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THE PHARMACOLOGY OF BASIC ESTERS OF THIAZOLE CARBOXYLIC ACIDS

BY

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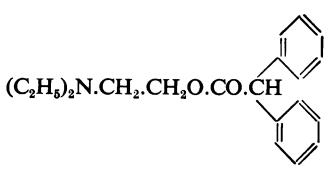
From Glaxo Laboratories, Ltd., Greenford, Middlesex

(Received March 2, 1946)

During an investigation into the pharmacological properties of thiazole compounds, several basic esters of 4-methyl-thiazole-5-carboxylic acid (II) and the diethylaminoethyl esters of 4-methyl-thiazole-5-acetic acid (III,R = CH_3), thiazole-5-acetic acid (III,R = H) and 2-amino-4-methyl-thiazole-5-carboxylic acid (IV) were prepared (Jones, Strachan and Robinson, 1946); these have now been examined pharmacologically. The close chemical relationship between these compounds and Trasentin (I) suggested that they might possess spasmolytic properties, and this has been confirmed *in vitro*. The appearance, moreover, of convulsions produced by the injection of non-toxic doses of these compounds into rabbits, mice, and guinea-pigs revealed stimulant properties on the C.N.S., which have been studied. Finally, the similarity in structure of diethylaminoethyl-2-amino-4-methylthiazole-5-carboxylate (IV) and procaine prompted us to investigate the local anaesthetic properties of this compound.



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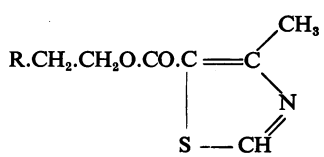

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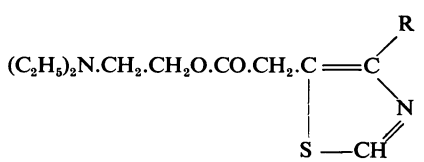

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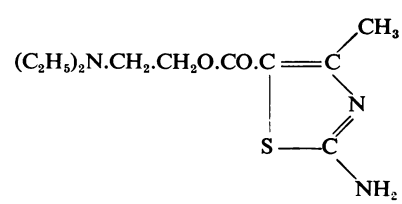

IV

NOTE.—Trasentin-6H is hexahydro-trasentin, i.e., one phenyl group of (I) replaced by cyclohexyl

EXPERIMENTAL

I. *Spasmolytic Properties*

1. *Action on isolated organs.*—The normal rhythm of the isolated ileum of the rabbit in Ringer's solution* was suppressed after a few minutes by a concentration of 1.25 mg./ml. diethylaminoethyl 4-methyl-thiazole-5-carboxylate (II,R = $(C_2H_5)_2N$) and was reduced, but not inhibited, by one-fifth of this concentration. In different experiments the tone fell, rose, or remained the same. Recovery took place within a few minutes after removal of the drug. Trasentin-6H, in a concentration of 2.5 μg./ml., produced no effect, but 12.5 μg./ml., i.e., 1/100 the concentration of the thiazole compound, produced a lowering of the tone with suppression of the rhythm.

The diethylaminoethyl ester, unlike Trasentin, which lowers muscle tone, raised the tone of the isolated uterus of the rabbit and guinea-pig in Ringer's solution. The effect was similar to that of posterior pituitary extract, except that the amplitude of the spontaneous contractions following this rise in tone was increased by the diethylaminoethyl ester, but remained the same with posterior pituitary extract. The increase of tone produced and maintained by 2 mg. of the ester was approximately the same as that produced by 0.05 I.U. posterior pituitary extract or by 60μg. atropine sulphate on guinea-pig uterus.

A concentration of 1.25 mg./ml. of the diethylaminoethyl ester contracted the bladder muscle of the guinea-pig.

2. *Action on isolated organs treated with acetylcholine (Ach).*—Preliminary tests on the Ach spasm of the isolated rabbit ileum in Ringer's solution showed that the esters listed in Table I had a weak spasmolytic action. Their activities were compared by measuring the degree of relaxation induced in a strip of ileum which was responding to a concentration of 0.4 p.p.m. Ach with constant submaximal contractions. The degree of

TABLE I

In vitro ACTIVITY AGAINST ACETYLCHOLINE (4×10^{-7}) IN RABBIT ILEUM

Compound. (10^{-4})	Per cent Relaxation of Contracted Muscle
Piperidinoethyl 4-methylthiazole-5-carboxylate	58
Diethylaminoethyl „ „	53
Dimethylaminoethyl „ „	50
β-Diethylaminopropyl „ „	48
Morpholinoethyl „ „	33
Diethylaminoethyl 2-methylthiazole-4-acetate	22

relaxation, expressed as the percentage reduction of this contraction, was measured for a standard dose of thiazole compound (see Table I). The piperidinoethyl ester of 4-methyl-thiazole-5-carboxylic acid (II,R = $C_5H_{10}N$) produced the maximum relaxation, followed by the dimethylaminoethyl ester (II,R = $(CH_3)_2N$) and, because the former was also the most active musculotropic compound, its spasmolytic activity was assayed against Trasentin by a method which involved "bracketing" doses of test and standard spasmolytic, as in the method of Dale for the assay of posterior pituitary extracts. It was found impossible to treat a single piece of gut with a sufficient number of doses of spasmolytic to make a randomized order feasible; this was due to the effects of Trasentin persisting for long periods and interfering with subsequent doses. Tested by this method the piperidinoethyl ester had approximately 1/1,000 of the activity of Trasentin.

