4-(4′-DIMETHYLAMINOSTYRYL) quinoline and some related compounds have been found to be markedly toxic for normal and neoplastic tissues.[1] In the previous report[2] it was shown that 4-DSQ interfered with the respiratory, phosphorylative and glycolytic processes of liver mitochondria and ascites tumour cells. In the present investigation a number of related styrylquinolines and analogues were examined in an attempt to correlate possible metabolic effects with the available biological data.

The following compounds were studied:* 4-(4′-aminostyryl) quinoline (I); 4-DSQ (II); 4-(4′-dimethylaminostyryl)-3-methylquinoline (III); 4-(4′-dimethylaminostyryl)-6,8-dimethylquinoline (IV); 1-(4′-dimethylaminostyryl)*iso*quinoline (V); 2-(4′-dimethylaminostyryl) quinoline (VI); 4-(4′dimethylaminostyryl)pyridine (VII); 4-(4′-dimethylaminostyryl)quinazoline (VIII); dihydro-4-(4′dimethylaminostyryl)quinoline (IX); 4-(4′-dimethylaminobenziledene-amino)quinoline (X); and 4-(4′-dimethylaminophenyl)azoquinoline (XI).

Their effect on the following enzymic processes was studied: the glutamate oxidation and the ATPase activity of mouse liver mitochondria, the DPN reduction by a crystalline beef liver glutamic dehydrogenase, and the respiration and glycolysis of mammary carcinoma ascites cells.

Table 1 contains a condensed summary of these studies.

The parent compound I, which was much more soluble than any of the other drugs tested, was markedly active in the mitochondrial and ascites cell† systems. Methylation of the free amino group (4-DSQ, II) and methyl substitution at position 3 of the quinoline nucleus (III) enhanced the activity. Compound V, in which the quinoline was replaced by an *iso*quinoline nucleus, showed approximately the same activity as 4-DSQ. However, compounds VI (2-DSQ), VII (quinoline replaced by pyridine) and

* The compounds used in this investigation were prepared in the department of chemistry at the Carson-Newman College (Jefferson City) by C. Cook, J. Dale, J. Fain, E. Franklin, A. Myers and J. Wilson under the direction of one of us (C.T.B.). Compound X was a gift from Dr. E. V. Brown of Seton Hall University.

† Throughout this study the S3A mammary carcinoma has been used.

TABLE 1. CONDENSED SUMMARY OF THE METABOLIC AND TUMOUR-INHIBITORY EFFECTS OF STYRYLQUINOLINES AND ANALOGUES

For details of methods compare the previous paper[2] and the present text. The solubility of the various compounds was measured colorimetrically (after incubation at 37°C in the absence of biological material the sediment and supernatant, obtained by centrifugation, were acidified after addition of extra ethanol. Aliquots were measured at the wave length of maximum absorption of each compound).

ATPase activity was calculated from the phosphate release from ATP after 20 min of incubation at 27°C. The compounds were tested at $1{\cdot}5 \times 10^{-4}$ M in the Warburg assays, at $1{\cdot}3 \times 10^{-4}$ M in the ATPase assay and at $1{\cdot}3 \times$ and $2{\cdot}0 \times 10^{-5}$ M in the spectrophotometric determination of the glutamic dehydrogenase activity. The data on tumour growth were collected from reference 1 and unpublished experiments.

Compound		Solubility	Crystall. glutamic dehydrogenase (DPN-reduction)	Liver Mitochondria		Ascites Tumour Cells			Relative activity against Lymphoma 8 and Walker 256 tumours
				Glutamate oxidation (O_2-consumption)	ATPase (phosphate release)	Respiration endogenous (O_2-consumption)	Glycolysis (Lactate production)		
							Anaerob.	Aerob.	
		per cent of added dose	per cent of controls						(75 mg/kg)
I: H_2N	CH=CH	80	100; —	0-5	400–500	30–50	150–200	150–200	moderately active
II: $(CH_3)_2N$	CH=CH	22	45; 36*	0–15	700–900	30–50	200–280	200	(very) active
III: $(CH_3)_2N$	CH=CH	20	69; —	10–20	700–900	20–50	200–250	200	very active
IV: $(CH_3)_2N$	CH=CH	15	—; 80	80–100	500	80–100	100–120	100–120	very slightly active
V: $(CH_3)_2N$	CH=CH	?	90; 80	0–10	500–700	20–30	200	200	(very) active
VI: $(CH_3)_2N$	CH=CH	30	90; 75	90–100	200–250	100	100–110	100–110	slightly active†
VII: $(CH_3)_2N$	CH=CH	36	100; —	90–100	110–150	100	100–110	100	very slightly active
VIII: $(CH_3)_2N$	CH=CH	25	—; 87	70	400	100	150	150	inactive
IX: $(CH_3)_2N$	CH_2—CH_2	?	—; 84	0–10	400	20–30	150	150	slightly active‡
X: $(CH_3)_2N$	CH=N	?	—; 60	20	500–600	30–50	150	150–175	inactive‡
XI: $(CH_3)_2N$	N=N	33	31: 26*	50-60**	700–800	60	150–190	150	inactive
o-aminoazotoluene		60	100; 90	50**	700–800	50–80	150–180	150–180	

VIII (quinoline replaced by quinazoline) were (much) less active in some or all respects. Hydrogenation of 4-DSQ led to a compound (IX), probably containing a $—CH_2CH_2—$bridge instead of the —CH=CH—, which markedly inhibited respiratory processes and was moderately active as a stimulator of glycolysis. Two other isosters of 4-DSQ, one of which contained an aldimine bridge (X) and the other an azo (XI) instead of the ethylene bridge, showed also moderate or significant activity. Finally methyl substitution at positions 6 and 8 of the quinoline nucleus of 4-DSQ led to compound IV which possessed a low activity, except for the effect on the mitochondrial ATPase. The latter finding provided the only exception in the rough parallel that was noted between the extent of the mitochondrial ATPase activation and the ability of the various compounds to enhance the glycolysis of the ascites cells (cf. [2]). The reason for this discrepancy is not known to us; it was not due to the impermeability of the cells since the anaerobic glycolysis of the homogenate system described in the previous paper,[2] was only 10 per cent higher in the presence of IV than in its absence.

In view of the limited solubility of the compounds it was not surprising that $7{\cdot}5 \times 10^{-5}$ M 4-DSQ (II) had approximately the same and $3{\cdot}0 \times 10^{-4}$ M concentrations of compounds VII and VIII exactly the same effects on the anaerobic glycolysis of the ascites cells as the corrresponding $1{\cdot}5 \times 10^{-4}$ M concentrations.

As shown in Table 1, the correspondence between the extent of the respiratory inhibition produced by the various compounds in the liver mitochondria and the ascites cells was close. The inhibitory effect of the drugs on the rate of DPN reduction by a crystalline preparation of beef liver glutamic dehydrogenase could not be correlated with the inhibitory effects on the more integrated oxidative systems. The inhibitory effects on the rate of reduction of DPN ($4{\cdot}7 \times 10^{-4}$ M)—are listed in Table 1 for 1·3 and $2{\cdot}0 \times 10^{-5}$ M concentrations of the various drugs.

The effect of the hepatic carcinogen *o*-aminoazotoluene on the various metabolic processes is included in Table 1. It is interesting to compare the latter effects with those obtained with compound XI, the other hepatic carcinogen studied in the present series, which was found to exert an extremely potent inhibitory effect of a non-competitive type on the rate of DPN-reduction by the glutamic dehydrogenase preparation.

The tumour-inhibitory activities of the drugs (75 mg/kg) are also compiled in Table 1.

The number of tumour-bearing rats which died from the treatment showed that the general toxicity paralleled the anti-tumour activity of the various compounds. It has been noted that especially the Walker rat 256 tumour and the rat Lymphoma 8 are readily inhibited by the active styrylquinolines and analogues, while other rat tumours were not so responsive.[1] Tumour-growth inhibition studies with the ascites tumour used in the present investigations have not been carried out. However, it has been found that ascites and solid tumours of mice in general responded not very favourably to the styrylquinolines.

A comparison between the biochemical and biological data of Table 1 shows that, among the drugs listed, 4-DSQ (II), the 3-methyl derivative (III) and the *iso*quinoline isoster (V) are the most toxic for the tumours and their hosts as well as for the metabolic processes studied. Compound I is moderately active biologically and markedly active when administered at a higher dose (100 mg/kg) to the intact animal, and its good solubility appears to favour the metabolic effects in the isolated systems. In

spite of its tumour-inhibitory capability, 2-DSQ (VI) exerted no significant metabolic effects. The aldimine (X), on the other hand, was markedly active in the present experiments but has been found inactive when tested in a 75 mg/kg dose against the Walker rat tumour. However, it should be emphasized that the metabolic effects obtained in the present study are only illustrative of the intrinsic affinities of the drugs for the particular enzymes tested. In the intact animal the pharmacodynamic properties of the drugs are of equal importance. Thus, the rapid degradation *in vivo* may convert a potentially effective compound, such as X, into a harmless product. Moreover, the possibility cannot be excluded that the drugs may interfere with yet other processes; compare for instance the nucleotoxic effects of the related aminostilbenes.[3]

The correspondence in effect of the active styrylquinolines and 2 : 4-dinitrophenol has been discussed in the previous paper.[2] The latter compound markedly interferes with the metabolism of tumour slices[4] and may inhibit tumour growth.[5] The present results suggest that a high inhibitory activity towards DPN-linked mitochondrial enzymes together with a dinitrophenol-like activity may, at least partly, be responsible for the rather acute toxic effects following the *in vivo* administration of some of the styrylquinolines.

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THE KINETIC BASIS OF ORGANOPHOSPHATE POISONING AND ITS TREATMENT

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Abstract—The biochemical lesion in organophosphate poisoning is inhibition of cholinesterase which results in auto-intoxication by accumulated acetylcholine. Possible therapeutic processes are antagonism of acetylcholine, direct detoxication of the organophosphate, or reactivation of the inhibited enzyme. In this paper a simple mathematical model of the physiology of poisoning and treatment has been constructed by kinetic analysis of the basic biochemical reactions involved in these processes, with the added assumption that an animal will die once the fraction of cholinesterase in its body has fallen below an arbitrary minimum. Formulae have been derived from the model which enable the therapeutic efficiencies of the different forms of treatment, either alone or in combination, to be calculated. Qualitatively, the experimental and calculated relative efficiencies of the different therapeutic processes are in good agreement.

IT is a fundamental tenet of pharmacology that drug action must ultimately be explicable on a molecular basis, but owing to the complexity of living processes it is rarely possible to attribute a pharmacological action, even qualitatively, to any precise chemical or biochemical reaction. Poisoning by organophosphates is one of the rare examples in which the underlying biochemical lesion is known with some degree of certainty. The highly specific biochemical mechanism, namely the inhibition of a single enzyme, has been studied kinetically *in vitro* and successful therapeutic measures have been devised from a knowledge of this mechanism. These features of organophosphate poisoning make it a suitable system in which to attempt a semi-quantitative account of a pharmacological result simply on a basis of chemical kinetics.

The mathematical model of poisoning and treatment described in this paper incorporates only the essential biochemical reactions involved and no attempt has been made to include such obviously important factors as *in vivo* distribution of toxic and therapeutic agents. Despite this neglect, the qualitative deductions from the model, particularly on the relative effects of different forms of treatment are often surprisingly consistent with experiment.

It is generally accepted that the lethal effects of organophosphates are due to the accumulation of acetylcholine resulting from the inhibition of cholinesterase (ChE).[1,2] Each active centre in this enzyme is inhibited by direct chemical combination with a single molecule of organophosphate[3] so that the minimum number of molecules required to kill would be roughly equal to the number of active centres of ChE at any vital cholinergic site in the body. However, organophosphates are normally administered by routes involving prior distribution via the blood to nearly all parts of the body before they can reach any of these vital sites, in which case the minimum lethal dose would be nearer that equivalent to all the ChE in the body. In fact,

organophosphates combine not only with ChE but also with other enzymes and proteins[4,5] and many of them are readily detoxicated by the body[6–9] so that although they are often still toxic in very small doses, these doses (expressed as molecular equivalents) are generally in considerable excess over the total body ChE.

Three types of drug have been used to treat organophosphate poisoning, (*a*) drugs which will antagonize the acetylcholine which accumulates when the body ChE is inhibited,[10] (*b*) drugs which will directly detoxicate the organophosphate[11] and (*c*) drugs which will reactivate the inhibited ChE.[12,13]

The basic processes involved in poisoning and these forms of treatment are:

(i) chemical combination of the organophosphate (P) with ChE (E) to give the inhibited enzyme (I);

(ii) all non-injurious reactions of the organophosphate with body tissues, including excretion;

(iii) detoxication of the organophosphate by a therapeutic agent (A);

(iv) restoration of active ChE by dephosphorylation of the inhibited enzyme with a therapeutic agent (B)*; and

(v) antagonism of the accumulated acetylcholine by atropine or related compounds.

The kinetics of processes (i), (iii) and (iv) have been elucidated *in vitro* and the dependence of the rates on the concentrations of the reactants firmly established.[15–17] It is not possible to separate clearly all the various processes collectively involved in (ii), but it is reasonable to group them as a summed "waste" reaction, the rate of which depends simply on the dose of organophosphate injected. This assumption has been found to be true for most types of drug.[18] Process (v) may be incorporated into the kinetic scheme as shown below without any detailed knowledge of its mechanism. Processes (i) to (iv) above may be described by the chemical equations:

(i) $\mathrm{P} + \mathrm{E} \longrightarrow \mathrm{I}$

(ii) $\mathrm{P} \longrightarrow$ non-toxic products

(iii) $\mathrm{P} + \mathrm{A} \longrightarrow$ non-toxic products

(iv) $\mathrm{I} + \mathrm{B} \longrightarrow \mathrm{E} +$ non-toxic products

The corresponding velocities are then given by:

(i) $v = -d\mathrm{E}/dt = k_1\mathrm{EP}$

(ii) $v = -d\mathrm{P}/dt = k_2\mathrm{P}$

(iii) $v = -d\mathrm{P}/dt = k_3\mathrm{P} = k_3\,\mathrm{AP}$

(iv) $v = \ \ d\mathrm{E}/dt = k_4\mathrm{I} = k_4'\,\mathrm{BI}$

Concentrations in the above and subsequent equations are represented simply by capital letters, the customary square brackets having been omitted for greater clarity.

The rate constant k_4 increases linearly with concentration B only when the solution is very dilute. In stronger solutions the rate approaches a maximum beyond which any increase in B leads to no significant increase in rate.[17] This fact is unimportant in the subsequent general theory but should be taken into account in any attempted calculation of the therapeutic effect of a specific reactivator at a specific dose.

In order to relate these chemical reactions to the physiology of poisoning the assumption is made that since ChE is a vital enzyme, once the fraction of this enzyme in an animal falls below a certain minimum as a result of combination with the

* Long term recovery from sub-lethal doses of poison is aided by the synthesis of new ChE by the body, but this process is almost certainly too slow to materially affect the quantity of these rapidly acting poisons required to kill.[14]

organophosphate, the animal will die. Kewitz and Nachmansohn[19] have suggested 2 per cent as a minimum essential level of brain ChE although they consider that future experiments may necessitate amending this figure. The results in the present paper have been worked out for values ranging from 0·1 to 10 per cent. The concentration of organophosphate required to just produce this degree of inhibition is taken to be the LD50. In the presence of an acetylcholine antagonist, an increased concentration of acetylcholine can be tolerated at nerve and nerve-muscle junctions without causing functional breakdown, so that the fraction of the body ChE essential for life to continue will be decreased. By postulating such a decrease, process (v), namely antagonism of acetylcholine, may be included in the kinetic scheme in a qualitatively correct manner without any further knowledge of its mechanism. Quantitatively the proposed decrease must be largely arbitrary.

Two further assumptions required before these reactions can be analysed kinetically are that the animal body is a homogeneous system containing appropriate doses of A and B, into which P is introduced instantaneously at zero time; and that the concentrations of A and B remain constant over the relatively short period of time during which the enzyme inhibition becomes maximal and the bulk of the organophosphate disappears.

Equations for therapeutic efficiencies for all the possible therapeutic procedures are derived first below. Their significance is then considered in the Discussion.

The set of basic reactions can be analysed in a closed mathematical form only when reaction (iv) does not occur. When all the reactions are included it is necessary to resort to approximate numerical methods. For convenience these two situations are dealt with separately.

Case I. The enzyme is not reactivated, i.e. reaction (iv) is absent.

When (i), (ii) and (iii) take place simultaneously the rate equations are

$$-d\mathrm{P}/dt = (k_2 + k_3 + k_1\,\mathrm{E})\,\mathrm{P}$$

and

$$-d\mathrm{E}/dt = k_1\,\mathrm{EP}$$

whence, by simple division

$$d\mathrm{P}/d\mathrm{E} = (k_2 + k_3)\,/\,k_1\,\mathrm{E} + 1$$

If at zero time, $\mathrm{P} = \mathrm{P}_0$ and $\mathrm{E} = \mathrm{E}_0$, this equation may be integrated to give

$$\mathrm{P} = \mathrm{P}_0 - ln\,(\mathrm{E}_0/\mathrm{E})\,(k_2 + k_3)\,/\,k_1 - (\mathrm{E}_0 - \mathrm{E}) \qquad (1)$$

If, as is generally the case experimentally, $\mathrm{P}_0 \gg \mathrm{E}_0$ (see p. 119), then

$$\mathrm{P} = \mathrm{P}_0 - ln\,(\mathrm{E}_0/\mathrm{E})\,(k_2 + k_3)\,/\,k_1 \qquad (2)$$

When all the organophosphate has disappeared,

$$ln\,(\mathrm{E}_0/\mathrm{E}_\infty) = k_1\mathrm{P}_0/(k_2 + k_3) \qquad (3)$$

where E_∞ is the final (and minimum) level of ChE reached. In the absence of any therapeutic agent (A), so that k_3 is zero, the LD50 of P (p in moles/1. rather than in the usual units of mg/kg) is given by

$$p = k_2\,ln\,(\mathrm{E}_0/\mathrm{E}_{min})k_1$$

where E_{min} is the minimum ChE level reached after an LD50 dose of organophosphate.

Since p is generally known but k_2 unknown, this equation can be more usefully expressed in the form

$$k_2 = k_1 p / ln\,(E_0/E_{min}) \tag{4}$$

Since the animal is assumed to die, once its ChE level falls below E_{min}, E_{min} may be defined as the "minimum essential ChE".