*The bath containing the isolated tissue had a capacity of approximately 40 ml., and this value was used in calculating the concentrations recorded in the text.

A concentration of 0.5 mg./ml. of the diethylaminoethyl ester completely suppressed the contractions produced by a concentration of 0.1 p.p.m. Ach, whilst 1.25 mg./ml. was required to antagonize the effect of 0.4 p.p.m. Ach. Under the same conditions, 5 μg./ml. Trasentin was sufficient to suppress the contractions produced by 0.4 p.p.m. Ach, so that Trasentin was apparently 250 times as active. The recovery of the normal rhythm after treatment with Trasentin was proportional to the degree of relaxation induced, whereas the recovery of rhythm by the ileum relaxed by the thiazole compound was not dependent on the degree of relaxation and was very irregular.

B. B. Dikshit (1938) has reported that isolated rabbit ileum kept in Ringer's solution for 98 hours at 1° C. is unable to synthesize Ach, and he considered this synthesis to be mainly a function of the nerve plexus in the intestinal wall. This may provide a method of inhibiting Auerbach's plexus and so producing a denervated preparation on which the action of plain muscle stimulators and spasmolytic substances can be tested free from interference by the nerve plexus. These preparations are not as sensitive to Ach as is the normal isolated gut, but good contractions can be obtained with 10 μg. Ach, which is the quantity used throughout this work to produce contractions of the isolated gut. Observations on four strips of ileum from two rabbits showed that the response to 0.1 p.p.m. Ach was completely inhibited by a concentration of 1 mg./ml. of the diethylaminoethyl ester, and that the contracted ileum was relaxed to twice its original length by 0.2 mg./ml.

A concentration of 0.2 mg./ml. of the dimethylaminoethyl ester also reduced the contraction to 0.1 p.p.m. Ach, but to a smaller extent than an equal concentration of the diethyl compound. In two further tests a concentration of 1 mg./ml. completely inhibited the action of 0.1 p.p.m. Ach on a strip of ileum from another rabbit. These results suggest that the spasmolytic action is exerted directly on the muscle and is not mediated through Auerbach's plexus.

3. *Action on isolated organs treated with histamine or barium chloride.*—The effects of each compound on the spasm produced in the isolated guinea-pig ileum suspended in Tyrode by a concentration of 2 p.p.m. histamine hydrochloride was tested at four different levels and the percentage relaxation plotted against dosage. The resulting graphs proved to be straight lines. The relative activities of the compounds, as set out in Table II, were calculated from the concentrations required to produce half-relaxation. The dimethylaminoethyl ester was the most active member of the series and had one-quarter the activity of Trasentin-6H.

TABLE II

In vitro ACTIVITY AGAINST HISTAMINE HYDROCHLORIDE (2×10^{-6}) IN GUINEA-PIG ILEUM

Compound	Concentration Producing Half Relaxation
Dimethylaminoethyl 4-methylthiazole-5-carboxylate	$3{\cdot}8 \times 10^{-5}$
Diethylaminoethyl ,, ,, ...	$6{\cdot}0 \times 10^{-5}$
Piperidinoethyl ,, ,, ...	$7{\cdot}2 \times 10^{-5}$
β-Diethylaminopropyl ,, ,, ...	$1{\cdot}7 \times 10^{-4}$
γ-Diethylaminopropyl ,, ,, ...	$2{\cdot}1 \times 10^{-4}$
Morpholinoethyl ,, ,, ...	$2{\cdot}7 \times 10^{-4}$
Diethylaminoethyl 2-amino-4-methylthiazole-5-carboxylate	$2{\cdot}2 \times 10^{-4}$
Diethylaminoethyl 2-methylthiazole-4-acetate	$1{\cdot}5 \times 10^{-3}$
Diethylaminoethyl thiazole-4-acetate	$1{\cdot}8 \times 10^{-3}$
Trasentin-6H	$1{\cdot}0 \times 10^{-5}$

The effects of the compounds on the barium chloride contractions of the isolated guinea-pig ileum resembled those produced on the rabbit ileum, except that the compounds

permanently disturbed the normal rhythmical contractions. The barium chloride contractions of the isolated rabbit's ileum were also inhibited.

4. *"In vivo" experiments.*—Attempts to obtain in the living animal the reactions shown by the isolated tissues have failed in every instance except for the heart rate. The movements of a loop of ileum in a rabbit were recorded by a kymograph needle connected to it by a thread. 10 mg. of the diethylaminoethyl ester failed to diminish the violent movements induced by 25 mg. $BaCl_2$ injected intravenously. Similar results were obtained in the guinea-pig after intracardial injections. A dose of 2.5 mg. intravenously produced a lowering of the heart rate in three rats, as recorded by the electrocardiograph, but this is probably a result of vagal activity following C.N.S. stimulation (see below), for this dose is close to the convulsive level by the intravenous route.

II. Central Nervous Stimulation

The convulsions produced by the dimethyl- and diethyl-aminoethyl esters of 4-methyl-thiazole-5-carboxylic acid were compared with those produced in rabbits by intravenous injection of leptazol and Trasentin-6H in quantities shown in Table III. The doses quoted in the Table were determined by injecting six rabbits with graded doses starting at the LD 50 and diminishing in size.

TABLE III

MINIMAL CONVULSIVE DOSES BY INTRAVENOUS INJECTIONS TO RABBITS

Substance	*Dose* (mg./kg.)
Diethylaminoethyl 4-methyl-thiazole-5-carboxylate	87.5
Dimethylaminoethyl 4-methyl-thiazole-5-carboxylate	62.5
Leptazol	10.0
Trasentin-6H	15.0

At the onset of a convulsion produced by either thiazole compound, the rabbit sits back on its haunches with its forelegs extended. This takes place within a minute. A clonic phase ensues with violent running motions of the fore and hind legs, and passes into a tonic phase with opisthotonus. At this point the animal loses its balance and becomes unconscious. The tonic phase lasts no longer than one minute and is followed immediately by a return of consciousness and clonic movements which pass off leaving the animal exhausted. During the first clonic phase and part of the tonic phase the respiration is completely inhibited, but as the latter passes off the rate of respiration increases and apparently also the amplitude. Rabbits convulsed with the dimethyl compound always recovered their normal sitting position within ten minutes, usually soon after they recovered consciousness. Convulsions produced by the diethyl compound, on the other hand, were followed by a long period of exhaustion and the animal rarely recovered its balance in less than fifteen minutes; sometimes it took as long as half an hour. These convulsions are identical with those produced by leptazol, except that it is not possible with the thiazole compounds to induce such violent clonic movements involving the whole of the trunk and limbs, and the convulsions do not last as long.

Observations were also made to discover whether any difference existed between the convulsions produced in intact frogs and in frogs in which the higher nervous centres were separated from the spinal cord. It was observed that with intact frogs the thiazole compounds and leptazol produced convulsions similar to those obtained with strychnine, whereas in pithed frogs the effect was less marked and lasted for a much shorter period than with strychnine. The results suggest

that the higher centres as well as the spinal cord are involved in the stimulation produced by the thiazole compounds and by leptazol.

J. W. Schultz, L. M. Tainter, and J. M. Dill (1939) describe a method of distinguishing between cortical and sub-cortical stimulation by measuring the total activity exhibited by rats during a period of seven hours. The rat, which has received a subcutaneous dose of the test substance, is suspended in a cage by a spring which oscillates as the animal moves about the cage. By recording the number and extent of the oscillations, an arbitrary measure of the amount of the activity exhibited in unit time can be recorded. The authors distinguish by this method between leptazol and picrotoxin, on the one hand, and nikethamide and caffeine on the other, the last two substances producing a marked increase in activity which, after nikethamide injections, extends over a period of six hours and, after injection of caffeine, over a period of three hours. Leptazol and picrotoxin, on the other hand, only produce a brief period of activity, extending to not more than one hour, and this may be partly due to convulsions. We have made similar observations with the thiazole compounds and amphetamine. The thiazole compounds did not appear to increase the total activity, but, indeed, appeared to depress it soon after the injection. It may, therefore, be concluded from the results reported in these three sections that central nervous stimulation by these compounds is primarily a medullary stimulation with involvement of the spinal cord.

Analeptic Activity

The results described in the previous three sections suggested that the thiazole compounds might antagonize the action of anaesthetics on the central nervous system. Tests were therefore made to ascertain their effect on: (*a*) the duration of anaesthesia, and (*b*) the toxicity of anaesthetics. Two different types of anaesthetics were chosen for the main investigations, namely, amytal sodium as a representative of the barbiturates, and paraldehyde which belongs to a different group of compounds, but produces anaesthesia of approximately the same duration.

(a) *Reduction of Anaesthetic Time*

From the dosage-mortality curve of amytal it was ascertained that no significant mortality would be expected in groups of animals receiving a dose equal to 60 per cent of the LD 50; as this dose produced a satisfactory period of anaesthesia, it was selected to produce the standard degree of anaesthesia. The same proportion of the LD 50 of paraldehyde was also found to be satisfactory.

Two analeptic drugs differing in their action on the nervous system were chosen for comparison with the two thiazole compounds, namely, picrotoxin and β-phenyl-*iso*propylamine sulphate (amphetamine). Picrotoxin is used as an antidote in barbiturate poisoning because it is safe in doses far exceeding those lethal to unanaesthetized animals, although its action is relatively short and somewhat irregular. Amphetamine, on the other hand, although as toxic to unanaesthetized as to anaesthetized animals, has a more prolonged action than picrotoxin (compared by the percentage recovery of mice), and is more effective at dose levels proportionately further removed from the LD 50 for unanaesthetized animals. It was, however, found impossible to obtain even an approximate value for the toxicity of amphetamine, and a search of the literature showed that other workers had experienced the same difficulty; as much as a tenfold difference is reported in the figures quoted by different authors using the same route for mice. It was therefore considered necessary to make an exhaustive study of the matter, the results of which finally enabled

us (Chance, 1946), after the conditions of the test had been sufficiently defined and controlled, to determine the toxicity of this substance with the same degree of accuracy as is usually encountered in biological assays. Meanwhile, a comparison was made of the two substances already mentioned with picrotoxin. Groups of sixteen mice received the standard dose of 120 mg./kg. by intraperitoneal injection followed immediately by a subcutaneous injection of the analeptic.