It is implicit in the derivation of (4) that the body ChE is uniformly distributed, but this need not be so. Provided $P_0 \gg E_0$ (the average initial ChE concentration), the rate of disappearance of the organophosphate and hence the concentration P depends only on the "waste" rate k_2, except where the local concentration of ChE is so high that the depletion of organophosphate in the immediate neighbourhood is too great to be compensated for by diffusion of fresh organophosphate into the region from outside. This means that for practical purposes the value of p is determined by the ratio E_0/E_{min} and not by the precise values of E_0 or E_{min}. In other words results calculated by assuming the body ChE to be uniformly distributed will be equally valid whether the enzyme is uniformly distributed or not. There is evidence[20] that not only is ChE non-uniformly distributed but also in many tissues it is non-uniformly accessible to different drugs. The purpose of the so-called "inaccessible" ChE is uncertain, it does not seem to contribute to synaptic or neuromuscular transmission. In this paper it will be assumed that all the essential ChE is "accessible".

The following formulae for therapeutic effectiveness may be derived from (3) and (4).

Firstly, if in the presence of an acetylcholine antagonist, the "minimum essential ChE" is reduced from E_{min} to E'_{min} and the LD50 is raised to p_a, then from (4),

$$p_a/p = ln\,(E_0/E'_{min})/ln\,(E_0/E_{min}) \tag{5}$$

Secondly, if in the presence of a given dose of A the LD50 is raised to p_b, then from (3)

$$p_b = ln\,(E_0/E_{min})\,\{(k_2/k_1) + (k'_3\,A/k_1)\}$$

Dividing throughout by p, and by use of (4), this becomes

$$p_b/p = 1 + ln\,(E_0/E_{min})\,(k_3\,A)\,/\,k_1 p \tag{6}$$

Thirdly, if p_c is the LD50 in the presence of a mixture of A and an acetylcholine antagonist which reduces the "minimum essential ChE" from E_{min} to E_{min}, then from (3)

$$p_c = ln\,(E_0/E'_{min})\,(k_2 + k'_3 A)/k_1$$

$$= \frac{ln\,(E_0/E'_{min})}{ln\,(E_0/E_{min})}\left\{\frac{k_2\,ln\,(E_0/E_{min})}{k_1} + \frac{k'_3\,A\,ln\,(E_0/E_{min})}{k_1}\right\}$$

Thus, by dividing throughout by p as above

$$\frac{p_c}{p} = \frac{ln\,(E_0/E'_{min})}{ln\,(E_0/E_{min})}\left\{1 + \frac{k'_3\,A\,ln\,(E_0/E_{min})}{k_1 p}\right\} \tag{7}$$

Case II. The system includes a reactivator, B. If all four reactions (i)–(iv) take place together, then

$$dE/dt = k_4 (E_0 - E) - k_1 EP \tag{8}$$

and

$$-dP/dt = (k_2 + k_3 + k_1 E) P \tag{9}$$

The solution of these two simultaneous equations may be simplified if $(k_2 + k_3) \gg k_1 E$. That this is true under conditions likely to be met experimentally may be shown as follows:

$$\text{From (4), } k_2 = k_1 p / ln\,(E_0/E_{min})$$

Now, for most toxic organophosphates $p \geqslant 0{\cdot}1$ mg/kg, which $\geqslant 10^{-6}$M, so that even if E_{min}/E_0 is as little as 0·1 per cent

$$k_2 \geqslant k_1 (10^{-6})/6{\cdot}9$$

But, the concentration of ChE in blood cells[21,22] is less than 10^{-8}M and this is probably true of the body as a whole, so that

$$k_2 \gg k_1 E \text{ and thus } (k_2 + k_3) \gg k_1 E$$

Thus (9) now becomes

$$-dP/dt = (k_2 + k_3) P, \text{ whence } P = P_0 e^{-(k_2 + k_3)t}$$

By substitution of this value for P, (8) becomes

$$dE/dt = k_4 (E_0 - E) - k_1 E P_0 e^{-(k_2 + k_3)t} \tag{10}$$

A further simplication may be made by introduction of the new variables

$$y\,(= E/E_0) \text{ and } x\,(= e^{-(k_2 + k_3)t})$$

so that:

$$\frac{dy}{dx} = \frac{k_1 P_0}{k_2 + k_3}\, y - \frac{k_4}{k_2 + k_3}\left(\frac{1 - y}{x}\right) \tag{11}$$

If $k_3 = 0$, i.e. reactivation is the only action of the therapeutic agent, k_2 may be replaced by $k_1 p / ln\,(E_0/E_{min})$ (see 4), so that

$$\frac{dy}{dx} = \frac{P_0\, ln\,(E_0/E_{min})}{p}\, y - \frac{k_4\, ln\,(E_0/E_{min})}{k_1 p}\left(\frac{1 - y}{x}\right) \tag{12}$$

Experimentally we are not primarily interested in the ChE level at every single instant of time, but in the minimum ChE level which is reached for any likely combination of rate constants and doses of A, B and P. These minima can best be obtained by integration of (11) or (12) for selected values of the rates and concentrations with graphical extension to any other desired values. Direct integration of the differential equation

$$f = dy/dx = Fy - G\,(1 - y)/x$$

is possible only in terms of "incomplete gamma functions" which must be obtained from published tables and it is simpler to use the numerical integration formula[23]

$$y = y_0 - (x - x_0) f\{x_0 + \tfrac{1}{2}(x - x_0), y + \tfrac{1}{2}(x - x_0) f_0\}$$

in order to get the minimum values of y. These are illustrated in Fig. 1 for various values of F and G.

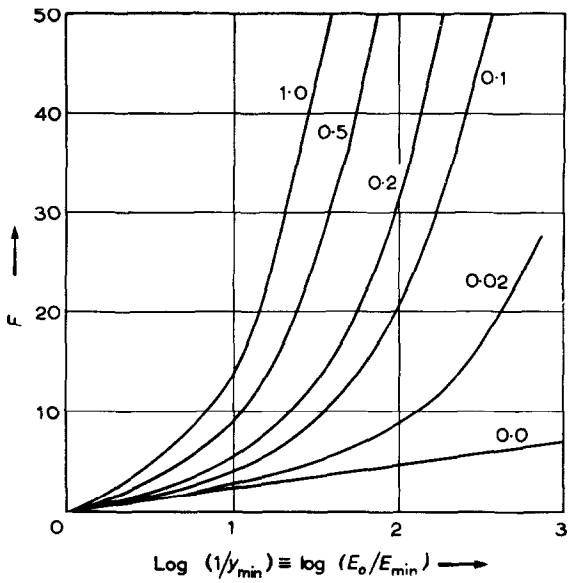


FIG. 1. Minimum ChE levels obtained by numerical solution of the equation $dy/dx = Fy - G(1-y)/x$. "The numbers adjacent to the curves are the corresponding coefficients G."

In order to use these results to calculate the effectiveness of particular reactivators against particular organophosphates the following procedure was adopted. From Fig. 1, the value of F corresponding to the values 0·02, 0·1, 0·2, 0·5 and 1·0 for G was read off for each of a series of minimum ChE levels between 0·1 and 10 per cent. Since, from (12), $G = k_4 \ln(E_0/E_{min}) / k_1 p$ and $F = P_0 \ln(E_0/E_{min}) / p$, a value of P_0/p can be obtained for the corresponding value of $k_4/k_1 p$ for each of these values of E_{min}. In Fig. 2, P_0/p is shown plotted against $k_4/k_1 p$. These values of P_0/p are the therapeutic efficiencies for a reactivator and will in future be denoted by p_d/p.

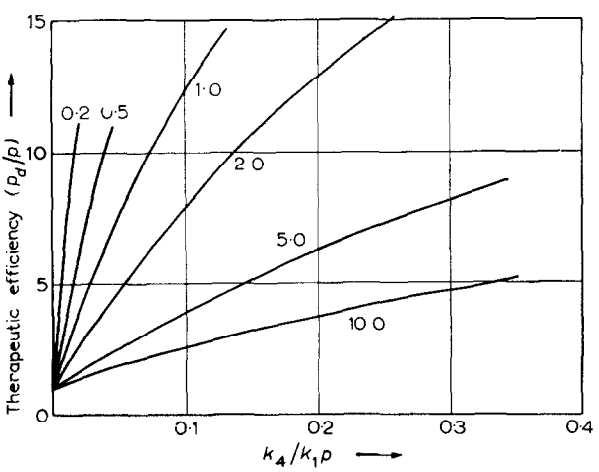


FIG. 2. Dependence of the therapeutic effect on the ratio of the reactivating power of the therapeutic agent to the inhibitory power of the organophosphate ($k_4/k_1 p$) for various minimum ChE levels (per cent). "The numbers alongside the separate curves give the minimum ChE level (%)."

When the results for each ChE level in Fig. 2 are plotted in the form log $(p_d/p - 1)$ against log (k_4/k_1p) a series of parallel straight lines is obtained (See Fig. 3). These lines may be described approximately by equations of the form log $(p_d/p - 1) = a + 0{\cdot}8$ log (k_4/k_1p). If the values of a are plotted against log (E_0/E_{min}) a further

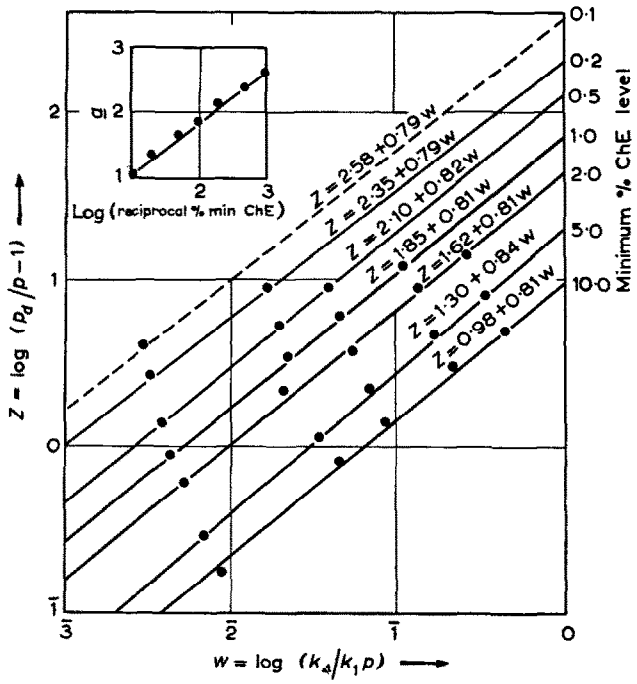


FIG. 3. Relation between the logarithms of the therapeutic effect and the ratio of reactivating power to inhibitory power.

straight line is obtained (see inset to Fig. 3) from which $a \approx 0{\cdot}2 + 0{\cdot}8$ log (E_0/E_{min}).

Thus,

$$\log (p_d/p - 1) \approx 0{\cdot}2 + 0{\cdot}8 \log (E_0\, k_4/k_1p\, E_{min})$$

or

$$p_d/p \approx 1 + 1{\cdot}6\,(E_0\, k_4/k_1p\, E_{min})^{0{\cdot}8} \qquad (13)$$

Since the derivation of this formula is empirical it should be used with caution for ChE levels outside the range 0·1–10 per cent, for values of k_4/k_1p above 0·5, or for therapeutic efficiencies greater than 20.

DISCUSSION

The main purpose of the above calculations is to see whether our qualitative ideas on how organophosphate poisoning and the various forms of treatment operate are quantitatively reasonable. The fundamental assumption of the theory is that once the ChE level in an animal falls below an arbitrary "minimum essential ChE", the animal will die. But, although inhibition of ChE is the biochemical lesion, the primary cause of death is respiratory failure to which at least three distinct physiological mechanisms

may contribute, namely inhibition of the respiratory centre, bronchoconstriction and neuromuscular block.[24] These mechanisms vary in importance from species to species and from toxic agent to toxic agent. It has been further assumed that the organophosphate and therapeutic agents are always uniformly distributed in the animal body. In these circumstances it must be accepted that the theory can give only a generalized picture and not one which can reliably predict what effect a particular therapeutic agent administered by a specific route will have against a particular organophosphate in a particular species. This reservation must be borne in mind in the following discussion in which, of necessity, specific experiments have been used for comparison with deductions from theoretical equations.

Each type of therapeutic procedure is considered below in turn.

(*a*) *Acetylcholine antagonists.* The likely therapeutic effect of compounds such as atropine can be calculated from (5). Since atropine antagonizes acetylcholine, less ChE will be needed in the presence than in the absence of atropine. Suppose the "minimum essential ChE" is reduced from E_{min} to E'_{min} thus causing the LD50 of the organophosphate to be raised from p to p_a. Then, the therapeutic effect is given by

$$p_a/p = 1n\,(E_0/E'_{min}) \,/\, 1n\,(E_0/E_{min})$$

For example, if the "minimum essential ChE" level is reduced from 10 per cent to 1 per cent, then p_a/p is 2; while if from 10 per cent to 0·1 per cent, then p_a/p is 3.

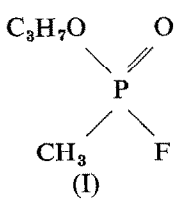
These values for p_a/p are of the same order as the experimentally determined effectiveness of acetylcholine antagonists,[10,25] and indicate that our arbitrary decrease in "minimum essential ChE" in the presence of atropine is quantitatively reasonable.

(*b*) *Detoxicating agents.* The therapeutic effectiveness of compounds of this class may be calculated from (6), namely

$$p_b/p = 1 + 1n\,(E_0/E_{min})\,k'_3\,A/k_1p$$

Two important conclusions emerge from this equation. Firstly, the therapeutic effect depends on k'_3/k_1, that is, on the relative reactivities of therapeutic agent and ChE with the organophosphate and not solely on the detoxicating power of the therapeutic agent. Secondly, the therapeutic effect will only be significant when $1n\,(E_0/E_{min})\,k'_3\,A/k_1p > 1$.

The most reactive compounds with organophosphates in neutral solution yet discovered are hydroxamic acids,[26] which have rate constants for reaction with Sarin (*iso*propyl methylphosphonofluoridate) (I) of about



(I)

10^2 1.mole^{-1}min.$^{-1}$. For Sarin[5] $k_1 > 10^7$ 1.mole^{-1}min.$^{-1}$ so that k'_3/k_1 is only about 10^{-5} which requires A/p to be about 10^5. Some hydroxamic acids have been tested as antidotes to Sarin but they were ineffective except in enormous doses (about 1 g/kg) which raised the LD50 about twice.[11,12] If the above rate constants and concentrations are substituted into (6) then p_b/p is found to lie between 1 and 2 for "minimum

essential ChE" levels between 10 per cent and 0·1 per cent, a result which is moderately consistent with experiment.

Unless much more reactive compounds can be discovered, drugs which act solely by detoxication of organophosphates are unlikely to have much potential therapeutic value.

(*c*) *Acetylcholine antagonists + detoxicating agents.* The effect of a combination of agents of these two types may be calculated from (7), namely

$$\frac{p_c}{p} = \frac{ln\,(E_0/E'_{min})}{ln\,(E_0/E_{min})} \left\{ 1 + \frac{k'_3\,A\,ln\,(E_0/E_{min})}{k_1 p} \right\}$$

The terms on the right-hand side of this equation represent the effects of the two separate agents when given alone, so that the combined effect is the result of each individual agent acting independently. It is evident that for a combined therapy of this kind to be decidedly advantageous both individual agents must be moderately effective when given alone.

There is little experimental evidence available to test this deduction but the results obtained by Askew[25] on the treatment of Sarin poisoning with atropine plus diacetyl monoxime are reasonably consistent with it. The biochemical basis of the therapeutic activity of diacetyl monoxime is not known with certainty but it probably acts primarily as a detoxicant (see section *e*).

(*d*) *Reactivating agents.* Unlike the situation in the previous three therapeutic procedures, when reactivation forms the basis of treatment it is impossible to derive any general formula for the therapeutic effect. However, as described earlier, over the limited range of "minimum essential ChE" levels of from 10 per cent to 0·1 per cent, therapeutic efficiencies (p_d/p) for any particular reactivator may be calculated approximately from (13), namely

$$p_d/p = 1 + 1{\cdot}6\,(E_0 k_4/k_1 p E_{min})^{0{\cdot}8}$$

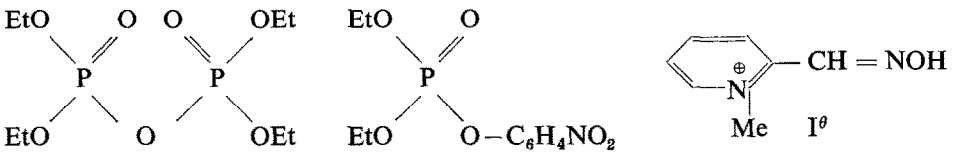
Several interesting general deductions may be made from (13).

Firstly, since the therapeutic effect depends on $(E_0/E_{min})^{0{\cdot}8}$ rather than on $ln\,(E_0/E_{min})$, the "minimum essential ChE" is far more important than in the therapeutic procedures described under (*a*)–(*c*). This dependence means that if the "minimum essential ChE" could be lowered in some way, for example by the presence of acetylcholine antagonists, the therapeutic effect might be greatly improved. This point is elaborated in section (*f*).

Secondly, the therapeutic efficiency is dependent not only on the rate of reactivation but also, inversely, on the product of the LD50 of the organophosphate and the rate at which it inhibits the enzyme. This product is simply a measure of the rate of spontaneous detoxication of the organophosphate (see equation 4). Although an approximate inverse relation between k_1 and p (i.e. making $k_1 p$ a constant) has been claimed for some organophosphates, this is not general.[27] This means that the therapeutic effect of a given reactivator against a range of organophosphates which give the same phosphorylated enzyme will not necessarily be constant; and also, that the ease of treatment of poisoning by organophosphates with different dialkylphosphorylating groups may not necessarily be directly related to the rate at which the corresponding dialkylphosphorylated enzymes can be reactivated.

This deduction may explain the observation[28] that TEPP (tetraethyl pyrophosphate,

II) is less amenable than E600 (diethyl *p*-nitrophenyl phosphate, III) to treatment by the reactivator PAM (pyridine-2-aldoxime methiodide, IV), although both organophosphates form diethylphosphoryl—ChE.



(II) (III) (IV)

These two compounds have almost identical toxicities but k_1 is 3 times as great for TEPP as for E600.[15] Unfortunately, evidence of this kind cannot be used as a direct test of the theory as the experimental data may be complicated by distributive effects, about which little is known, and which are often used to rationalize experimental results for which no other explanation is available.

A third consequence of (13) also arises from the change in dependence on E_{min} from logarithmic to almost linear when a reactivator is exerting a significant therapeutic effect. In so far as the toxicity of an organophosphate varies from animal to animal in a given species owing to minor variations in the "minimum essential ChE", this change in dependence will result in a decrease in the slope of the mortality-dose of organophosphate curve.

Experimentally a decrease in the slope of the response curve has been observed when oximes were used in the treatment of organophosphate poisoning[29] whereas no

TABLE 1. THE LIKELY EFFECTIVENESS (LD50 RATIO, p_a/p) OF PYRIDINE-2-ALDOXIME METHIODIDE (PAM, IV), IN THE TREATMENT OF POISONING BY SARIN (I), E600 (III) OR DFP (V).