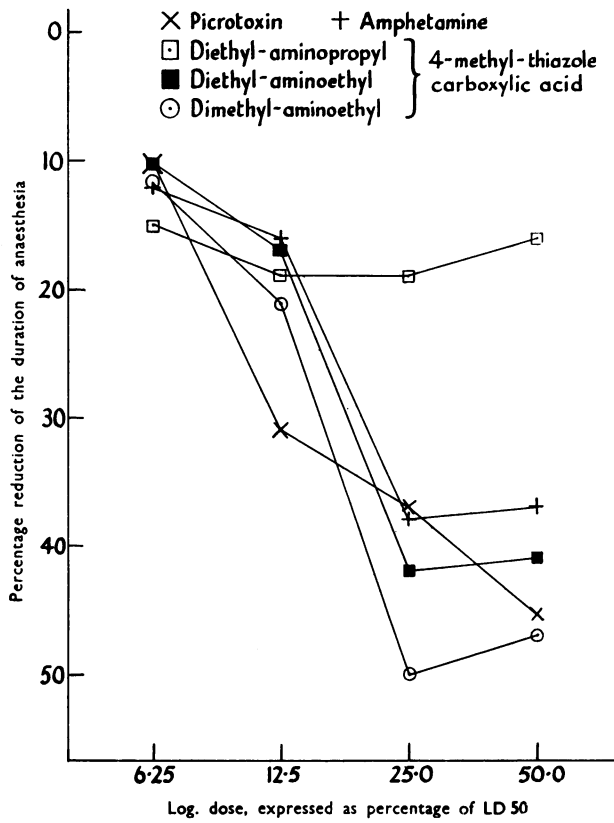

FIG. 1.—Shortened duration of amytal anaesthesia.

Both thiazole compounds, like picrotoxin, reduced the duration of anaesthesia produced by the standard dose of amytal (Fig. 1). The reductions in the duration of anaesthesia, brought about by equivalent proportions of the LD 50 of the two thiazole compounds and picrotoxin, were approximately equal; when the logarithm of the dose was plotted against the reduction in anaesthesic time the relationship was not linear for the thiazole compounds, though possibly so for picrotoxin.

The thiazole compounds were, however, less effective than picrotoxin against paraldehyde, and acted somewhat differently (Fig. 2). Over the same range of doses, expressed as proportions of the LD 50, the dimethyl compound was less

effective than the diethyl compound, which had an optimal activity at a level equal to 25 per cent of its LD 50. These compounds are clearly less active than picrotoxin, but their action is nevertheless pronounced, and against amytal their action is indistinguishable from that of picrotoxin when compared at the same proportions of their respective LD 50's.

After the investigation into the factors affecting the toxicity of amphetamine had been completed, two more compounds became available. These were the hydrochlorides of diethylaminopropyl 4-methyl-thiazole-5-carboxylate

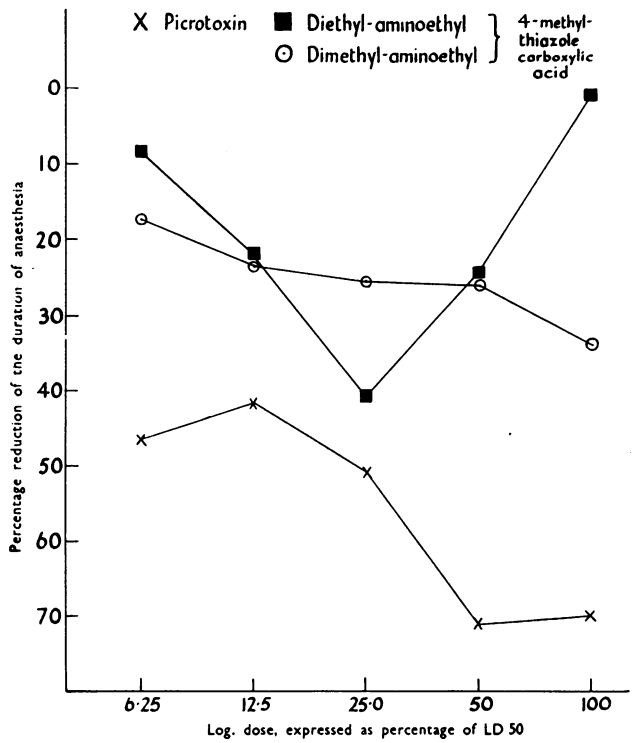

FIG. 2.—Shortened duration of paraldehyde anaesthesia.

(II,R = $(C_2H_5)_2N.CH_2$) and diethylaminoethyl 2-amino-4-methyl-thiazole-5-carboxylate (IV). The former produced convulsions on injection into mice, and was therefore tested for analeptic activity, together with the hydrochlorides of the diethyl- and dimethylaminoethyl esters of 4-thiazole-5-carboxylic acid. On this occasion amphetamine sulphate was also included in the comparison. The combined results are shown in Fig. 1.

(b) *Effect on the Toxicity of Amytal*

The LD 50 of amytal sodium on "Swiss" mice had been measured previously and found to be 200 mg. per kg. body-weight. When redetermined on a group of twenty mice it appeared to be slightly higher, and it is possible that the original estimate may have been

somewhat low. A dose of 200 mg./kg. was, however, chosen as the toxic dose in these experiments, and gave a mortality of 43 per cent.

The values of LD 50 for the diethyl and dimethyl compounds are 700 and 500 mg. per kg. respectively, for strychnine 1 mg. per kg., and for picrotoxin 5 mg. per kg. Each substance was tested by subcutaneous injection into a group of not more than ten mice immediately after intraperitoneal injection of the standard dose of amytal. Both the amytal and the various amounts of the test substances were administered in equal volumes of solution. The procedure was completed in a total of four minutes, so that each received the antidote at the onset of anaesthesia, which for amytal occurs two minutes after intraperitoneal injection. In each investigation carried out on one day the same number of animals was used for each dose of all four substances, and simultaneously the same number of animals received the standard dose of amytal alone. The whole investigation was then repeated until all doses of each substance had been tested on forty animals.

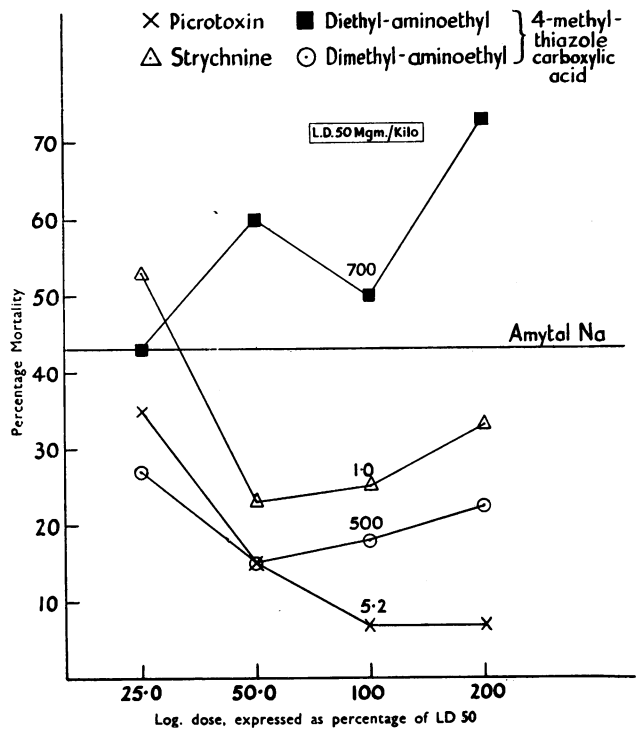

FIG. 3.—Effect of picrotoxin, strychnine and the dimethyl- and diethylaminoethyl esters of 4-methyl-thiazole carboxylic acid on the toxicity of amytal sodium (200 mg./kg.) in mice.

Fig. 3, in which the dose, expressed as the logarithm of the percentage of the LD 50, is plotted against the percentage mortality for each substance, shows that the mortality to the standard dose of amytal was 43 per cent—a slightly lower value than expected. Reductions in mortality are statistically significant when the mortality was 20 per cent or less. The three highest doses of picrotoxin produced a significant lowering of mortality from the standard dose of amytal, though the lowest did not. With strychnine, reductions of mortality due to the

LD 50 of amytal were produced at three dose levels (200, 100 and 50 per cent of the LD 50 of strychnine), but they were not statistically significant ; nor was a significant increase in mortality shown with the smallest dose of strychnine. The curve for the dimethyl compound shows that it behaves very like strychnine, but is, if anything, more active ; significantly lower mortalities were produced by 50 and 100 per cent LD 50 doses. The diethyl compound, on the other hand, increased the mortality due to amytal, significantly at the highest dose (200 per cent of its LD 50).

III. Local Anaesthetic Activity

Since it was thought possible that the amino substituted thiazole, diethylaminoethyl 2-amino-4-methyl-thiazole-carboxylate might possess local anaesthetic properties similar to those of procaine, it was tested for local anaesthetic activity by the method of Chance and Lobstein (1944). It was inactive at a concentration of 1 in 100, as was also diethylaminoethyl 4-methyl-thiazole-carboxylate.

Discussion

The piperidinoethyl, dimethylaminoethyl, diethylaminoethyl and β-diethylaminopropyl esters of 4-methyl-thiazole-5-carboxylic acid had approximately the same activity in inhibiting the contractions produced by acetylcholine in the isolated ileum (neurotropic action) but were only about 1/1,000 as active as Trasentin ; the morpholinoethyl ester of 4-methyl-thiazole-5-carboxylic acid and the diethylaminoethyl ester of 2-methyl-thiazole-4-acetic acid were even less active. The compounds did not produce mydriasis. Their effect on spasm induced by barium chloride or histamine (musculotropic action) was of the same order as that of Trasentin. In this respect the piperidinoethyl, dimethylaminoethyl and diethylaminoethyl esters were more active than the morpholinoethyl or the β- and γ-diethylaminopropyl esters of 4-methyl-thiazole-5-carboxylic acid ; the two isomeric propyl esters showed about the same activity. The diethylaminoethyl esters of 2-methyl-thiazole-4-acetic acid and thiazole-4-acetic acid had only a fraction of the activity of the corresponding ester of 4-methyl-thiazole-5-carboxylic acid, demonstrating the importance of direct attachment of the carboxylic group to the nucleus. The activity of the diethylaminoethyl ester of 2-amino-4-methyl-thiazole-5-carboxylic acid was 1/30 that of the corresponding ester of 4-methyl-thiazole-5-carboxylic acid, so that the introduction of an amino group materially reduced the musculotropic activity.

The diethylaminoethyl ester of 4-methyl-thiazole-5-carboxylic acid, although resembling Trasentin in its action on the isolated ileum, had a different effect on the isolated uterus of the rabbit or guinea-pig and, instead of relaxing the muscle, increased the tone in a manner similar to posterior pituitary extract or atropine. The substance was less potent, however, 2 mg. producing the same response as 0.05 I.U. posterior pituitary extract or 60 μg. atropine sulphate. This ester had no effect on the ileal contractions induced by barium chloride or histamine hydrochloride *in vivo*.

Some of the esters were found to be stimulants of the central nervous system, producing convulsions in mice and guinea-pigs. Some of them reduced the duration of narcosis induced by amytal and paraldehyde, though the effect was markedly different with the different compounds.