Toxic Agent	LD50* (mg/kg)	k_1†	PAM (mg/kg)	k_4'†	$\frac{k_4' B M_P}{k_1 p M_B}$‡	Effectiveness (p_a/p) for a "minimum essential ChE" level (per cent) of		
						10	1	0·1
Sarin	0·21[a]	$1·5 \times 10^{7}$[c]	10	500[e]	0·0010	1·04	1·25	2·59
			20		0·0020	1·07	1·44	3·75
			50		0·0048	1·14	1·87	6·50
E600	0·7[b]	$1·1 \times 10^{6}$[d]	10	1000[f]	0·015	1·35	3·21	15·0
			20		0·031	1·62	4·89	25·6
DFP	4·0[b]	10^{5}[c]	10	30[f]	0·0006	1·03	1·17	2·04
			20		0·0012	1·05	1·29	2·84
			50		0·0030	1·10	1·60	4·80

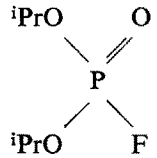
* The LD50's are for mice (*sub cut.*) and are taken from a[25] and b[13].

† Rate constants k_1 and k_4' are for erythrocyte ChE and are in units of 1. mole^{-1} min.$^{-1}$. Values of k_1 are from c[5] and d[15], while values for k_4' are estimated for very dilute solution at 37° from results in e[30] and f[17].

‡ M_B and M_P are the molecular weights of PAM and the organophosphates and convert weight concentrations for B and *p* into molar concentrations.

such consistent decrease in slope was found when acetylcholine antagonists were used as therapeutic agents.[10]

In Table 1, (13) has been used quantitatively to calculate the likely therapeutic effect of PAM against poisoning by Sarin, by E600, or by DFP (di-*iso*propyl phosphorofluoridate, V).



(V)

Experimentally, PAM is more effective against poisoning by E600 than against poisoning by DFP,[28] but is relatively ineffective against Sarin poisoning except in conjunction with atropine.[25]

(*e*) *Reactivating agent + detoxicating agent.* Since many hydroxamic acids and oximes will detoxicate organophosphates as well as reactivate the inhibited enzyme, it is of interest whether the ability to detoxicate would significantly enhance the therapeutic effect due to reactivation alone. This question can be answered qualitatively by consideration of (11) and Fig. 1. For a reactivator with a fixed rate of reactivation (k_4) the lethal concentration of organophosphate (P_0) at any "minimum essential ChE" is given by $P_0 = (k_2 + k_3)F/k_1$, where F may be read from Fig. 1 for the appropriate values of k_4, $k_2 + k_3$ and E_{min}. If the compound has no detoxicant action k_3 is zero and P_0 is the same as p_d in section (*d*), but if it will also detoxicate, P_0 will tend to increase as k_3 increases. But, when $k_2 + k_3$ increases, if the "minimum essential ChE" level is to remain unaltered, the corresponding decrease in the coefficient G ($= k_4/(k_2 + k_3)$) will, as can be seen from Fig. 1, necessitate a fall in F. In other words the LD50 (P_0) increases not in proportion to $k_2 + k_3$ but considerably more slowly. But, if there were no reactivation, P_0 would, from (3), increase linearly with $k_2 + k_3$, so that the added effect of detoxication in conjunction with reactivation is less than that of detoxication alone. This situation may be contrasted with the combined effect of detoxication and an acetylcholine antagonist which is equivalent to the effect of each agent acting independently (see section *c*).

A further question arising out of a compound having two distinct therapeutic properties is whether one or other will predominate in the overall therapeutic effect. If $(p_b/p - 1)$ and $(p_d/p - 1)$ are used instead of p_b/p or p_d/p as measures of therapeutic efficiency, then from (6) and (13), the relative effectiveness (q) of detoxication to reactivation is given by

$$q = \frac{ln\,(E_0/E_{min})\,(k_1 p)^{0\cdot 8}\,k_3\,A}{1\cdot 6\,(E_0/E_{min})^{0\cdot 8}\,k_4{}^{0\cdot 8}\,k_1\,p}$$

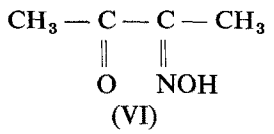
If E_{min} is 1 per cent, this becomes

$$q = k_3\,A/14\,k_4{}^{0\cdot 8}\,(k_1 p)^{0\cdot 2}$$

For most toxic organophosphates k_1 is about 10^6 1.mole^{-1} min.$^{-1}$ while p is about 10^{-6} to 10^{-5} moles 1.$^{-1}$, so that for such compounds $(k_1 p)^{0\cdot 2}$ does not differ greatly from 1. Thus, only if k_3' A is about 10 k_4 ($\approx 10\,k_4'$ B, see p. 116) will the therapeutic

effect of detoxication be comparable with that of reactivation. As, if both properties are possessed by the same compound, A and B are the same, the second order rate constant (k_3') for reaction with the organophosphate must exceed that (k_4') for reactivation by a factor of 10 before detoxication is likely to predominate in the overall therapeutic effect.

For most oximes and organophosphates the rate constants are such that the effect of reactivation will far outweigh that of detoxication, but in one instance, the treatment of Sarin poisoning by diacetyl monoxime (VI) and related compounds[12] the reaction rate is over 10 times the rate of reactivation. In this case the theory would suggest, in



(VI)

agreement with the available physiological evidence, that detoxication is the more important effect. Quantitatively the therapeutic success of diacetyl monoxime is not explicable on either basis, and the compound may have some other, as yet unknown, pharmacological action which is therapeutically beneficial.

(*f*) *Reactivating agent + acetylcholine antagonists.* In section (*d*) it was pointed out that the therapeutic effect of reactivating agents was very sensitive to the "minimum essential ChE" level and that if this level could be lowered the therapeutic efficiency could be greatly improved. Such a lowering would result from the administration of acetylcholine antagonists together with the reactivator. The resulting synergism may be demonstrated mathematically as follows.

Let p be the LD50 of the organophosphate alone, p_a that in the presence of the acetylcholine antagonist, p_d that in the presence of the reactivator, and p_f that in the presence of both. Also let E'_{min} be the "minimum essential ChE" in the presence of the acetylcholine antagonist. Then from (5) and from (13)

$$p_a/p = ln\,(E_0/E'_{min})/ln\,(E_0/E_{min})$$

$$p_d/p = 1 + 1{\cdot}6\,(E_0/E_{min})^{0{\cdot}8}\,(k_4/k_1p)^{0{\cdot}8}$$

and

$$p_f/p_a = 1 + 1{\cdot}6\,(E_0/E'_{min})^{0{\cdot}8}(k_4/k_1p_a)^{0{\cdot}8}$$

whence

$$p_f/p = (p_a/p)\,[\,1 + 1{\cdot}6\,(E_0/E_{min})^{0{\cdot}8}(k_4/k_1p)^{0{\cdot}8}$$

$$(E_{min}/E'_{min})^{0{\cdot}8}\,\{\,ln\,(E_0/E_{min})/ln\,(E_0/E'_{min})\}^{0{\cdot}8}] \qquad (14)$$

The terms on the right-hand side of (14) are the therapeutic effect of the acetylcholine antagonist alone, multiplied by the therapeutic effect of the reactivator alone but in which the non-unity term has been multiplied by the factor $(E_{min}/E'_{min})^{0{\cdot}8}\,\{ln\,(E_0/E_{min})/ln\,(E_0/E'_{min})\}^{0{\cdot}8}$. As E'_{min} decreases, i.e. with more effective antagonists, $(E_{min}/E'_{min})^{0{\cdot}8}$ will increase far more rapidly than $\{ln\,(E_0/E'_{min})\}^{0{\cdot}8}$ so that the above factor will increase rapidly and the synergism will increase enormously.

The combined therapeutic effect of any particular group of drugs can be computed theoretically from (14) as required. The calculation is illustrated below for a few instances from Table 1.

(i) Suppose the acetylcholine antagonist will reduce E_{min} from 10 per cent to

1 per cent, then $p_a/p = 2$. If this same antagonist is given in conjunction with 20 mg/kg of PAM, which, theoretically, will raise the LD50 of E600 1·62 times, the combined effect is given by

$$p_b/p = 2\,[1 + 1{\cdot}6\,(10)^{0{\cdot}8}\,(0{\cdot}031)^{0{\cdot}8}\,(10)^{0{\cdot}8}\,\{ln\ 10/ln\ 100\}^{\,0{\cdot}8}] = 6{\cdot}5$$

If the acetylcholine antagonist were still more effective and would reduce E_{min} from 10 per cent to 0·1 per cent, then $p_a/p = 3$, $p_d/p = 1{\cdot}62$ and $p_f/p = 34$.

(ii) If Sarin were the organophosphate treated with 20 mg/kg of PAM, then if E_{min} were reduced from 10 per cent to 1 per cent,

$$p_a/p = 2,\ p_d/p = 1{\cdot}07 \text{ and } p_f/p = 2{\cdot}5.$$

If E_{min} were reduced from 10 per cent to 0·1 per cent, then

$$p_a/p = 3,\ p_d/p = 1{\cdot}07 \text{ and } p_f/p = 5{\cdot}2.$$

These specimen calculations illustrate the main theoretical features of this synergism, namely that the combined effect can in some cases be enormously greater than either individual effect, and that with a moderately good antagonist, synergism could still be found with a reactivator which, given alone, is almost inactive.

Experimentally[13,25,28,31] there is a very marked synergism between the reactivator PAM and the acetylcholine antagonist atropine in the treatment of poisoning by a wide range of organophosphates. This synergism has been attributed to localization effects, the oxime being assumed to be active mainly peripherally and against neuromuscular block while atropine counteracts the central effects of the organophosphate. While such distributive effects are obviously important, no amount of theoretical synergism being any help if neither agent can reach the sites at which the organophosphate exerts its lethal action, the mere existence of synergism is not in itself evidence for a difference in site of action of the two therapeutic agents. Nevertheless, distributive effects could strongly reinforce the intrinsic synergism, so that selection of a mixture of reactivators and acetylcholine antagonists such that at least one compound of each class would penetrate to every vital site at which the organophosphate acts, might prove more fruitful than a search for more potent single compounds.

The theory developed in this paper has been applied exclusively to organophosphate poisoning. Precisely similar lines of reasoning could be applied to any other form of poisoning in which the biochemical lesion is specific and capable of being studied kinetically "in vitro".

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THE EFFECT OF PYRIMIDINE NUCLEOSIDES THYMIDINE, CYTIDINE AND URIDINE ON MORPHOLOGICAL CHARACTERISTICS ON CELLS GROWN IN VITRO

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Abstract—Nucleolus behaviour under the separate influence of the nucleosides thymidine, cytidine and uridine is described. These substances are able to induce structural changes in the nucleolus without interfering with the mitotic process. The conclusions are discussed in the light of other research.

THE effect of various nucleosides and nucleotides on living cells "in vitro" was studied in detail by Hughes by means of phase-contrast cine-micrography. Nucleolar fragmentation and, in some cases, pre-prophase inhibition were recognized. Among the substances examined by Hughes,[1] such as adenosine, guanosine and adenilic acid, adenosine was the only one able to break up nucleoli, and to prevent cells from entering pre-prophase. Lettré,[2] and Lettré and Siebs[3] described the formation of filamentous structures in the nucleoli under the influence of adenosine.

In our investigations, carried out on substances able to antagonize pyrimidine analogues (Di Marco and Gaetani,[4] Di Marco and Necco[5]), we examined the effects of the nucleosides thymidine, cytidine and uridine on living cells *in vitro*, with respect to the structural transformations in the nucleolus.

MATERIAL AND METHOD

The fibroblasts used in these investigations were obtained from explants of hearts, taken from eight-day chick embryos. The cultures were grown for 24 hr on coverslips, and then transferred to chambers adapted for phase-contrast microscopy.

While observing the cultures, the respective nucleosides, dissolved in Hank's saline, was added to them until a final concentration of 10 mM was reached. The observation of the cells was continued for a 3 hr period after the addition of the substances. The behaviour of the nucleolus under the influence of each nucleoside was recorded photomicrographically.

The position of the cells in the outgrowth was noted and the culture was fixed and stained. The same cells were identified in the permanent preparation and compared with their appearance during life.

RESULTS

When thymidine (Schwartz's) 10 mM in Hank's saline, was added to a culture,

during the following 60 min most of the nucleoli became larger and looser, showing an irregular outline.

Under the influence of 10 mM cytidine (N. B. Co.) solution, neutralized with N/10 NaOH, we could follow the transformation of the nucleolus from a dense to a filamentous structure (Plate I). Within 20 min of the application of this substance, a kind of swelling of the nucleolus could be observed, together with the appearance of a fine granular structure within it.

During the first 20 min after the application of 10 mM uridine (N. B. Co.), we found loose nucleoli, and "holes" became visible. Nucleolar unfolding increased within 60 min and went on for a further 60 min, when the culture was fixed and stained. (Plate II)

DISCUSSION

Bernhard, Haguenau and Oberling[6] only once observed "vacuolar" or "alveolar" nucleoli in a rat spleen. Several attempts to repeat this observation have not succeeded. According to Vincent and Huxley,[7] there were most probably metabolic products of nucleoli in these vacuoles, since it is not possible, within the vacuole, to elicit the reactions of nucleic acid, proteins, etc.

Brachet,[8] in a recent study, demonstrated that the structure and chemical composition of nucleoli are strictly related to the energetic conditions of the cytoplasm. In fact, reducing the production of energy in the cytoplasm by inhibitors of oxidative phosphorylation (dinitrophenol, Na usnate) it is possible to induce an inbalance of RNA contents in the different cellular components (nucleolus, nucleoplasm and cytoplasm). Using this technique in Acetabularia mediterranea, a unicellular giant alga, the nucleus assumes a spherical shape, strongly vacuolated and poor in RNA.

As far as the filamentous structures in nucleoli are concerned, the literature confirms that they are easily observable by treatment with particular substances. Nucleosides and nucleotides were the compounds most studied from this point of view; and all of them showed their ability to induce a moderate degree of nucleolar fragmentation. In some cases, filamentous structures were formed in a material which was formerly homogeneous. Most probably the effect on the nucleoli is a specific one for these substances.

The morphological changes in the nucleolus occur as readily as they disappear after removal of the substances that induced them (Hughes[1]). Thus, it seems probable that such compounds easily enter the cells and that, on the other hand, the structural changes are easily reversible.

Therefore, the particular sensitivity of nucleolar material in the presence of nucleosides suggests that, in the nucleolus, an equilibrium exists between the precursors (nucleosides or other more simple precursors) and nucleic acids.

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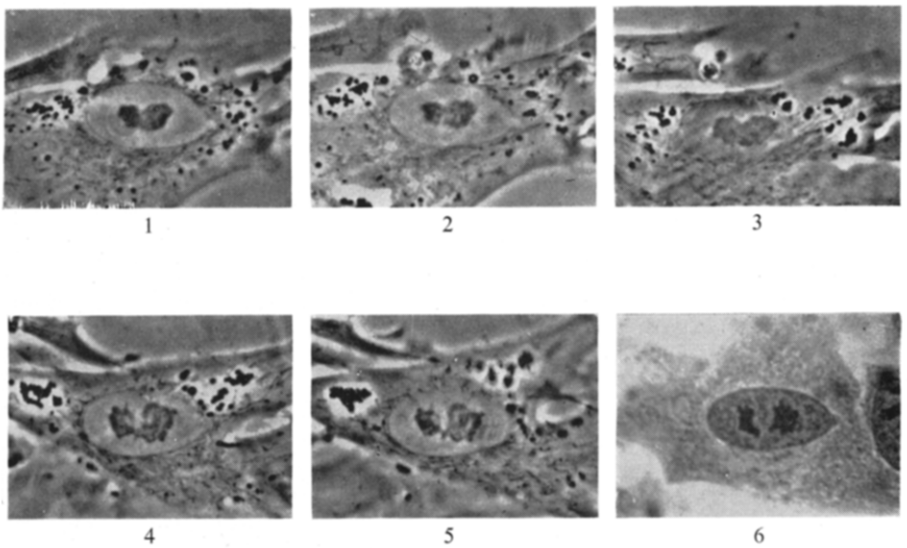
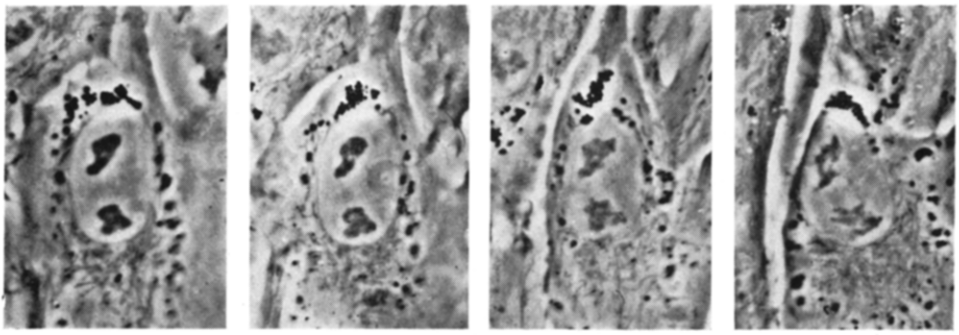


PLATE I. Unfolding of the nucleolus.
Numbers 1–5. Living cell 5, 20, 60, 110 and 120 min respectively after the addition of cytidine (10mM).
Number 6. The same cell. Fixation after 170 min.



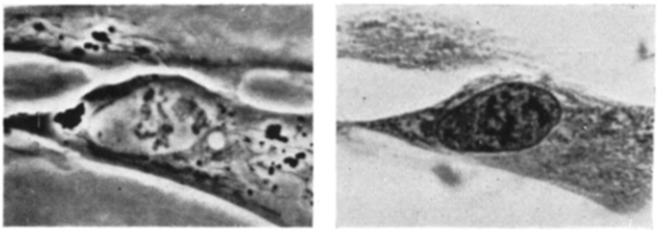


PLATE III. Nucleolar unfolding.

Numbers 1–4. Living cell 5, 20, 60, and 120 min respectively after the addition if uridine
Number 1a. Living cell 130 min after addition of uridine (10mM).
Number 2a. The same cell. Fixation after 170 min.

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COMPARATIVE UTILIZATION BY SARCOMA-180 CELLS IN CULTURE OF ^{14}C-LABELLED URACIL, 6-AZAURACIL, AND THEIR RIBOSIDES*

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Abstract—The riboside of 6-azauracil-2-^{14}C, 6-azauridine-2-^{14}C, a potent inhibitor of the reproduction of sarcoma-180 cells in culture, is accumulated in the acid-soluble extractives of the cells, while 6-azauracil-2-^{14}C, which is inactive as an inhibitor of the growth of these cells, is taken up extremely poorly. Similarly, the radioactivity of uridine-2-^{14}C and uridine-2-^{14}C-5′-phosphate is readily assimilated by these tumour cells, in contrast to that of uracil-2-^{14}C, which is utilized only to a limited extent. Accordingly, the difference in the inhibitory activity of 6-azauracil and its riboside, azauridine, on the growth of sarcoma-180 is attributed to the relative capacities of these neoplastic cells to assimilate uracil and its ribose-containing derivative, uridine, upon which capacities the assimilation of the analogues are dependent.

STUDIES of the metabolism of pyrimidines have suggested that effective antagonists of this class of intermediates could be useful not only in advancing knowledge of the functions of various pyrimidine-containing compounds, but also as inhibitors of processes essential for cellular reproduction. Of particular interest have been studies[1] showing that neoplastic change in liver may be associated with an increased capacity to incorporate uracil into ribonucleic acid (RNA). However, it is now evident from the work of Canellakis[2,3,4] that at least two factors may be involved in this altered behaviour of liver. Thus, in hepatomas and in regenerating liver tissue (following partial hepatectomy) there is a marked reduction in the capacity to catabolize uracil, a circumstance which can lead to an increase in the concentration of uracil available to hepatic cells for anabolic utilization, and thus to the appearance of uracil in RNA. However, in addition to this interplay of catabolic and anabolic systems, another factor appears to be involved, for despite the constantly diminished capacity of regenerating liver tissue to degrade uracil, a marked increase in the incorporation of the pyrimidine into hepatic RNA appears about 48 hours subsequent to partial hepatectomy. It may be assumed that this factor plays a significant role in the anabolic processes of neoplastic cells derived from liver and, possibly, from other tissues.[4] In any case, these and other observations have provided a rational basis for the development of antagonists of pyrimidine-metabolism as potential carcinostatic agents, e.g., 6-azauracil (*as*-triazine-3:5-dione) and 5-fluorouracil (2:4-dioxo-5-fluoropyrimidine).*

* It is pertinent to mention, with respect to the nomenclature of pyrimidines, that according to the older numbering system, *as*-triazine-3:5-dione would be termed 4-azauracil; however, the newer system, recommended by the American Chemical Society and used by Chemical Abstracts, has been employed in our laboratories. The numerical prefix ("6-") probably should be employed regularly, although this has not been done with the abbreviation (i.e. AzU), since the corresponding symmetrical triazine, 5-azauracil (*s*-triazine-2:4-dione), in the studies so far carried out, has not shown promise of biological utility.

6-Azauracil (AzU) inhibits the growth of several experimental tumours in mice, including sarcoma-180, and the lymphomas, L-1210 and L-5178.[5,6] However, when tested on cells reproducing *in vitro*, AzU, in concentrations as high as 5μmoles/ml, has no significant inhibitory activity on the growth in culture of mouse fibroblasts or HeLa cells,[7] sarcoma-180,[8] L-5178-Y[9] and neoplastic mast cells (P-815). This lack of toxicity for the tumour cells in culture suggested that a metabolite of AzU, formed by the liver or other normal tissues of the host, might be responsible for the inhibitory action of the analogue *in vivo*. One of the major metabolites of AzU formed by anabolic reactions after administration of the analogue to mice is its riboside, azauridine (AzUR).[10] On the growth of cells in culture this derivative, in contrast to AzU itself, has a high inhibitory activity; this has been observed with sarcoma-180,[8] L-5178-Y[9] and P-815 mast cells. Furthermore, the dosage of AzUR (which is not cleaved to AzU by the mouse) required to inhibit the growth of several experimental tumours in mice is only one-tenth to one-twentieth that of AzU.[6]

In order to elucidate the biochemical mechanisms responsible for this marked difference in the inhibitory activity of AzU and its riboside for tumour cells, the relative utilizations by sarcoma-180 cells in culture of ^{14}C-labelled uracil, uridine, uridine-5′-phosphate (UMP), AzU and AzUR have been examined and the findings are presented in this communication.

MATERIALS AND METHODS

The compounds studied were labelled as follows: uracil-2-^{14}C, uridine-4-^{14}C, uridine-4-^{14}C-5′-phosphate, 6-azauracil-2-^{14}C (*as*-triazine-3:5-dione-3-^{14}C) and its riboside, 6-azauridine-2-^{14}C.*

The culture techniques employed were those described previously:[8] 200,000 sarcoma-180 cells, suspended in 2 ml of Eagle's medium[11] containing 10 per cent horse serum, were introduced into each culture flask (Earle's T-15)[12] and allowed to multiply for four days. After the first 24 hours the medium was replaced by Eagle's medium containing 5 per cent *dialysed* horse serum, and this medium was renewed daily. During the third and fourth days of the incubation the sarcoma-180 cells were exposed to a ^{14}C-labelled compound (added to the medium in a final concentration of 0·2 μmoles/ml). A generation-time of approximately 48 hours was obtained, resulting in a cell-count of about 400,000 after two days and 800,000 after four days. It is important to note that, during this relatively short time, the growth of the cultures exposed to the labelled AzUR was not significantly inhibited. At the end of the incubation period the individual cultures were treated as follows: the cell layer was washed repeatedly at 3°C with fresh medium, and removed from the glass surface of the culture flasks by treatment with 1 per cent ethylene-diamine tetraacetate ("Versene"). Subsequent to the addition of cold trichloroacetic acid (TCA) (final concentration, 5 per cent), 1 ml of a 10 per cent homogenate of mouse liver was added (to serve as a carrier for the minute amounts of cellular material). Finally, the TCA-precipitate was fractionated according to the procedure of Schneider;[13] this permitted the separation of the low-molecular weight pyrimidine-derivatives (acid-

* We are grateful to our colleagues of these laboratories: P. K. Chang, for the synthesis of 6-azauracil-2-^{14}C [18]; R. E. Handschumacher, for a generous supply of 6-azauridine-2-^{14}C; and E. S. Canellakis, for assistance in the enzymic conversion of orotic acid-4-^{14}C to uridine-4-^{14}C and UMP-4-^{14}C.

soluble fraction) from the products of nucleic acid-hydrolysis. The radioactivity of the fractions was measured in a liquid-scintillation counter* and the results (Table 1) are expressed in per cent of the total radioactivity added to the medium.

TABLE 1. INCORPORATION OF ^{14}C-LABELLED NUCLEIC ACID PRECURSORS AND THEIR ANALOGUES INTO SARCOMA-180 IN CULTURE

	Percentage of total counts recovered	
	in acid-soluble fraction	in nucleic acid fraction
Uracil	0·17	0·30
Uridine	1·36	3·64
Uridine-5′-phosphate	1·47	4·11
6-Azauracil	0·03	0·00
6-Azauridine	0·43	0·00

The results given in the table are from a typical experiment which is representative of several others. The data presented were derived from cultures run in parallel, and each of the values is an average of the results obtained from two cultures.

RESULTS

As shown in Table 1, free uracil was incorporated relatively poorly by the sarcoma cells, while uridine and UMP were taken up to a much greater extent, both into the acid-soluble fraction and into the total nucleic acids. AzU was assimilated exceedingly poorly, as indicated by the very small amounts extracted by cold TCA. However, its riboside, AzUR (or derivatives thereof), appeared in the acid-soluble fraction in an amount which was approximately one-third that of the corresponding metabolite, uridine, and to a much higher degree than uracil. On the other hand, neither form of the analogue was incorporated into the fraction containing the nucleic acids.

DISCUSSION

These studies indicate that sarcoma-180 cells have a relatively inefficient system for the utilization of free uracil. Although this utilization is believed to involve the formation of UMP, probably from uridine as an intermediate, this has not been definitely established under these conditions. In any case, the riboside of uracil is utilized efficiently by the cells and the presence of a uridine kinase is strongly indicated. It is suggested that AzUR serves as a relatively good substrate for this kinase, though not as efficiently as does uridine. In fact, the conversion of AzUR to its phosphorylated derivatives has been demonstrated by Handschumacher[14] in bacterial cells and by Pasternak and Handschumacher[15] in lymphoma L-5178-Y. For the relatively inefficient uracil-utilizing system of sarcoma-180, AzU appears to be a very poor substrate; as a result, these tumour cells accumulate this analogue to an insignificant extent. The situation resembles that which was observed by Handschumacher in AzU-resistant

* Aliquots in aqueous solution (in volumes not exceeding 0·5 ml) were added to 20 ml of a mixture of absolute ethanol and toluene (1:2) containing 0·27 per cent 2:5-diphenyloxazole and 0·0033 per cent 1:4-di-(2-(5-phenyloxazole))-benzene, and measurements of radioactivity were made in a liquid scintillation counter (Technical Measurements Company, New Haven). Appropriate internal standards were run as tests of the degree of quenching of light-emission.

strains of *Streptococcus faecalis*, since these cells, although permeable to AzU, were unaffected by it, but their growth was markedly inhibited by AzUR. It was shown that the resistant bacterial cells which were selected by AzU were those which had, in contrast to the parent organisms, a very limited capacity to utilize free uracil-2-^{14}C, but which retained the ability to utilize uridine-2-^{14}C efficiently,[16] and their sensitivity to inhibition by AzUR.

It is of considerable interest that neither AzU nor its riboside was incorporated to a significant degree into the nucleic acids of the cells. Thus, the inhibitory action of AzUR on the reproduction of sarcoma-180 cells in culture (which is readily observed in more prolonged experiments),[8] almost certainly cannot be attributed to the formation of so-called fraudulent nucleic acids. From the recent studies of Handschumacher[14] and Pasternak and Handschumacher,[15] which have demonstrated that azauridine-5′-phosphate (AzUMP) blocks the conversion of orotidine-5′-phosphate to UMP, it appears reasonable to suggest that the mechanism of action of AzUR, mediated by its phosphorylated derivative, is to block one step in the pathway of conversion of orotic acid into the various pyrimidine-containing compounds of the cell. Thus, AzUR may be regarded primarily as a specific inhibitor of the intracellular acquisition of pyrimidines synthesized *de novo*. AzU, which has not been significantly inhibitory to the growth of those cells studied in culture, nevertheless must be converted to ribose-containing derivatives in order for carcinostatic activity to be exerted *in vivo*; such a conversion has been demonstrated to occur in liver tissue.[17] In addition, it has been shown that of AzU-2-^{14}C administered either to man or mouse, from 2 to 5 per cent appears in the urine as the riboside.[17]

A point of ancillary significance is the very slightly, but consistently, higher uptake of isotopic carbon from UMP-2-^{14}C as compared to that of uridine. Studies with doubly labelled compounds (^{32}P as well as ^{14}C) have not been done; accordingly, it is not possible to attribute these findings to assimilation of the intact nucleotide by these sarcoma cells. However, it has also been observed that the inhibitory activity of AzUR on the growth of sarcoma-180 cells in culture is more effectively antagonized by the 5′-phosphates of uridine and cytidine than by the corresponding ribosides, and, in preliminary experiments by Fischer[9] with L-5178-Y cells in culture, AzUMP appears to be significantly more active as an inhibitor of cellular reproduction than is AzUR.

Acknowledgements—We wish to express our gratitude to the Jane Coffin Childs Memorial Fund for Medical Research for a grant which permitted the equipment of a laboratory for tissue-culture studies. These investigations were supported by a grant from the Public Health Service (C-2817). Also, one of us (R.S.) is grateful for post-doctoral fellowship support by the Stiftung für Stipendien auf dem Gebiete der Chemie, Switzerland, and from a Research Training Grant (C-5012) of the Public Health Service. An abstract of the findings described in this paper will appear in the Proceedings of The International Cancer Congress, London, July 6–13, 1958.

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SOME STUDIES WITH ANALOGUES OF KINETIN

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(*Received* 24 *June* 1958)

Abstract—Compounds of similar structure to kinetin (6-furyl-amino-purine) have been studied with regard to their action on animal or human cells in tissue cultures. Of the compounds, two are derivatives of biogenic amines, 6-puryl-tryptamine and 6-puryl-histamine. The first induces disturbances of mitosis of normal and malignant, animal and human cells, while the second compound is cytotoxic against tumour cells, but showed no effect on normal fibroblasts.

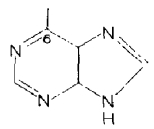
IN 1955, Miller *et al.*[1] described the isolation of a new plant growth factor, kinetin. Kinetin is essential for cell divisions of plant cells in tissue culture in a medium of partly known composition together with β-indolyl acetic acid. Chemically, it proved to be 6-furyl-aminopurine (I).[2]

With a preparation of kinetin, given to us by Skoog in 1955, we could detect no growth-promoting effect on tissue cultures of normal or malignant, animal or human cells or an increase of the number of mitoses. Other investigators have found a growth-promoting effect of kinetin also on animal cells. Orr and McSwain[3] describe a growth stimulation by kinetin in human epithelium and human fibroblasts; and Japanese authors[4] found an increase of the mitotic rate in cells of the Yoshida tumour of the rat *in vivo* under the influence of kinetin. Guttman and Buck[5] likewise confirmed a positive effect on the division of paramaecium caudatum. On the other hand neither we[6] nor Biesele[7] have seen any growth-stimulating effect of kinetin in repeated experiments with tissue cultures or with animal tumours *in vivo*. These negative results cannot exclude that a factor like kinetin is of importance for the induction of mitosis of animal cells. The tissue cultures and the tumours (the cells or their environment) may contain already an optimal quantity of such a factor so that a further addition is without effect. For a decisive test cells must be grown in a medium free from kinetin; and until this is done the question whether kinetin is an essential growth factor for animal cells as well as for plant cells remains open.

This paper is concerned with the action of compounds having a structure similar to kinetin and sixty N6-substituted adenine derivatives have been tested. The results with 6-puryl derivatives of tryptamine and histamine will be reported in detail.

Most of the compounds which are closely related in chemical structure to kinetin, like kinetin itself, show no perceptible effect on animal and human cells *in vitro*. Examples are such compounds in which the furan group is substituted by the phenyl-, thienyl-, or -pyridyl group (II, $n = 1$, III, IV). Skinner and Shive[8,9,10] found an inhibitory effect of the 6-phenyl-alkyl-aminopurines (II, $n = 1$–5, 7) in the regeneration of amputated tentacles of hydra which increases with the length of the alkyl

side chain. We could perceive no effect on animal and human cells *in vitro* with the substances kindly given to us by Dr. Skinner. In addition, we prepared the 6-cetyl-amino-purine (V)[11] which was also inactive.



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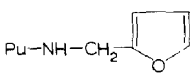

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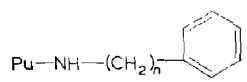
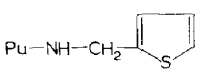


II $n = 1\text{–}5, 7$



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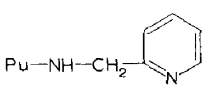

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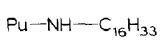

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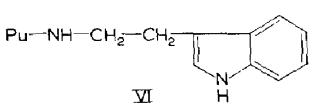

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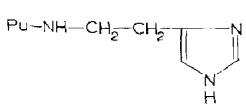

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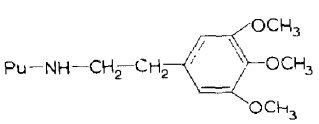
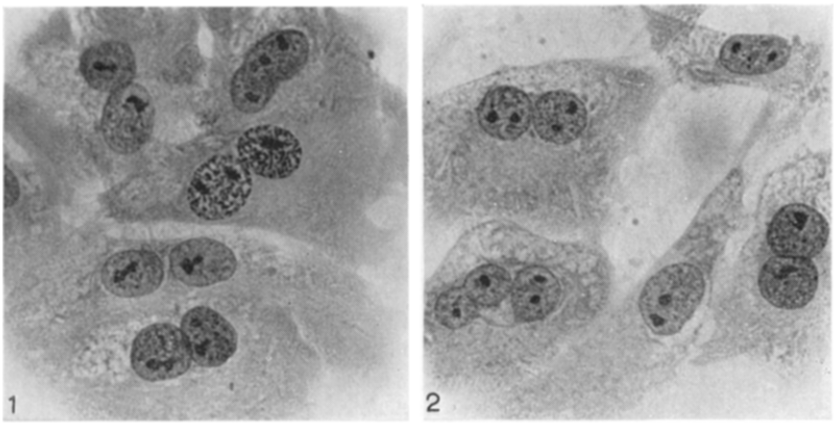


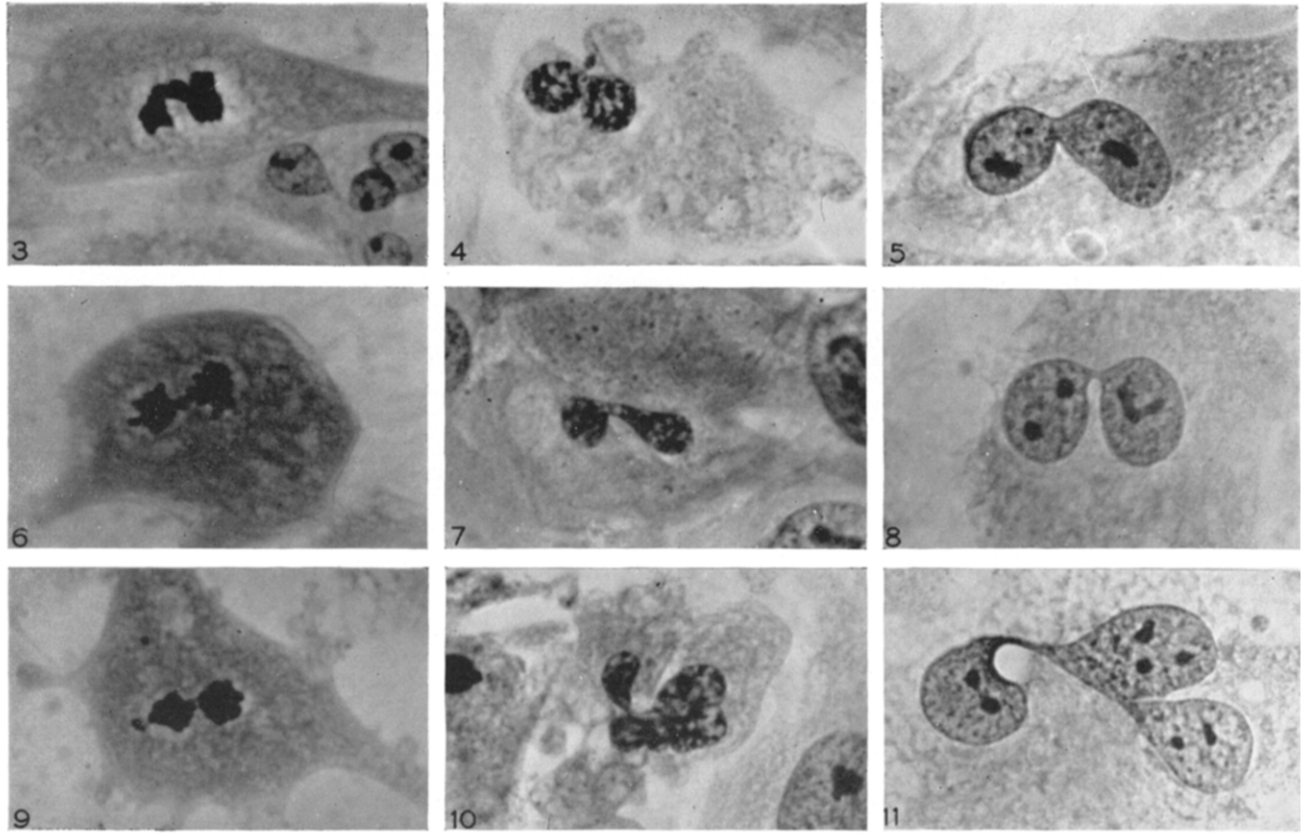
VIII

The first compound which proved to be effective was the 6-puryl tryptamine or 6-N-β-indolyl-ethyl adenine (VI).[6] This compound produces disturbances of mitosis in normal as well as in malignant cells. There is a delay in the separation of the chromosome sets and an inhibitory effect on the plasma division, resulting in numerous cells with two nuclei (see Figs. 1–2). If the chromosome sets are not completely separated there takes place all the same a reconstruction of resting nuclei but the junction between the reconstructing nuclei gives the impression of amitotic nuclear divisions (see Figs. 3–11). In various tumours examined, manifold modifications of the process of mitosis can be seen, which consist of the scattering of chromosomes, in the development of the so called "metaphase a trois groupes", and in other disturbances (see Figs. 12–13). The activity of the compound resembles in its mode of action that of adenine, examined by Hughes,[12] who observed a similar effect on fibroblasts with a 400 times larger dose (calculated on a molar basis). If we combine this compound