Trasentin and Trasentin-6H are convulsants and respiratory stimulants (Graham and Lazarus, 1940), whilst atropine also stimulates the medulla and higher cerebral centres, though at the usual clinical dosage its effects are manifest only as moderate respiratory stimulation and slight vagal excitation. The thiazole compounds are less potent stimulants than leptazol or Trasentin. They act on the medulla, and the effect resembles that of leptazol rather than that of strychnine. The diethylaminoethyl ester of 4-methyl-thiazole-5-carboxylic acid was slightly effective in antagonizing the effect of a lethal dose of amytal, but the dimethylaminoethyl ester was not. Neither compound was as effective as leptazol in inducing central nervous stimulation, the lethal dose being close to the convulsive dose.

SUMMARY

1. The basic esters of 4-methyl-thiazole-5-carboxylic acid and of thiazole-5-acetic acid possess spasmolytic and analeptic properties.

2. The spasmolytic activity of these compounds is exhibited only *in vitro*, and is most marked against histamine spasm. A comparison of the different substances revealed that the dimethylaminoethyl ester of 4-methyl-thiazole-5-carboxylic acid was the most active, having an activity against histamine spasm approximately one-quarter that of Trasentin-6H; the diethylamino- and piperidinoethyl esters were only a little less active than the dimethylaminoethyl ester.

3. The diethyl- and dimethylaminoethyl esters and the γ-diethylaminopropyl ester of 4-methyl-thiazole-5-carboxylic acid produced in guinea-pigs and rabbits convulsions similar to those caused by leptazol and compounds acting on the mid-brain, although there was evidence of spinal involvement. These esters have analeptic activity, demonstrated by their ability to reduce the duration of anaesthesia in mice. The diethyl- and dimethylaminoethyl esters of 4-methyl-thiazole-5-carboxylic acid are, however, inferior to picrotoxin in this respect; not only is the reduction of paraldehyde anaesthesia less marked than the reduction of amytal anaesthesia, but antidotal activity is exhibited by the dimethyl compound over a smaller dose range than that found for picrotoxin.

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THE TESTING OF DRUGS AGAINST EXOERYTHROCYTIC FORMS OF *P. GALLINACEUM* IN TISSUE CULTURE

BY

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(Received April 4, 1946)

In 1938 James and Tate described a tissue phase of *P. gallinaceum* in chicks which they called the exoerythrocytic phase of the parasite, and in 1945 Hawking succeeded in growing these forms in tissue culture. At the time of writing, similar forms have not been demonstrated in mammalian malaria, but it is likely that they do exist, and that the relapses common in benign malaria are due to their presence.

The tissue culture technique has been used as an *in vitro* method for testing the effect of drugs against the tissue phase of *P. gallinaceum*. It is hoped that this test can be adapted so that the effect of drugs against the tissue phase of all types of Plasmodia can be investigated. A drug which was active against the tissue phase of the malaria parasite in this test would probably be a causal prophylactic and have a beneficial effect on the relapse rate of benign tertian malaria.

METHOD

Ten-day-old chicks were injected with sporozoites of *P. gallinaceum* and killed 7–8 days later, when they were heavily infected with tissue forms of the parasite. In a typical experiment the spleen from such an infected chick was removed aseptically, minced finely in Tyrode's solution, and set up in Carrel flasks, each of which contained 3–4 coverslips, using the method described by Hawking (1945). The fluid phase consisted of Tyrode's solution containing 20 per cent (v/v) chick serum and 3–4 per cent (v/v) chick embryo extract; 0.05 per cent phenol red was added to indicate changes in the *p*H of the medium. Penicillin was also added to make a final concentration of 3 units per ml. as an additional safeguard against possible contamination by bacteria. (Later 0.5 unit/ml. was used.) Drugs to be tested were dissolved in Ringer's solution, sterilized by boiling, and appropriate concentrations made by serial dilution. 2.5. ml. volumes of the fluid phase were then run into the flasks containing the infected spleen explants, followed by 0.5 ml. volumes of the drug solutions—the control flasks receiving 0.5 ml. volumes of sterile Ringer solution in place of drug. In this way a series of flasks was set up in which the same infected material was in contact with varying concentrations of several different drugs.

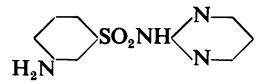
Under these conditions the final concentration of the drug in the flasks is less than the nominal concentration owing to the absorptive powers of the plasma and spleen explants used in the course of the experiments; the ratio of the nominal concentration to the final concentration may also vary because the amounts of spleen and plasma used were not

constant. This factor probably accounts for the disparity between some of the results obtained in different experiments using the same nominal concentrations of a particular drug. Frequent changing of the medium would probably obviate this effect, but it would be consuming of time and materials. In our experiments the medium was changed every 5 or 6 days when the *p*H of the medium became too low for cell growth to proceed favourably.

In the drug-free control flasks many parasites in all stages of schizogony were present after 7–10 days incubation at 37° C. When this was observed all the flasks in a series were sampled by withdrawing one or more of the coverslips from each aseptically, fixing them in methyl alcohol, staining with Giemsa, mounting, and examining them microscopically. In this way the effect of the drugs under test on the growth of the parasites in tissue culture could be observed. In cases where no parasites could be demonstrated microscopically, the result was confirmed by inoculation of aliquots of the fluid phase (0.5 ml. – 1.0 ml.) taken from such flasks into chicks and observation of the presence or absence of subsequent infection.