FIGS. 1 and 2. Chicken fibroblasts, 45 hr under the action of 6-puryl-tryptamine (25 μg/ml). Bi- and tri-nucleated cells. Bouin's fixation. Hemalum (Mayer). 500×

facing p. 138

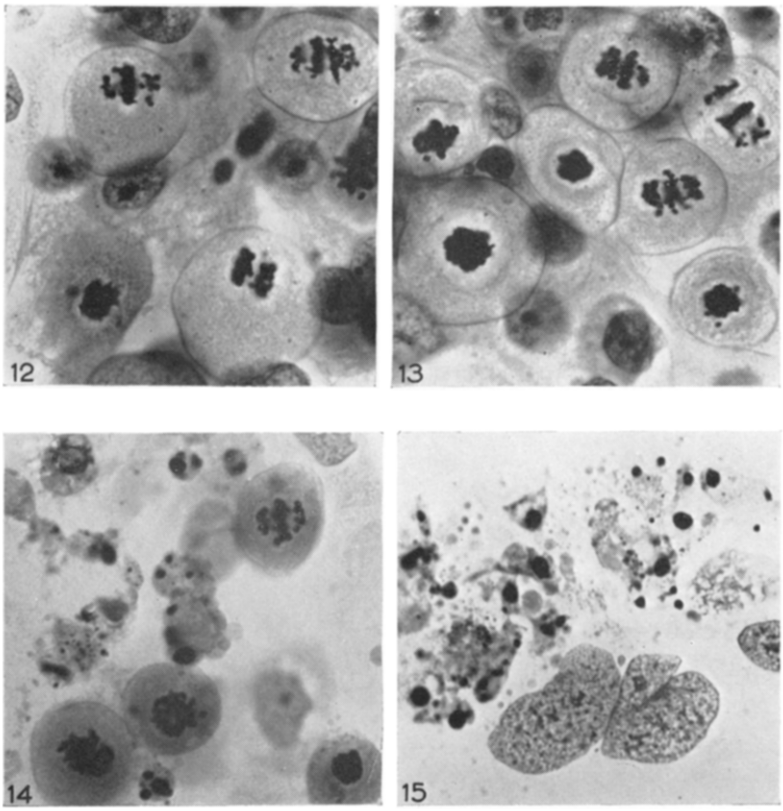


FIGS. 3–11. Chicken fibroblasts, 24–48 hr under the action of 6-puryl-tryptamine (25 μg/ml Bouin's fixation. Hemalum (Mayer). 1000×

FIGS. 3, 6, 9. Incomplete separation of the chromosome sets.

FIGS. 4, 7, 10. Beginning of reconstruction (pseudo-amitosis).

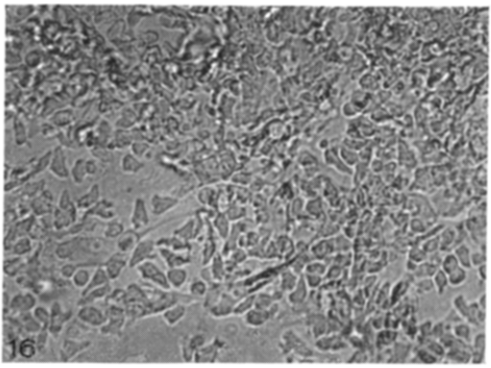
FIGS. 5, 8, 11. Completere construction and enlargement of the nuclei (seemingly amitosis).

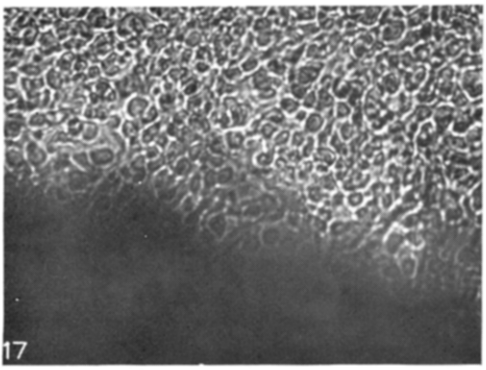


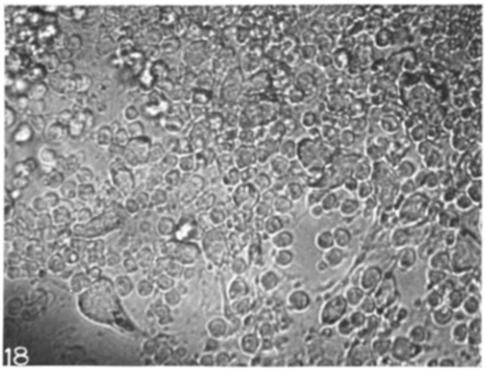
FIGS. 12–15. Tumour cells (HeLa-strain). Bouin's fixation. Hemalum (Mayer). 500×

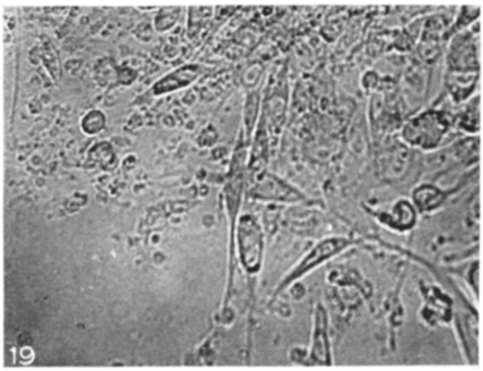
FIGS. 12, 13. Twenty-four hours after addition of 6-puryl-tryptamine (25 μg/ml).

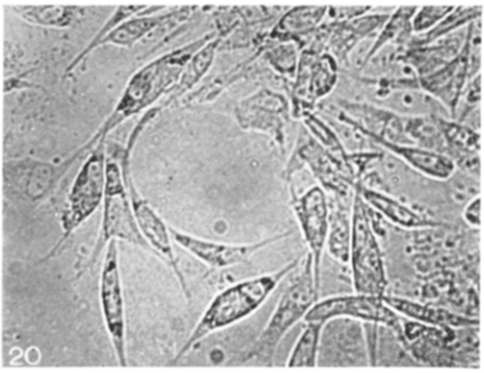
FIGS. 14, 15. Forty-eight hours after addition of 6-puryl-histamine (200 μg/ml).











FIGS. 16–20. Living cells of the Yoshida sarcoma of the rat, grown on a carpet of normal rat fibroblasts in a Carrel flask. A series of five images taken from a time lapse picture. 200 ·

FIG. 16. Untreated culture. The rat fibroblasts are covered by a dense layer of the rapidly moving ameboid tumour cells.

FIG. 17. Three minutes following the addition of some crystals of 6-puryl histamine, located in the lower part of the picture. Increasing by the tumour cells stop any further movement.

FIG. 18. Three hours later. The crystals are almost completely dissolved (see slight shadow to the lower left). The degeneration of the sarcoma cells continues while the normal rat fibroblasts gradually become visible.

FIG. 19. Twenty-four hours later. Normal rat fibroblasts start moving through the field. The tumour cells show complete disintegration.

FIG. 20. Forty-eight hours later. The debris is overgrown by normal rat fibroblasts.

with kinetin, the effect of the indole derivative is not antagonized. It is not yet defined how the action of (VI) can be interpreted from the biochemical point of view.

Searching for other effective compounds we found in the 6-puryl-histamine or 6-β-imidazolyl-ethyl-adenine (VII) another active substance.[13] This substance was reported by Skinner as ineffective in the regeneration test with hydra and it proved to be ineffective also in our hands with normal fibroblasts *in vitro*, even at the highest possible concentration. But it also produces strong cell-destroying effects on tissue cultures of sarcomas and carcinomas (see Figs 14–15), which are already injured by a dose of 100 μg/ml.

The Yoshida rat tumour can only be cultivated *in vitro* as a permanent culture if in the same culture normal fibroblasts are present.[14] These cells by themselves would die out within 10 days, but we have kept them alive now for 5 years in combination with fibroblasts in the tissue culture. When 6-puryl-histamine at 100 μg/ml is added to such a mixed culture, the tumour cells are destroyed and a pure culture of normal fibroblasts remains. Earlier experiments by A. Schleich[15,16] have shown that even a single cell of this tumour multiplies, when explanted in a culture of rat fibroblasts, and that after some time the cells of the Yoshida tumour overcrowd the culture of fibroblasts. From the fact that the mixed cultures of fibroblasts and Yoshida tumour cells after treatment with 6-puryl-histamine prove free from tumour cells after further cultivation, one can conclude that all tumour cells have been destroyed. This phenomenon has also been studied by motion pictures* and some frames from this film are shown in Figs. 16–20.

Not all biogenic amines acquire activity on substitution with the puryl group, e.g. the 6-puryl derivatives of phenyl ethyl amine (II, $n = 2$) or mescaline (VIII) had no effect in tissue culture.

Biesele [17, 18] has found, with purine itself and with 2:6-diamino-purine a stronger effect on sarcoma cells than on normal fibroblasts. With 6-puryl-histamine one can see a further increase in the selective effect on tumour cells in comparison with normal fibroblasts. The selective action of this compound can only be demonstrated in tissue culture; but these results are sufficiently encouraging to warrant further studies.

MATERIAL AND METHODS

Substances. The compounds were prepared by condensation of 6-methylmercapto purine[19] or 6-chloro purine[11] with the corresponding amines.

Compound I was received from Dr. F. Skoog, Madison, Wisc.; another sample was prepared here by Dr. H. Endo.

II, $n = 3$, 4, 5 and 7, and IV were received from Dr. Skinner, Austin, Texas.

II, $n = 1$ and 2, III and VI were prepared here by Dr. H. Endo.

V, VII and VIII were prepared here by Diplomchemiker H. Ballweg.

m.p. = melting point.

V: m.p. 152–53° (154–55° [11]).

VI: m.p. 241°.

VII: m.p. 256–57° (256° [10]).

VIII: m.p. 202°.

Tissue cultures. The methods generally used here have been described earlier[20,21]. To the medium of hanging drop cultures the compounds are added, either in solution

* This film has been demonstrated in March and April 1958 in eight places in USA.

or in solid form. With fibroblasts, the addition is done at the time of explantation, with tumours 2 days after the explantation in hanging drop cultures. The cells of the Yoshida tumour of the rat are cultivated together with rat fibroblastes in Carrel flasks.

In untreated cultures of chicken fibroblasts, we have found an average percentage of mitoses after 24 hr of 3·85 and after 48 hr of 1·92 (values from 300,000 cells from 300 cultures for each time). With the addition of 6-puryl histamine VII (100 μg/ml) the percentage of mitoses was 3·2 after 24 hr (controls 3·4) and 1·6 after 48 hr (controls 1·7). Under the action of 6-puryl tryptamine VI (25 μg/ml) the percentage of mitotic figures was 10·0 besides 10·8 per cent of bi- or poly-nucleated cells after 24 hr (controls 3·6 per cent mitoses). After 48 hr the values were 6·7 per cent mitoses and 22·0 per cent of bi- or poly-nucleated cells (controls 2·5 per cent mitoses) and after 72 hr 3·0 per cent mitoses and 29·9 per cent of bi- or poly-nucleated cells (controls 0·6 per cent mitoses).

Acknowledgements—We have to thank Mrs. Lydia Döring, Mr. August Mayer, Mrs. Ingeborg Martinius, Mrs. Eva Milewski and Dr. Anna Suchetzky for their valuable technical assistance.

We like to express again our thanks to Dr. F. Skoog, Madison, Wisc., and to Dr. Skinner, Austin, Texas, for placing some compounds at our disposal.

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HEPATIC PHOSPHORYLASE AND EPINEPHRINE HYPERGLYCAEMIA*

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(*Received* 10 *July* 1958)

Abstract—The activities of various hepatic enzymes of hexose metabolism were studied in normal rats. The activities and their standard errors, expressed in micromoles of substrate reacting per minute per gramme wet-weight of liver at 30°, were found to be: glucose-6-phosphatase, 6·5 (± 0·34); fructose-1:6-diphosphatase, 5·0 (± 0·29); glucose-6-phosphate dehydrogenase, 1·1 (± 0·14); 6-phosphogluconic dehydrogenase, 2·6 (±0·44); fructokinase, 4·5 (± 0·62); phosphorylase (towards glycogen), 12·3; phosphorylase (towards glucose-1-phosphate), 4·4. The high relative activity of phosphorylase, as well as its reversibility, raised serious questions as to the role of phosphorylase activation in epinephrine-induced hyperglycaemia. Furthermore, no alteration in the activity of this enzyme was detected when hyperglycaemia was prevented by administration of dihydroergotamine, or was produced by injection of epinephrine. These findings suggested that epinephrine administration must bring about, in addition to phosphorylase activation, events which alter the equilibrium conditions to those greatly favouring glucose formation or which activate an alternative pathway for glycogen breakdown.

INTRODUCTION

OVER the past decade Sutherland and his colleagues have reported a series of investigations which have shed considerable light on the biochemical mechanisms involved in epinephrine and glucagon hyperglycaemia. In early experiments[2,3] an increase in glucose output was observed when rabbit liver slices were incubated with G-1-P‡ or G-6-P, a finding which suggested that phosphorylase is the rate-limiting enzymatic step in the glycogen to glucose pathway. Addition of epinephrine or H-G (glucagon) to such slices caused increases in intracellular G-1-P and G-6-P concentrations indicating that these hormones increased phosphorylase activity. This hypothesis was confirmed[4] by measuring the phosphorylase activity of liver slices incubated for various periods of time at 37°, since the considerable decrease in the activity of this enzyme which occurred upon incubation could be restored to approximately its original level by further incubation in the presence of epinephrine or H-G.

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† Scholar in Medical Science of the John and Mary Markle Foundation.

‡ The following abbreviations are employed: G-1-P for glucose-1-phosphate, G-6-P for glucose-6-phosphate, 6-PG for 6-phosphogluconate, FDP for fructose-1:6-diphosphate, TPN for triphosphopyridine nucleotide, DHE for dihydroergotamine, DNP for 2:4-dinitrophenol, G-6-Pase for glucose 6-phosphatase, G-6-P dehydrogenase for glucose-6-phosphate dehydrogenase, 6-PG dehydrogenase for 6-phosphogluconic dehydrogenase, FDPase for fructose 1, 6-diphosphatase, H-G for glucagon, and μmole for micromole.

These observations were further clarified by the demonstration and purification of two enzymes, which specifically inactivated and reactivated liver phosphorylase by respectively removing and adding a phosphate group. Thus, it appeared that the phosphorylase activity measurable at any given time was the resultant of the action of these opposingenzymes.[5-7] Very recently Sutherland and his colleagues[8] have shown that the addition of pharmacological quantities of either epinephrine or glucagon to the particulate fraction of a liver homogenate induces formation of a unique adenine-containing dinucleotide which, if added to the supernatant fraction, causes reactivation of inactive phosphorylase. This new nucleotide is apparently identical with a cyclic dinucleotide synthesized and described by Markham[9]*. This finding, obviously a discovery of the first magnitude, indicates that phosphorylase activation by epinephrine is not a direct effect but rather one which is secondary to the synthesis of this nucleotide.

This report is concerned with the relative activities of the various potentially rate-limiting enzymes of hepatic hexose metabolism in the rat and raises a number of questions regarding the role of phosphorylase activation in epinephrine hyperglycaemia.

MATERIALS AND METHODS

Materials

Dipotassium G-1-P, barium G-6-P and barium FDP were purchased from the Schwarz Laboratories. Barium 6-PG was purchased from the Sigma Chemical Company. The barium salts were converted to potassium salts by treatment with potassium sulphate. Glycogen was purchased from the Pfanstiehl Laboratories, TPN from the Pabst Laboratories, epinephrine from Parke Davis & Company and dihydroergotamine methanesulphonate from the Sandoz Company. The other materials used were of the highest purity available commercially. All water used was doubly distilled, the second distillation being carried out in an all-glass still.

Normal rats used in these studies were females of the Holtzman strain weighing 150–250 g. They were fed laboratory chow *ad libitum*.

Determinations

Blood sugar concentrations were determined using tail vein blood by the anthrone procedure[10] after deproteinization with zinc sulphate and barium hydroxide.[11] Orthophosphate was measured by the Lowry-Lopez[12] and King modifications[13] of the Fiske-Subbarow procedure. TPN reduction was measured at 340 mμ in a Beckman DU-spectrophotometer with a photomultiplier attachment.

Preparation of liver fractions

Animals were anaesthetized with pentobarbital sodium (40 mg/kg) intraperitoneally. Thirty minutes later they were decapitated, their livers were removed, blotted dry on filter paper, and divided into two weighed portions. All subsequent operations were performed at ice-bath temperatures and all homogenizations were done with Potter-Elvehjem homogenizers. A portion of liver was immediately homogenized in a

* Recently, molecular weight determination have shown this proposed structure to be erroneous, (Sutherland, E. W. and T. W. Rall, *J. Biol. Chem.* **232,** 1077, 1958). Lipkin, D. *et. al.* now suggest that the true structure is a cyclic mononucleotide, adenosine 3′, 5′-phosphoric acid.

solution containing 0·15 M potassium chloride, 0·01 M sodium fluoride, and 0·001 M adenylic acid and brought to a final dilution of 1:10. This homogenate* was used for assays of phosphorylase activity in both directions. The remainder of the liver was homogenized in and brought to a 1:5 dilution with 0·15 M potassium chloride. A portion of this homogenate was used for the G-6-Pase assays and the remainder was centrifuged at 18,000 *g* for 30 min at 0°. The precipitate was homogenized and again centrifuged as described, after which the supernatant fluids were combined and brought to a 1:10 dilution (1 g of liver/10 ml) with 0·15 M potassium chloride. This was employed in the assays of G-6-P dehydrogenase, 6-PG dehydrogenase, FDPase, and fructokinase. Centrifugations were performed in an International refrigerated centrifuge, model PR-2, with a high-speed attachment.

Enzyme assay procedures

In order to make comparisons possible all enzymatic activities are expressed as μmoles of substrate disappearance or product formation per minute per gramme wet weight of liver or per 100 grammes of animal under optimal conditions at 30°.

With the exception of the dehydrogenase assays, the enzymatic activities were determined in duplicate and at two enzyme concentrations equivalent to 20 and 40 mg of liver (e.g. 0·1 and 0·2 ml of a 1:5 homogenate). G-6-P and 6-PG dehydrogenases were measured using the equivalent of 10 mg of liver. In each assay appropriate blanks and controls were included to enable corrections for zero time conditions and non-specific enzyme action. Phosphorylase and G-6-Pase assays were performed immediately after homogenization because of the relative instability of these enzymes.

Assays

G-6-Pase[14], phosphorylase-toward glycogen[15], FDPase[16], G-6-P dehydrogenase, 6-PG dehydrogenase,[17] and fructokinase[18] were assayed with minor modifications, according to procedures described by previous workers.

Phosphorylase-toward G-1-P

The reaction mixture contained 1 per cent glycogen; potassium phosphate buffer, 0·05 M, pH 6·5; sodium citrate buffer, 0·03 M, pH 6·5; sodium fluoride, 0·01 M; potassium chloride, 0·03 M, and whole liver homogenate in a final volume of 1 ml. The reaction was started by addition of liver homogenate and stopped with heat after 30 min incubation. The tubes were chilled and inorganic orthophosphate was precipitated by the addition of magnesia mixture after which G-1-P was determined as orthophosphate liberated by boiling with 1 M hydrochloric acid for 7 min.

RESULTS

Relative activities of enzymes of hexose metabolism

Tables 1 and 2 present the activities of a number of hepatic enzymes of hexose metabolism determined in our laboratory. Certain of these enzymes (individually or in various combinations) have been measured elsewhere under comparable conditions

* When reaction mixtures containing this homogenate were incubated for 30 min at 30° prior to the addition of G-1-P (substrate), no change in the activity of phosphorylase occurred, a finding which indicates that this enzyme is not inactivated during the period of assay. Also, maintenance of this homogenate in the absence of the other components of the reaction mixture for 60 min at 2° caused no loss of activity, whereas incubation at 30° for 30 min resulted in nearly complete inactivation of the enzyme.

TABLE 1. ACTIVITIES OF HEPATIC G-6-PASE, FDPASE, G-6-P DEHYDROGENASE, 6-PG DEHYDROGENASE, AND FRUCTOKINASE IN NORMAL RATS*

Enzyme	No. of rats	Specific activity† (per g wet-weight of liver)	Specific activity† (per 100 g of rat)
		Mean ± *S.E.*	*Mean* ± *S.E.*
G-6-Pase	17	6·5 ± 0·34	21·5 ± 1·73
FDPase	21	5·0 ± 0·29	15·3 ± 0·90
G-6-P dehydro.	10	1·1 ± 0·14	3·5 ± 0·46
6-PG dehydro.	5	2·6 ± 0·44	10·6 ± 1·93
Fructokinase	7	4·5 ± 0·62	14·9 ± 2·36

* Normal rats listed in Tables 3–5 are not included in this Table. Rats used were females of the Holtzman strain weighing 150–200 g.

† Expressed in μmoles of substrate reacting per minute at 30°. In 17 animals the assays for G-6-Pase, FDPase, and phosphorylase in both directions were performed on the same livers. In other cases, only one or two enzymes were assayed per liver. The values obtained in these assays did not differ signifi-cantly from those obtained when all enzymatic activities were assayed.

TABLE 2. HEPATIC PHOSPHORYLASE ACTIVITIES IN NORMAL RATS*

Direction of phosphorylase	No. of rats	Specific activity† (using 20 mg of liver per assay tube)	Specific activity† (using 40 mg of liver per assay tube)	Extrapolated‡ specific activity	Activity (expressed in μmoles/min per 100 g of rat§)
		Mean ± *S.E.*	*Mean* ± *S.E.*		
Glycogen	44	9·8 ± 0·37	7·3 ± 0·25	12·3	37·8
G-1-P	34	3·3 ± 0·17	2·2 ± 0·14	4·4	13·1

* Normal rats listed in Tables 3–5 are not included in this table. Rats used were females of the Holtzman strain weighing 150–200 g.

† Expressed in μmoles of substrate reacting per minute per gram wet-weight of liver at 30°.

‡ Mean specific activity at two enzyme concentrations extrapolated to zero-concentration.

§ Calculated from extrapolated specific activities.

and the activities reported are in good agreement with the above.[17,19–24] These activities represent the maximal rates attained under optimal conditions of pH, substrate concentration, etc., and offer little information on the actual velocities at any given moment under steady-state conditions *in vivo*. For example, these measurements do not take into account the Michaelis constants of these enzymes. Small changes in substrate concentration in the Michaelis range could cause large changes in reaction velocities. However, it seems more than coincidence that these maximal activities are of a similar order of magnitude, particularly those enzymes which function in the direction of glucose formation. A similar pattern of activities was observed in other species studied in smaller numbers, i.e. cat, dog and rabbit.

Under the assay conditions employed, all the enzymatic reactions studied except phosphorylase obey zero order kinetics. Phosphorylase (Table 2) is a readily reversible enzyme and its apparent rate decreases as the reactions proceed. The presence of fluoride in the reaction mixture prevents both inactivation of phosphorylase[6] and depletion of G-1-P (substrate or product depending upon the direction measured) by phosphoglucomutase action. Phosphorylase, therefore, follows "pseudo" first-order kinetics[15] and must receive special consideration if its activity is to be compared with those of the other enzymes. Under the conditions used in these studies, increasing the

amount of enzyme decreases the apparent specific activity of phosphorylase. Similarly, if this measurement is made over varying time intervals at a uniform enzyme concentration, decreasing values are obtained. When the enzyme concentration is varied over an eight-fold range, the apparent specific activity decreases in approximate linearity with increasing amounts of enzyme. Also, when the enzyme concentration is constant and the assay time varied, the apparent specific activity decreases in approximate linearity with time. However, these conditions may not hold in the intact animal in which, for example, after formation, G-1-P might be converted rapidly to G-6-P by phosphoglucomutase action. Under equilibrium conditions strongly favouring glycogen breakdown and glucose formation, the steady-state velocity of phosphorylase in the direction of G-1-P might approach the initial velocity. Therefore, the most valid measurement for comparative purposes is the specific activity determined at zero time or zero enzyme concentration. If one extrapolates to zero the mean specific activities measured at two concentrations of enzyme (Table 2), the specific activity in μmoles/min per g wet-weight of liver of phosphorylase becomes 12·3 in the direction of glycogen and 4·4 in the direction of G-1-P.*

Since G-6-Pase is very labile, measurements were made with homogenates rather than with isolated microsomes, a circumstance which increased the error from non-specific phosphatase action. When phenyl phosphate replaced G-6-P as the substrate, orthophosphate was liberated at rates of 1·5 to 2·0 μmoles/min per g wet-weight of liver.† Although one cannot compare these phosphate esters directly as substrates for non-specific phosphatases, these observations suggested that the mean observed specific activity of G-6-Pase should be corrected downward to approximately 4·5 to 5·5 μmoles/min per g wet-weight of liver. This value is in substantial agreement with those reported elsewhere[19,20,22,24]. On the other hand, phosphatase action occurring during the measurement of phosphorylase activity in the direction of G-1-P would destroy some of the product of the reaction and result in an observed specific activity lower than the true value.

Although measurements of phosphoglucomutase and phosphohexoseisomerase activities are omitted from these studies, work done in other laboratories indicates that their activities are much greater than those of the enzymes examined.[22,25]

The above studies were done on anaesthetized rats and it was assumed that phosphorylase activation due to intrinsic epinephrine release did not occur. However, this possibility did exist and the following studies were performed to assay enzymatic activities when epinephrine hyperglycaemia was either induced or prevented.

Influence of method of sacrifice on blood sugar concentration

The effect of the method of sacrifice upon the blood sugar concentration was examined in a series of preliminary studies (unpublished observations in this laboratory by L. Rhein). Decapitation of unanaesthetized animals almost invariably caused an immediate increase in the blood sugar concentration. While anaesthetization 30 min before decapitation decreased the incidence of hyperglycaemia, many animals still

* If one assumes a hypothetical first order reaction and extrapolates to zero the mean specific activities determined over different time intervals, a closer approach to linearity is obtained with semilogarithmic graphing. If the data of Table 2 are plotted in this fashion, slightly higher figures are obtained (13·3 and 4·8 μmoles/min per g wet-weight of liver).

† Since FDPase assays are performed with homogenates from which most cellular particles have been removed by high-speed centrifugation, the error due to non-specific phosphatase action is much less.

displayed this phenomenon. If a lobe of liver was removed under deep pentobarbital anaesthesia, hyperglycaemia still occurred in a few animals. However, the intravenous administration of 5·6 mg of DHE per kg, prior to removal of the liver in anaesthetized rats, prevented hyperglycaemia in all animals studied.

TABLE 3. EFFECT OF EPINEPHRINE AND DHE ON G-6-PASE AND PHOSPHORYLASE ACTIVITIES

Group	No. of rats	Blood sugar conc. (in mg per cent)		Enzymatic activities		
		10 min	70 min	G-6-Pase	Phosphorylase	
					Glycogen formation	G-1-P formation
1. Control	6	108 ± 9·9	103 ± 7·0	5·5 ± 0·5	9·9 ± 0·8	3·8 ± 0·4
2. DHE-treated	6	98 ± 3·2	90 ± 5·5	5·4 ± 0·9	10·8 ± 0·7	3·9 ± 0·3
3. Epinephrine-treated	6	102 ± 5·7	155 ± 12·5	5·9 ± 0·9	10·5 ± 0·6	3·8 ± 0·2
4. Epinephrine- and DHE-treated	6	102 ± 9·0	102 ± 16·0	5·6 ± 0·4	9·3 ± 0·9	3·9 ± 0·4

Rats anaesthetized at zero-time (Pentobarbital Sodium, 40 mg/kg, intraperitoneally). In the groups given drugs, the DHE (5·6 mg/kg, intravenously) was given at 10 min and the epinephrine (0·1 mg/kg, intraperitoneally) at 40 min. Tail vein blood samples were withdrawn at 10 and 70 min (just prior to liver removal). Enzymatic activities are expressed in μmoles of substrate reacting per minute per g wet-weight of liver at 30°. Four rats (one from each group) were run simultaneously in each experiment. All figures given represent mean ± the standard error of the mean.

Effect of DHE and epinephrine on hepatic enzymatic activities

To investigate the action of epinephrine and DHE on liver phosphorylase, four groups of rats were treated as described in Table 3. No significant changes were observed in blood sugar concentrations after 70 min of pentobarbital anaesthesia except in the animals (group 3) treated with epinephrine alone. Administration of DHE prior to epinephrine (group 4) blocked the hyperglycaemic response to this hormone. No significant changes in the activities of G-6-Pase or phosphorylase were seen in these groups.

Effect of removal of hepatic lobes on phosphorylase activity and blood sugar concentration

It was possible that the trauma due to the removal of the livers (Table 3) caused a release of epinephrine or a sympathetic discharge which overcame the DHE blockade and caused an activation of inactive phosphorylase. The experiments described in Table 4 were performed to check this possibility. After administration of DHE, two lobes were removed at different time intervals and blood sugar concentrations were determined before and after the removal of each lobe. The phosphorylase and G-6-Pase activities of each lobe were measured. No changes were observed in the enzymatic activities of the two lobes and, more importantly, blood sugar concentrations remained the same or decreased after the lobectomies, indicating that any epinephrine release induced by trauma had not resulted in escape from the DHE blockade. In control experiments in which DHE was omitted, hyperglycaemia usually occurred after removal of the first hepatic lobe, a finding which indicated that blood sugar increases could still occur despite the decrease in liver mass. Also, epinephrine-induced hyperglycaemia still occurred after lobectomy (Table 5).

TABLE 4. EFFECT OF REMOVAL OF HEPATIC LOBES ON PHOSPHORYLASE ACTIVITY, G-6-PASE ACTIVITY, AND BLOOD SUGAR CONCENTRATIONS AFTER THE ADMINISTRATION OF DHE*†

Rat #	Blood sugar (10 min mg %)	Blood sugar (50 min mg %)	First lobe (60 min)			Blood sugar (70 min mg %)	Second lobe (80 min)			Blood sugar (90 min mg %)	Blood sugar (110 min mg %)
			G-6-P ase	Phosphorylase			G-6-P ase	Phosphorylase			
				Glycogen	G-1-P			Glycogen	G-1-P		
1	115	92	6·5	8·2	2·2	93	6·5	6·6	2·3	69	77
2	106	106	7·7	9·8	3·3	100	6·7	10·6	3·1	94	88
3	121	102	5·2	6·7	3·4	104	5·6	6·8	2·3	94	94
4	140	112	5·1	6·7	3·3	88	5·4	5·7	2·5	92	86
5	138	116	7·1	7·4	3·6	100	6·0	8·2	3·5	82	66
6	138	114	7·0	9·5	3·5	88	5·0	11·7	4·3	animal	died
Mean (± S.E.)	126 (± 6·0)	107 (± 3·8)	6·4 (± 0·5)	8·0 (± 0·6)	3·2 (± 0·2)	95 (± 2·9)	5·8 (± 0·3)	8·2 (± 1·1)	3·0 (± 0·3)	86 (± 5·5)	82 (± 5·0)

* Rats were anaesthetized at zero-time (Pentobarbital sodium, 40 mg/kg, intraperitoneally). DHE (5·6 mg/kg intravenously) was given at 10 min and hepatic lobes removed at 60 and 80 min. Tail vein blood samples were withdrawn at 10, 50, 70, 90, and one hundred and ten min.

† Enzymatic activities are expressed in μmoles of substrate reacting per minute per gram wet-weight of liver at 30° For each assay the equivalent of 20 mg of liver were used per reaction tube.

TABLE 5. PHOSPHORYLASE ACTIVITIES AND BLOOD SUGAR CONCENTRATIONS SHORTLY AFTER EPINEPHRINE ADMINISTRATION*

Rat #	Rat (wt g)	Liver lobe (wt g)	Blood sugars (mg. %)				Phosphorylase activity† towards glycogen in hepatic lobe removed at 10 min
			0	8 Min	15 Min	25 Min	
1	235	1·6	124	128	137	148	11·3
2	238	1·3	90	90	97	151	12·0
3	245	1·3	94	109	108	126	9·8
4	234	1·2	90	88	108	136	11·2
5	234	1·2	100	88	109	117	10·2
Mean (± S.E.)			100 (± 6·5)	101 (± 8·0)	112 (± 6·8)	136 (± 6·5)	10·9 (± 0·4)

* Animals were anaesthetized at zero-time (Pentobarbital sodium 40 mg/kg intraperitoneally). Epinephrine (0·1 mg/kg intraperitoneally) was administered at 5 min. Hepatic lobes were removed at 10 min and tail vein blood samples withdrawn at zero, 8, 15, and 25 min.

† Enzymatic activities are expressed in μmoles of substrate reacting per minute per gram wet-weight of liver at 30°. For each assay the equivalent of 20 mg of liver was used per reaction tube.

Early effects of epinephrine on phosphorylase activity

In groups 3 and 4 of Table 3 hepatic lobes were removed 30 min after epinephrine administration. The question arose as to whether an instantaneous increase in phosphorylase activity occurred which had returned to normal by the time these livers were examined. Therefore, experiments were performed in which epinephrine was administered and the livers removed shortly after the onset of hyperglycaemia (Table 5). Two minutes after the intraperitoneal administration of epinephrine marked accelerations in heart and respiratory rates were noted. In hepatic lobes removed 5 min after drug administration, no significant increases in phosphorylase activities were detected, although definite hyperglycaemia occurred in each animal.

G-6-Pase activity after epinephrine administration

Because an increase in G-6-Pase activity would offer an attractive explanation of epinephrine hyperglycaemia, experiments were done in which rats were given varying hyperglycaemic doses of epinephrine, and hepatic G-6-Pase activities were determined at different intervals (3–30 min) after drug administration. In agreement with the results shown in Table 3 and with those of other workers,[19,20,22] the G-6-Pase activities did not differ significantly from those of control animals examined concurrently.

DISCUSSION

The above data raise several questions regarding the function of hepatic phosphorylase activation in epinephrine hyperglycaemia. Firstly, hepatic phosphorylase is a readily reversible enzyme, its maximal activity in the direction of glycogen being 3 times greater than that in the direction of G-1-P (Table 2). An increase *per se* in the activity of such a reversible enzyme should only speed the attainment of an equilibrium determined by the concentration of the reactants and should have no influence on the direction of the equilibrium.* Thus, under equilibrium conditions which favour

* We, as well as other workers, have directed attention to this difficulty earlier.[1,26–30] Recently, Theorell and Bonnichsen demonstrated apparent changes in equilibrium constants due to large increases in enzyme concentrations;[31] however, we have not observed this phenomenon with the varying concentrations of phosphorylase used in this study.

glycogen deposition (as in the post-prandial state), one would expect to observe more rapid glycogen formation after epinephrine administration. However, this does not occur, as shown by Teng *et al.*,[32] who observed that liver slices under strongly glycogenic conditions actually produced less glycogen when epinephrine was added to the incubation medium. Secondly, in normal rats maximal hepatic phosphorylase activity in the direction of G-1-P closely approximates that of G-6-Pase which renders questionable the concept[2,3] that phosphorylase, on the *basis of its activity alone*, is the rate-limiting step in the glycogen to glucose pathway. Furthermore, we are unable to demonstrate any changes in phosphorylase activity either when hyperglycaemia was induced by epinephrine or prevented by DHE administration.