The antimalarial action of the following compounds was investigated:

1. Sulphathiazole
2. Sulphadiazine
3. 2-(*m*-aminobenzenesulphonamido)-pyrimidine (N′-2-pyrimidylmetanilamide in U.S.A.)



4. Sontoquin (3-methyl-4-diethylamino*iso*amylamino-7-chloroquinoline sulphate)
5. Streptomycin
6. Streptothricin

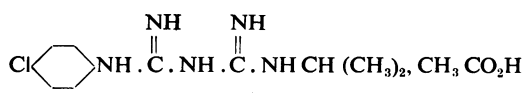
Compounds 3–6 were kindly supplied by the secretary of the Board for the Co-ordination of Malarial Studies, Washington.

The potency of streptomycin was stated to be 200 units per mg., and that of streptothricin to be 400 units per mg. In tests carried out in this laboratory using *B. coli* (strain No. 88, Dr. Felix), it was found that 0.05 mg. of streptomycin and 0.025 mg. of streptothricin just prevented the growth of the organism in 1 ml. of Hartley's broth.

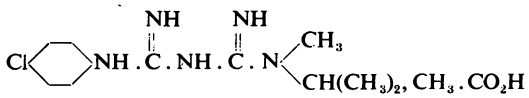
7. Quinine bisulphate
8. Mepacrine hydrochloride
9. Pamaquin dihydrochloride

This salt was kindly made for us by Dr. T. S. Work from the hydroxynaphthoate of pamaquin.

10. Paludrine acetate (N_1-*p*-chlorophenyl-N_5-*iso*propylbiguanide acetate)



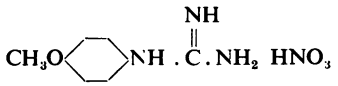
11. M4430. (N_1-*p*-chlorophenyl-N_5-methyl*iso*propylbiguanide acetate)



Compounds 10 and 11 were kindly supplied by Dr. C. M. Scott, of Imperial Chemical (Pharmaceuticals), Ltd.

12. Stilbamidine. (Diamidinostilbene nitrate)
Kindly supplied by Dr. Wien of May & Baker, Ltd.

13. *p*-Anisylguanidine nitrate. (V313)



This compound was made by Dr. H. King, F.R.S. It is the most active member of a series of guanidines and biguanides made for testing on malarial infections (King and Tonkin, 1946).

The concentrations quoted in the tables refer to the compounds or the salts of the compounds as listed above.

RESULTS

Results obtained in tests on these compounds using the tissue culture technique are given in Table I. Many of these compounds were toxic to macrophages, and a specific inhibitory effect of a drug on the exoerythrocytic forms of *P. gallinaceum* could only be recorded when the cells were observed to grow satisfactorily in the drug concentration used, while the parasites failed to grow.

Compounds of the sulphonamide type (sulphathiazole, sulphadiazine, and *m*-aminobenzenesulphonamidopyrimidine) had a considerable range of activity; i.e., the cells grew in high concentrations of the drugs, whereas the parasites were only able to grow in very low concentrations. *m*-Aminobenzenesulphonamidopyrimidine was slightly more toxic to macrophages than sulphathiazole and sulphadiazine. *p*-Anisylguanidine nitrate, a compound not containing the sulphonamide group but similar in structure to paludrine and M4430, also exerted considerable antiparasitic activity and was not toxic to the cells in the culture.

Of the other compounds tested, some activity was shown by streptomycin and streptothricin. The former was non-toxic to the cells in tissue culture in high concentrations and exerted an anti-parasitic effect when the concentration was above 100 mg./100 ml. (=200 units per ml.). Streptothricin was more toxic to the cells than streptomycin, but inhibited parasitic growth over a small range of concentrations not toxic to cells. The only other compound in the series to show any anti-parasitic activity was quinine. In all experiments except the second one (see Table I), the cell growth in contact with the higher concentrations

TABLE I

THE EFFECT OF DRUGS ON THE EXOERYTHROCYTIC FORMS OF *P. Gallinaceum* IN TISSUE CULTURE

++Heavy growth of parasites. +Good growth of parasites.
±No parasites seen microscopically but chicks infected.
−No parasite growth. T toxic, and Sl.T slightly toxic, to cells. CP Poor growth of cells.

Drug	No. of Expt.	Concentration in mg./100 ml.										Remarks
		30·0	20·0	15·0	5·0	0·5	0·1	0·05	0·02	0·01	0·005	0·0	
Sulpha-thiazole	1	Sl.T		Sl.T								++	
	2				−	−	±					++	Approx. minimal effective concentration 0·05 mg./100 ml.
	3		Sl.T									++	
	4						−	−				+	
	5						−					+	
	6						−	−	+	+	++	++	
		10·0	5·0	2·5	1·0	0·5	0·2		0·1	0·05	0·0		
Sulpha-diazine	1	−	−	−	−	−					+		Approx. minimal effective concentration 0·1 mg./100 ml.
	2						−		−	−	+		
	3								+		+		
		5·0	1·0	0·5	0·1	0·05	0·025		0·01	0·005	0·0		
m-Amino-benzene-sulphon-amido-pyri-midine	1	CP−	−		−						+		Approx. minimal effective concentration 0·1 mg./100 ml.
	2				−				+	+	+		
	3		−	−	−	+*	+*				++		
	4				−	+	+		+		++		
	5			−	−				+*		+		
		25·0	12·5	5·0	2·5	2·0	1·0		0·5	0·0			
Quinine bisulphate	1	T	T							++			
	2				−		+		++	++			
	3						+			+			Approx. minimal effective concentration 2 mg./100 ml. Toxic to cells.
	4					CP−				++			
	5					CP−	±			++			
	6					CP−	CP−			++			
	7			T			+			++			
	8			T	CP−		−			+			
	9						CP−		+	++			
	10					CP−	+		+	+			
		5·0		1·0		0·5	0·2		0·1	0·0			
Mepacrine	1	T		T		±			+	++			
	2					−				++			Inactive
	3			T		T			++	++			
	4					+	+		+	++			
		0·5		0·1		0·05	0·02		0·01	0·0		? Slight action	
Pamaquin	1	T		T		−			−	+		Approx. minimal effective concentration 0·05 mg./100 ml. Very toxic to cells.	
	2			+*		+*	+			+			
	3			T		−				+			
	4					+*	+			+			
		2·0	1·0	0·5	0·2	0·1	0·05	0·02	0·01	0·005	0·0		
Paludrine	1		T	CP−		CP−	CP−				+		
	2						+		+	+	+		
	3			±	+	+	+	+			+		No definite action
	4	−	CP±		+	++	++				++		
	5	CP−	−	+							+		
	6	T	+*	+*							++		