Under our assay conditions the enzymes (Tables 1, 2) involved in glucose biosynthesis in the rat have activities of approximately 5 μmoles/min per g wet-weight of liver. If one considers a normal rat weighing 200 g with a 6 g liver and an extracellular fluid space of 35 ml,[33] the liver of this animal, if functioning at the above activity, could add to the bloodstream 30 μmoles or 5·4 mg of glucose per min. Furthermore, if one assumes instantaneous equilibration throughout the extracellular fluid space and relatively slow distribution to the intracellular fluid space, glucose released into the bloodstream at this rate would cause an increase in the blood sugar concentration of about 15 mg per cent per min. It must be recalled that our assays are performed at 30°, whereas the temperature of the rat is closer to 40°. The enzymatic rates at this higher temperature would be approximately doubled; theoretically this could raise the blood sugar concentration 30 mg per cent per min. In the hyperglycaemia produced by epinephrine or glucagon, rates of increase of blood sugar concentration are approximately 2–5 mg per cent per min (Table 5).[34] Thus, if phosphorylase activity were to decrease by 50 per cent, as in the liver slice experiments of Sutherland *et al.*,[4] there would still be more enzymatic activity available than is actually employed in epinephrine hyperglycaemia.

An explanation is required for the discrepancy between the above findings and the early studies of Sutherland *et al.*[2,3] with regard to the rate-limiting role of phosphorylase in the glycogen to glucose pathway. The above phosphorylase measurements were performed with homogenates or cell fractions prepared in the presence of fluoride and represent maximal enzymatic activities present at a given time, whereas Sutherland's studies employed rabbit liver slices to which such substrates as G-1-P and G-6-P were added. Since it is generally agreed that hexose phosphates penetrate intact cells little if at all, such experiments alone may not provide the basis for firm conclusions. Also, the studies employing rabbit liver slices were performed prior to the discovery of the inactivating enzyme and such precautions as fluoride addition were not taken to prevent phosphorylase inactivation during the course of the experiments. In view of later studies with liver slices,[4] in which phosphorylase activity was observed to fall markedly during incubation, it seems certain that significant inactivation must also have occurred during those experiments in which the apparent rate-limiting function of phosphorylase was observed.[2,3]

The possibility exists that, as a result of the methods of preparing the tissues for assay, the phosphorylase activities reported in this paper are significantly higher than those in the cell. Homogenization in the presence of fluoride might cause a build-up of active phosphorylase by inhibiting the inactivating enzyme while not affecting the reactivating system. Although it is difficult to rule out this possibility conclusively,

several considerations made it seem unlikely. The phosphorylase activity of liver slices lost by incubation at 37° was restored by administration of either epinephrine or glucagon but not by homogenization in the presence of fluoride.[34] It has been shown[35] that exposure of liver slices either to low temperatures or to DNP, 10^{-4} M, prevents the reactivation of phosphorylase by epinephrine. All operations described above, between removal of the liver from the body and the assay procedure, were carried out at ice-bath temperatures. Furthermore, in two animals treated with DHE the hepatic phosphorylase activities (towards glycogen) were determined, and the homogenization of the livers was carried out in a medium containing both fluoride (0·01 M) and DNP (10^{-3} M). The activities, 10·2 and 9·9 μmoles/min per g wet-weight of liver, respectively, were within the normal range recorded above (Table 2).

It seems apparent that other, as yet obscure, events must occur upon epinephrine administration which change the equilibrium conditions to those strongly favouring glucose formation or which activate a second alternative pathway for glycogenolysis. Evidence is already at hand which suggests several possibilities. For example, Leloir and Cardini[36] have recently discovered a new hepatic mechanism for glycogen synthesis; in this reaction uridine diphosphate glucose rather than G-1-P serves as a substrate. Also, alterations in the concentrations of Na^+ and K^+ can exert marked effects on glycogen storage and breakdown.[37] Particularly suggestive in this regard is the rapid liberation of K^+ from the liver after epinephrine administration.[38] Changes in the availability of orthophosphate may profoundly influence the direction of the phosphorylase equilibrium. Addition of Ba^{++} to reaction mixtures promotes glycogen synthesis,[37,39] probably by removing orthophosphate as an insoluble barium salt, thus shifting the equilibrium towards glycogen. Also, the size and physical state of the glycogen molecule can markedly influence phosphorylase activity.[40] Since metabolic changes such as these may be localized to a particular area of the complex architecture of the cell, they might be difficult to detect by gross chemical determinations.

Although the above factors place in question the physiological role of phosphorylase activation, it is difficult to conceive of Nature evolving and retaining so elaborate a mechanism for the control of an enzyme without good reason. However, further investigation will be necessary in order to explain fully this important phenomenon.

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SPECIES, STRAIN AND SEX DIFFERENCES IN METABOLISM OF HEXOBARBITONE, AMIDOPYRINE, ANTIPYRINE AND ANILINE*

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Abstract—Species and strain differences in response to hexobarbitone and presumably to antipyrine, amidopyrine and other drugs can be expressed largely in terms of the activities of the drug-transforming enzymes in microsomes. However, variation in the inherent sensitivity of the central nervous system may also be a factor. The sex difference in the rat in response to hexobarbitone and presumably to other drugs is also a reflection of the activity of the enzyme system in liver microsomes. Sex hormones have a role in regulating sex difference in drug metabolism. The guinea pig and mouse show no sex difference in the metabolism of hexobarbitone. Implications of these findings to drug action and to the development of new drugs are discussed.

IT IS well known that various animal species react differently to the same drug. This variability in drug response makes it difficult to extrapolate results of animal experiments to man. In addition, it creates a serious obstacle to the contruction of new therapeutic agents, since a drug may be active in one or more animal species yet be relatively ineffective clinically. The converse is also possible—a drug inactive in animals may be effective in man, though this possibility is largely a hypothetical one since compounds which are not active in animals are rarely selected for clinical trial.

Some species differences in drug action are qualitative in nature; for example, morphine depresses man, dogs and rats, but stimulates cats, horses and goats. However, most species differences are in duration of drug action, due to variations in rate of drug inactivation§ or in sensitivity of the "receptor site".

In addition to species variability, there are sex differences in drug response. Holck *et al.*[2] reported that female rats anesthetized with certain oxy-barbiturates sleep considerably longer than males, but other species of animals do not show this sex difference. A number of other studies describe sex differences; for example, female

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§ It is known that other drugs are oxidized at variable rates by liver microsomes of various animal species: deamination and demethylation of sympathomimetic drugs;[21] dealkylation of aromatic ethers[22] and demethylation of narcotic analgesics.[23] In addition, the enzymes that reduce chloramphenical[24] and prontosil[25], which are also present in microsomes, have a wide spectrum of activity in various species.

rats show a greater response to red squill[3], strychnine[4], nicotine[2], picrotoxin[5] and sulfanilamide.[6]

The present study was undertaken to determine whether variability in drug metabolism is an important factor in species, strain and sex differences in the duration of action of hexobarbitone. The data show that differences in the response to hexobarbitone are largely a function of the rates of metabolism *in vivo* and that these rates are inversely related to the activity of the hexobarbitone metabolizing enzyme. Variations in rate of metabolism are also described for amidopyrine, antipyrine and aniline.

MATERIALS AND METHODS

Animals used in these studies were rats (Sprague-Dawley and several other inbred strains), guinea pigs (Hybrid), mice (N.I.H., general purpose), rabbits (New Zealand White), and mongrel dogs. Only mature, female animals were used in the studies of species differences. Drugs were administered intravenously to dogs and intraperitoneally to other species, in the following doses: hexobarbitone sodium, 50 mg/kg to dogs and 100 mg/kg to other species; antipyrine, 75 mg/kg to dogs and 50 mg/kg to other species; aniline (free base) 50 mg/kg to all species.

Determination of biologic half-life of Drugs. After waiting one or more hours for approximate diffusion equilibrium between plasma and tissues to be established, blood samples (oxalated) were drawn at various time intervals. The plasma level of each drug declined linearly when plotted against time on semi-log paper. Since negligible amounts of the drugs were excreted in urine, the rates of decline reflected the rates of bio-transformation. The time required for the plasma levels of the drug to decline by one-half was estimated graphically and expressed as the biologic half-life. Since it was not practical to draw successive blood samples from mice, the biologic half-life of drugs in these animals was calculated from the amount of drug that disappeared from the whole body in 30 or 60 minutes. The mice were stunned and then homogenized in a Waring blender with 100 ml of water. The mixture was filtered through gauze and the filtrate analyzed for the various drugs.

Duration of action of hexobarbitone. The duration of action or "sleeping time" of hexobarbitone in rodents was defined as the time between injection and restoration of the righting reflex; in the dog as the time until the animal was able to stand unassisted.

Preparation of tissue samples for enzyme studies. All steps in the preparation of tissue samples were carried out at 0° to 3° C. Animals were stunned or anesthetized, exsanguinated, and the livers immediately removed and homogenized in two volumes of 0·2 M phosphate buffer, pH 7·4, with a Potter-Elvehjem type of glass homogenizer. A liver supernatant fraction, free of unbroken cells, nuclei and mitochondria was prepared by centrifugation of the homogenate at 9,000 × g for 30 minutes in a Servall centrifuge; this supernatant fraction containing the microsomes was used for the enzyme studies.

Measurement of enzyme activity. Two ml. of the supernatant fraction were incubated with 50 μmoles nicotinamide, 75 μmoles $MgCl_2$, 0·1 μmole of TPN, substrate (1 to 3 μmoles hexobarbitone or 4 μmoles amidopyrine) and 0·2 M phosphate buffer, pH 7·4, to make a final volume of 5·0 ml. The reaction mixture was incubated for one hour in a Dubnoff metabolic shaking incubator in an atmosphere of air.

The activity of the hexobarbitone oxidizing enzyme was assayed by measuring

disappearance of substrate while the activity of the enzyme that demethylated amidopyrine was assayed by measuring the resulting product, 4-aminoantipyrine.

Hormone treatment. Female rats were injected subcutaneously with 3·8 mg of testosterone propionate in 0·15 ml sesame oil every other day for seven weeks; male rats were injected intramuscularly with 17 μg of oestradiol benzoate in 0·05 ml sesame oil every other day for the same period. Control rats were given sesame oil.

Chemical methods. Hexobarbitone,[7] 4-aminoantipyrine,[8] antipyrine[9] and aniline[10] were estimated by methods described previously.

The designation $\pm$ signifies the standard deviation of the mean.

RESULTS

Species differences in rates of metabolism

A number of mammalian species varied greatly in their ability to metabolize hexobarbitone, antipyrine and aniline (Table 1). Although mice metabolized these drugs much more rapidly than did the other animals, there was no clear-cut relationship between species size and the rate of bio-transformation.

TABLE 1. SPECIES DIFFERENCE IN METABOLISM OF HEXOBARBITONE, ANTIPYRINE AND ANILINE

Species	Biologic Half-life in Minutes		
	Hexobarbitone	Antipyrine	Aniline
Mouse	19 ± 7 (12)†	11 ± 0·25 (6)†	35 ± 4 (6)†
Rat	140 ± 54 (10)	141 ± 44 (6)	71 ± 1 (3)
Guinea Pig	—	110 ± 27 (5)	45 ± 8 (7)
Rabbit	60 ± 11 (9)	63 ± 10 (7)	35 ± 22 (6)
Dog	260 ± 20 (8)	107 ± 20 (8)	167 ± 66 (6)
Man*	360	600	—

* Biologic half-life of antipyrine calculated from published data;[28] of hexobarbitone from unpublished data (J. J. Burns and E. M. Papper).
† Figures in brackets refer to number of animals.

The question of the relationship between the duration of pharmacologic action of a drug and its rate of disappearance was investigated using hexobarbitone because its period of action is easily determined. There was a striking relationship between "sleeping time" and the biologic half-life (Table 2). For instance, hexobarbitone disappeared so rapidly in mice that the effect of a large dose lasted only a few minutes, while in dogs the drug had a much longer half-life and an action that persisted for hours.

Further studies were made to relate species differences in pharmacologic response to the activity of the hexobarbitone metabolizing enzyme in microsomes.

There was an inverse relationship between the activity of the enzyme system and the duration of drug action; the greater the enzyme activity the shorter was the duration of response (Table 2).

It is particularly noteworthy, however, that while the mice, rats and rabbits recovered the righting reflex at plasma levels of about 60 μg of drug per ml, the effects in dogs and man persisted until the levels had declined to about 20 μg per ml (Table 2). These

TABLE 2. SPECIES DIFFERENCE IN DURATION OF ACTION AND IN METABOLISM OF HEXOBARBITONE. DOSE OF BARBITURATE: 100 MG/KG FOR MOUSE, RABBIT AND RAT, AND 50 MG/KG FOR DOG

Species	Duration of Action	Biologic Half-life	Plasma Level of Hexobarbitone on Awakening	Relative Enzyme Activity
	minutes	minutes	μg/ml	μg/gm/hr.
Mouse (12)†	12±8	19±7	89±31*	598±184
Rabbit (9)	49±12	60±11	57±12	196±28
Rat (10)	90±15	140±54	64±8	134±51
Dog (8)	315±105	260±20	19±4	36±30
Man‡	—	360	20	—

* Micrograms per gram of tissue. Tissue levels are about 50 per cent higher than plasma levels.
† Figures in brackets refer to number of animals.
‡ Unpublished data (J. J. Burns and E. M. Papper).

results suggest that the longer duration of hexobarbitone action in dog and man is due not only to the slower rate of bio-transformation, but also to a greater sensitivity of the central nervous system to the action of the drug.

Species differences were also observed in the demethylation of amidopyrine. Table 3 illustrates the variable capacity of enzymes in liver microsomes to oxidatively demethylate amidopyrine (4-dimethylaminoantipyrine) to 4-amino-antipyrine.[8]

TABLE 3. SPECIES DIFFERENCE IN DEMETHYLATION OF AMIDOPYRINE BY LIVER MICROSOMES

Species	Relative Enzyme Activity
	μg/gm/hr.
Mouse	170±30 (10)*
Rat	58±17 (13)
Guinea Pig	60±7 (6)
Rabbit	39±10 (5)
Dog	29±3 (2)

* Figures in brackets refer to number of experiments

Strain difference

Pronounced variation in the biologic half-life of antipyrine was demonstrated in eight inbred strains of rats. For example, the half-life was 114 ± 28 min in five female rats of the M 520 strain compared to 282 ± 20 min in six female rats of the Buffalo strain.

Sex difference

When male and female rats were given 100 mg of hexobarbitone per kg, the females slept about four times as long as the males. A comparison of the plasma levels showed that the drug was metabolized much more rapidly in males than in females. In accord with this finding, the liver enzyme system of males showed considerably higher

activity than that of females in oxidizing hexobarbitone (Table 4a). Both sexes recovered from the barbiturate at about the same plasma level (about 60 μg/ml), a finding which indicates that variability in the central nervous system sensitivity was not a factor in the sex difference.

TABLE 4a. SEX DIFFERENCE IN DURATION OF ACTION AND IN METABOLISM OF HEXOBARBITONE IN RATS

Sex	Sleeping Time	Plasma level at 60 minutes	Relative Enzyme Activity
	minutes	μg/ml.	μg/gm/hr.
Female	90±15 (10)*	65±8	134±51
Male	22±5 (11)	23±9	682±102

* Figures in brackets refer to number of animals in each series

The observed sex variation prompted an investigation of the influence of the sex hormones. Male rats were given oestradiol and female rats testosterone for a period of seven weeks before administration of a single dose of hexobarbitone. Oestradiol treatment markedly increased the "sleeping time" in males. Correspondingly, it decreased the rate of metabolism of the drug *in vivo* and lowered the capacity of the liver microsomal system to metabolize hexobarbitone (Table 4b). In contrast,

TABLE 4b. INFLUENCE OF OESTRADIOL IN MALE RATS

	Sleeping Time	Plasma level at 60 minutes	Relative Enzyme Activity
	minutes	μg/ml.	μg/gm/hr.
Control	22±5 (11)*	23±9	682±102
Oestradiol	84±22 (9)	62±13	177±33

TABLE 4c. INFLUENCE OF TESTOSTERONE IN FEMALE RATS

	Sleeping Time	Plasma level at 60 minutes	Relative Enzyme Activity
	minutes	μg/ml.	μg/gm/hr.
Control	90±15 (10)*	65±8	134±51
Testosterone	38±17 (14)	37±14	543±123

* Figures in brackets refer to number of animals in each series

testosterone treatment significantly shortened the duration of action in females, increased the rate of drug metabolism *in vivo* and enhanced the capacity of the liver microsomal system to metabolize hexobarbitone (Table 4c).

A sex difference was also demonstrated in the metabolism of antipyrine and amidopyrine in rats. The biologic half-life of antipyrine averaged 141 ± 44 min in six

female rats compared to 70 ± 24 min in eight male rats. Amidopyrine was demethylated by the liver microsomal system of females at the rate of 58 ± 17 μg/gram of tissue per hr (average of 13 animals) compared to 137 ± 50 μg for males (13 animals).

There was no sex difference in the duration of action of hexobarbitone in guinea pigs and mice; furthermore, the hormones did not induce an appreciable change in the disappearance rate of the barbiturate in mice.

Sex difference at various ages

Up to the age of 4 weeks there was no sex difference in the duration of action of hexobarbitone in rats, but at five weeks there was an abrupt decrease in the recovery time of male animals. The maximal difference between the sexes appeared in seven weeks when 100 mg of hexobarbitone per kg elicited a response lasting 22 min in male rats and 66 min in females.

DISCUSSION

Studies presented in this paper give considerable insight into factors that determine species differences in drug action. The wide species variation in response to hexobarbitone reflects in large part the variability in its rate of metabolism *in vivo*. As might be expected, the duration of action of the barbiturate bears an inverse relationship to the activity of the liver microsomal enzyme[5] that inactivate the drug. Accordingly, species differences in duration of hexobarbitone action can be expressed largely in terms of the activity of a microsomal enzyme system. Species differences also extend to the metabolism of other drugs. Thus, the diversity in activities of the enzyme systems in liver microsomes that demethylate amidopyrine and hydroxylate antipyrine account for the wide species variation in their metabolism. Presumably there is a wide species difference in the response to these compounds.

Mice in general metabolize drugs more rapidly than do other animals. This probably explains the relatively low toxicity of many drugs in this species. In man the converse is usually true, though there are a number of exceptions.

The sensitivity of the responding tissue may also be a factor in species difference. Thus, the central nervous system of dog and man is more susceptible than that of rodents to the action of hexobarbitone. Mice recover their righting reflex when about 85 mg of hexobarbitone per kg is still present in the body; whereas a dose of 85 mg/kg is highly toxic to dogs and man.

Differences in the metabolism of antipyrine in several strains of rats suggests that inheritance is an important factor in drug metabolism. Jay[11] reported a three-fold variability in the response to hexobarbitone among several inbred strains of mice, but a remarkably uniform response by individual mice of a given strain. In contrast, he noted that members of a non-inbred strain vary considerably in their response to the drug. Assuming that the differences are a function of drug metabolism it would again suggest the importance of inheritance in drug metabolism. It is not surprising that a heterogenous species like man has considerable biochemical variability. Two revealing examples of this are seen in the metabolism of dicumarol[12] and of ethyl biscoumacetate (Tromexan),[13] which show a ten-fold variation in rates of metabolism in different individuals. The diversity of therapeutic and toxic responses to these anticoagulants as well as to other drugs is probably largely due to individual differences in the activities of the metabolizing enzymes.

It is also apparent that the sex difference in rats in response to hexobarbitone, and presumably to antipyrine and amidopyrine, is due to differences in the activity of the metabolizing enzymes.* Females are not actually more sensitive than males to hexobarbitone, but are merely unable to metabolize it as rapidly. The effect of oestradiol treatment in lowering the hexobarbitone metabolizing enzyme activity of males, and of testosterone in raising that of females, indicates that sex hormones may play an important role in modifying the activities of these enzymes. This is also suggested by the similarity in response of sexes to hexobarbitone until the animals reach the age of about five weeks, after which an abrupt sex difference occurs. Since the change occurs only in the male rats, it suggests that increased production of androgens is the dominant factor in determining the sex difference. Other workers have also reported that the "sleeping time" of immature rats is the same for both sexes after hexobarbitone administration.[2,14]

It is of considerable interest that mice and guinea pigs exhibit no sex difference in the rate of metabolism of hexobarbitone, and that the administration of sex hormones induces no appreciable change. This marked sex difference in the rats, but not in mice and guinea pigs poses an interesting problem in endocrinology.

The fate of a drug in different species may become rather complicated if metabolism proceeds along two or more pathways, since the relative importance of a particular pathway may vary from one species to another. For example, amphetamine can undergo N-demethylation, hydroxylation or deamination *in vivo*.[15,16] Deamination is a major route of metabolism of this drug in rabbits, but this reaction is virtually absent from rats and dogs. In the dog, demethylation is the major pathway and hydroxylation is negligible, while in rats, hydroxylation is an important pathway. Another example of competing reactions is described by Parke and Williams,[17] who suggest that ortho- and para-hydroxylation of aniline may involve different enzymes. These investigators have shown that rabbits form large amounts of the para-, but little of the ortho-derivative. In contrast, cats form large amounts of ortho- and little of the para-derivative. Of particular interest, are pathways which are present in most species, but completely missing in others; for example, dogs cannot acetylate primary amines[18] and cats cannot convert phenols to glucuronides.[19]

The aforementioned considerations show how species variation in drug metabolism complicates the designing of new drugs, since the rate or pathway of metabolism of compounds in man is difficult to predict from animal data. As yet, it is not possible to relate rates and pathways of drug metabolism to chemical structure even in a single animal species. But recent studies indicate that the number of enzyme systems responsible for drug metabolism are probably relatively few in number;[20] and it may become possible to predict the fate of a drug from structural and physical properties. But these predictions would be valid only for an inbred strain and would not apply to the species in general. Far more difficult will be the application of information obtained in animals to problems in human therapy. It will be difficult to correlate the rate of drug metabolism with chemical structure in man until more is known concerning the nature of the various drug enzymes and the extent of individual variability in their activities.

* Other workers have shown a sex difference in the metabolism of drugs by rat liver microsomes. The microsomes of male rates are more active than those of females in demethylating various narcotic analgesics;[23] in contrast, microsomes of female rats are more active in converting the insecticide parathion to the toxic metabolite paraoxon.[26] In addition, Robillard *et al*,[27] have noted that liver slices of male rats metabolize pentobarbital more rapidly than do liver slices of females.

TABLE 5. SEX DIFFERENCE IN THE DURATION OF HEXOBARBITONE ACTION IN RATS AT VARIOUS AGES

Age	Duration of Action in Minutes	
	Females	Males
weeks		
3	66±13 (5)*	67±3 (4)*
4	62±13 (4)	62±20 (4)
5	66±7 (5)	31±3 (5)
7	74±15 (4)	24±3 (4)
9	67±15 (5)	22±4 (6)
14	66±18 (8)	19±6 (8)

* Figures in brackets refer to number of animals.

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STUDIES WITH REVERSIBLE INHIBITORS OF MONOAMINE OXIDASE: HARMALINE AND RELATED COMPOUNDS

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Abstract—Harmaline and related compounds have been shown to be potent inhibitors of monoamine oxidase effecting 50 per cent inhibition of serotonin metabolism at 10^{-6} M. Inhibition is reversible both *in vitro* and *in vivo*. Procedures have been presented for evaluating the effectiveness of monoamine oxidase inhibitors both *in vitro* and *in vivo*.

INHIBITORS of the enzyme monoamine oxidase (MAO) are no longer mere research tools but are finding application in therapeutics, particularly in the psychological and cardiovascular disorders. The compound which has been most widely used in this respect is isopropyl-isonicotinyl hydrazine (iproniazid).[1] As a result of the clinical findings with this compound there has developed a great interest in MAO inhibitors, and many new compounds of this type are being introduced.

The majority of these new MAO inhibitors are hydrazine derivatives, in analogy to iproniazid. There is no doubt that many more potent agents will be found with the hydrazine element acting as the "prosthetic group". In fact, one such compound, β-phenylisopropylhydrazine (PIH), has many times the activity of iproniazid.[2] However, the inhibition produced by the hydrazines is not a competitive and reversible one, and it has been shown that the effects of iproniazid last for many days following the disappearance of the drug from the body.[3] No doubt such long lasting, irreversible effects are in many cases desirable. However, from the experimental as well as the therapeutic standpoint it would be equally desirable to obtain reversible MAO inhibitors with short action *in vivo*. Potent reversible inhibitors have been described;[4] of these, the harmala alkaloids are the most active. This paper describes the actions of one of the harmala alkaloids, harmaline, both *in vitro* and *in vivo*, and presents the procedures which may prove generally useful for the laboratory evaluation of MAO inhibitors.

METHODS

Monoamine oxidase assays. These were carried out by measuring the disappearance of serotonin, as previously reported.[5] Serotonin was assayed spectrophotofluorometrically by the procedure of Bogdanski *et al.*[6] Harmaline is also a fluorescent base

and is extracted together with serotonin. Fortunately, its fluorescent characteristics in 3 *N* HCl are quite different from those of serotonin (activation maximum: serotonin 295 mμ, harmaline 380 mμ; fluorescence maximum: serotonin 550 mμ, harmaline 480 mμ). When 5–10 mg of harmaline were injected into rats the amounts of harmaline in the tissues were not sufficient to interfere with the serotonin assay. When larger amounts of harmaline were used, it was necessary to extract the alkalinized tissues with 15 ml of $CHCl_3$ before carrying out the usual *n*-butanol extraction for serotonin.

Harmaline assay. One ml of tissue homogenate in a glass-stoppered centrifuge tube was made alkaline by addition of 0·1 ml of 20 per cent Na_2CO_3 and 2 ml of 0·5 *M* borate buffer, pH 10. Fifteen ml of $CHCl_3$ were added and the tube was shaken and then centrifuged. Ten ml of the $CHCl_3$ fraction were transferred to another tube containing 2 ml of 0·1 *N* HCl. After agitation and centrifugation the aqueous layer was transferred to a cuvette and its fluorescence measured in the spectrophotofluorometer (activation 380 mμ, fluorescence 480 mμ) and compared to standards carried through the entire extraction procedure. Recoveries of harmaline added to tissues were quantitative. Further details of procedures for assay of harmala alkaloids will be published elsewhere.[7]

RESULTS

Inhibition of MAO in tissue homogenates

The effects of harmaline and related harmala alkaloids (Fig. 1) on MAO of rat liver are shown in Table 1. A comparison is made with iproniazid and PIH. It is apparent

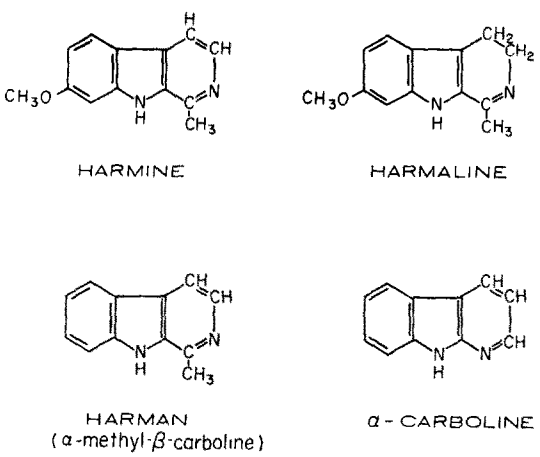

FIG. 1. Harmaline and related compounds.

that the harmala compounds are much more potent than either of the hydrazine compounds. However, direct comparison is rendered difficult in view of the different nature of the inhibitions. Iproniazid must be preincubated with enzyme under oxygen in order to be effective,[8] whereas harmaline, being a competitive inhibitor, needs no such preincubation. Following 20 minutes of preincubation with mitochondria the

TABLE 1. INHIBITION OF MAO IN HOMOGENATES OF RAT LIVER

Compound	Inhibition at 10^{-3} M per cent	Conc. producing 40–60 per cent inhibition
Harmaline	100	10^{-6}
Harmine	100	10^{-6}
1, 2, 3, 4-Tetrahydroharmine	97	10^{-5}
Harman	98	5×10^{-6}
D, L-Tetrahydroharman-3-carboxylic acid	0	—
1-3, 4-Dihydroharman-3-carboxylic acid	21	—
α-Carboline	47	—
β-Phenylisopropylhydrazine	100	5×10^{-6}
Iproniazid	90	3×10^{-4}

Procedure: The incubation mixture contained 1·5 ml. of rat liver homogenate (equivalent to 500 mg. of tissue), 0·5 ml. of 0·05 M phosphate buffer (pH 7·4), 1 mg. of serotonin, inhibitor and water to yield a total volume of 3·5 ml. Incubation was carried out in air at 37° C. for 40 minutes.

inhibition produced by iproniazid could not be reversed by washing the particles with saline. However, the inhibition produced by harmaline was readily reversed by this procedure (Table 2).

TABLE 2. REVERSIBILITY OF HARMALINE INHIBITION

Compound	Per cent inhibition	
	Before washing	After washing
Harmaline 10^{-5} M	92	38
Iproniazid 10^{-3} M	84	79

Procedure: Rat liver mitochondria (equivalent to 10 gm of liver) were incubated with the inhibitor at 37° C for 30 min at pH 7·4. The mitochondria were then washed four times with 5 ml of isotonic KCl. Control omitting inhibitor were incubated and washed in the same manner. Following this the washed mitochondria were made up to the original volume. Mitochondria equivalent to 1·5 gm of liver were then incubated with serotonin as described in Table 1.

Effects of harmaline in vivo

The following experiment was designed to investigate the ability of harmaline to inhibit MAO *in vivo* and to determine the duration of its effects. Harmaline was administered to rats intraperitoneally. At stated intervals several animals were killed. The livers and brains were homogenized and assayed for both MAO activity and harmaline content.

As shown in Fig. 2, MAO inhibition was strong for 2–3 hr and fell to negligible levels within 6 hr. As would be expected for a reversible inhibitor the harmaline levels in the tissues parallelled the degree of inhibition. This is quite different from the

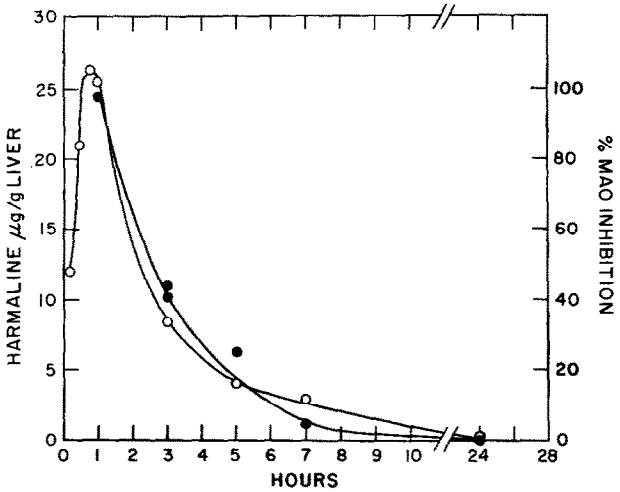

FIG. 2. Relationship between tissue levels of Harmaline and MAO inhibition. Harmaline was administered intraperitoneally (5 mg/kg) into rats and the animals were killed at stated intervals. The livers were removed and homogenized in water (3° C): 1 g of liver plus 2 ml of water. Aliquots were taken for assay of MAO activity as described in Table 1 and for harmaline assay.

○——○ Harmaline; ●——● MAO Inhibition

effects observed with iproniazid where the inhibition persists for many days following the disappearance of the compound from the body.[3]

Effect of harmaline on conversion of serotonin to 5-hydroxyindoleacetic acid (5HIAA) in vivo

One mg of serotonin was injected intraperitoneally into mice (18–20 g); these included controls and animals which had been given harmaline (10 mg/kg) intravenously 10 minutes before the experiment. At the end of one hour the mice were homogenized* and assayed for both serotonin and 5HIAA. It is apparent (Table 3) that

TABLE 3. EFFECT OF HARMALINE ON CONVERSION OF SEROTONIN TO 5HIAA IN THE INTACT MOUSE

Exp.	Interval following serotonin injection	Serotonin metabolized	5HIAA formed
	hr	mg/animal	mg
Control animals	1	1·68	0·23
	2	1·76	0·23
Harmaline animals	1	1·20	0·02
	2	1·60	0·03

Mice were given 15 mg of harmaline per kg intraperitoneally. After one hour these animals and untreated controls were given 2 mg of serotonin intraperitoneally. The animals were killed at stated intervals following the serotonin injection and homogenized in 100 ml of 0·1 N HCl and assayed for serotonin and 5HIAA.

* Urine and faeces produced during the experimental period were included in the homogenization procedure.

although equivalent amounts of serotonin disappeared in both cases the conversion to 5HIAA was markedly inhibited in those animals pretreated with harmaline.

Effect of harmaline on endogenous levels of amines

If a MAO inhibitor is effective it can increase levels of endogenous amines in tissues. The level of brain serotonin is particularly sensitive to the effects of MAO inhibitors, as was previously shown with iproniazid.[9] When harmaline was administered to rats in doses of 5–15 mg/kg the brain serotonin level was found to increase (Fig. 3). The

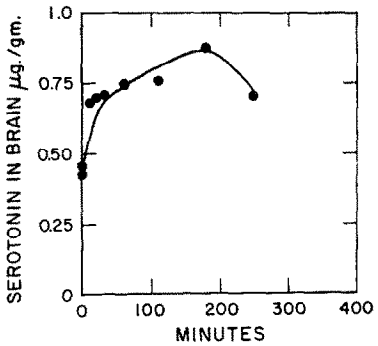

FIG. 3. Effect of Harmaline on Brain Serotonin Levels. Harmaline was administered in doses ranging from 5–15 mg/kg. Animals were killed at stated intervals and the brains were assayed for serotonin. The 0-time control value represents the average obtained on 27 animals. From 10–60 min the values represent the averages obtained with 9 animals. Subsequent figures represent the averages obtained on 6 animals.

magnitude of the increase indicates that harmaline can effectively inhibit the metabolism of serotonin. The rapidity of the increase may be taken as an index of the rapid turnover of serotonin in the brain.[10]

DISCUSSION

If one compares the inhibitors of MAO with those of cholinesterase then harmaline can be compared in its reversible mode of action to physostigmine, and iproniazid to the irreversible inhibitor, di-isopropyl fluorophosphate (DFP). In the laboratory both types of MAO inhibitors will prove useful. However, it should be kept in mind that comparisons of reversible and irreversible agents *in vivo* are not a simple matter. Each agent has its advantages and disadvantages. From a clinical standpoint, long-lasting effects may be desirable. However, we know little about the function of MAO and of the possible clinical consequences of its inactivation for long periods of time. Furthermore, hydrazines will most likely react irreversibly with other biological sites. Experience with cholinesterase inhibitors has shown that the reversible agents such as prostigmine are clinically far safer to use and easier to control.

It is of interest that harmaline, harmine and other harmala alkaloids were used therapeutically as early as 1920, for various disorders for which iproniazid is now suggested.[11] This was before MAO was even known. That harmaline does effectively inhibit MAO in man, in doses which have been used therapeutically, has been shown by Sjoerdsma, Gillespie and Udenfriend.[12] Many of the effects reported here for harmaline have already been observed with harmine, harman and tetrahydronorharman. These and other analogues may prove to be as interesting as harmaline.

The limited number of representatives of carboline compounds, tested hitherto for MAO inhibition, does not offer a simple rationale for the construction of active inhibitors. However, a few guiding principles become discernible at this time: (i) fully aromatic β-carbolines are very active, while α-carboline is relatively inactive; (ii) di- and tetrahydro-β-carbolines are active (the fact that they contain the elements of a tryptamine substituted in the α-indole position and at the nitrogen in the side chain may contribute to their activity as competitive inhibitors); (iii) substitution in the benzene ring or at C(2) in the pyridine ring does not significantly alter the degree of inhibition; and (iv) the α-amino acid "precursors" of tetrahydroharman and of harmaline are inactive.

In these circumstances the re-investigation of the therapeutic possibilities of this class of compounds, utilizing dosages which effectively inhibit MAO, is acquiring new interest. Whether or not the harmala alkaloids turn out to be clinically useful these studies indicate that it is possible to obtain potent reversible MAO inhibitors which function as such *in vivo* both in experimental animals and in man.

The methods used to detect and quantitate the effectiveness of MAO inhibitors presented here are quite simple. They should facilitate the search for new and more active MAO inhibitors. When used in conjunction with the newly devised serotonin metabolism test,[12] inhibitors may be found which will also be therapeutically useful.

Acknowledgments—The activities of several MAO inhibitors were determined by Dr. Masayori Ozaki who is continuing further studies along these lines. Harmaline and harmine were kindly supplied through the courtesy of Dr. F. A. Hochstein, Chas. Pfizer and Co. D:L-Tetrahydroharmon-3-carboxylic acid and 1-3-4-di hydroharman-3-carboxylic acid were put at our disposal by Professor R. Tschesche, Hamburg. Iproniazid was supplied by Hoffman-La Roche, New Jersey, and β-phenylisopropylhydrazine by Lakeside Laboratories, Wisconsin.

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BOOK REVIEWS

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Three sections are clearly and logically defined: Part I, Occurrence and metabolism, Part II, Peripherial actions; Part III, Central actions.

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This book will help many people to penetrate more deeply in the difficulties of the problems opened by HT and to solve them, as usual, one after the other until the story becomes logical and consistent.

Z. M. BACQ

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