TABLE I *continued*

Drug	No. of Expt.	Concentration in mg./100 ml.											Remarks
		5·0		1·0		0·5	0·2		0·1		0·0		
M4430	1	T		CP–					+		++		
	2			T		CP+	++		++		++		Inactive
	3					CP+	++				++		Toxic to cells
	4					T	++				++		
		5·0	2·0	1·0		0·5	0·2		0·1	0·05	0·0		
p-Anisyl guanidine nitrate	1	CP–									+		Approx. minimal
	2			–		–	+			+	+		effective concen-
	3	–	–								+		tration 0·5 mg./
	4	–	–	–			++		+		++		100 ml.
		0·5	0·25	0·1		0·05	0·025		0·01		0·0		
Sontoquin	1	T		T							++		Inactive
	2	T	T	T		T					++		Very toxic to
	3					+	+		+		++		cells
	4	T	CP++	++		++					++		
		1·0		0·5		0·2			0·01		0·0		
Stilba-midine	1	–							+		+		
	2	CP–									++		Inactive
	3	CP–		CP+		+					+		
		500	250	100	50	25	5·0	2·5	2·0	0·4	0·1	0·01	0·0
Strepto-mycin	1								+		+	+	+
	2								++	++			
	3				+	+	+	+					+
	4	–	–		+								++
	5		–	+	+								+
		25·0	12·5	10·0		5·0	2·5		1·25		0·5	0·0	
Strepto-thricin	1	–	–				–		+		+	+	
	2	T		–								++	
	3						+		++		++	++	
	4						–		+		+	+	

Approx. minimal effective concentration 2·5 mg./100 ml. (= 10 units per ml.)

*A few parasites were seen in early samples taken from flasks but none in later ones; they appeared to have died off during the course of the experiment.

of the drug was poor, owing to the toxicity of quinine for cells of the macrophage type. It was therefore not possible to demonstrate a clear-cut anti-parasitic activity with this compound, but probably it exerts a slight effect on the growth of exoerythrocytic forms of *P. gallinaceum*.

No activity was exerted by mepacrine, sontoquin, M.4430, or stilbamidine. Pamaquin and paludrine, both of which were expected to show activity, were also relatively ineffective. Both compounds were toxic to the cells, so that it is possible that if higher concentrations could have been tested, some anti-parasitic activity might have been detected.

TABLE II

THE INHIBITION OF THE ANTIPARASITIC ACTION OF SULPHONAMIDES

++Heavy, + good growth of parasites. — No growth of parasites. ?— A few disintegrating parasites seen but chicks not infected.

Drug	Conc. mg./100ml.	Inhibitor	Conc. mg./100ml.	Number of Experiment 1	2	3	4	5	Ratio of conc. drug/ inhibitor
Sulpha-thiazole	5·0	PAB	0·5	++	++	++			
			0·1		++			+	
			0·05		++			+	500
			0·02					+	
			0·01					+	
			0·005					—	
			0·0	—	—		—	—	
	0·0	PAB	0·0	++	++	+	++	++	
			10·0			+			
	5·0	MAB	5·0	—					
			0·0	—					
	0·0	MAB	0·0	++	+				
			10·0		+				
m-Aminoben-zene-sulphon-amido-pyri-midine	1·0	PAB	1·0	—					
			0·0	—					
	0·0	PAB	0·0	++					
	0·5	PAB	10·0		+*				0·1
			5·0		+		—		
			1·0				—		
			0·5			—			
			0·1			—			
			0·0		—	?—	—		
	0·0	PAB	0·0		+	++	++		
			10·0		+				

* A few parasites were seen in early samples taken from the flasks, but none in later ones; they appeared to have died off during the course of the experiment.

Inhibition of Anti-parasitic Activity

Experiments were also set up to demonstrate the inhibition of the anti-parasitic activity of compounds of the sulphonamide type. The drugs used were sulphathiazole and *m*-aminobenzenesulphonamidopyrimidine, and the corresponding inhibitors were *p*-aminobenzoic acid (PAB) and *m*-aminobenzoic acid (MAB) (Table II). 0.01 mg./100 ml. PAB inhibited the activity of 5 mg./100 ml. sulphathiazole, one molecule of the inhibitor therefore being equivalent to approximately 270 molecules of the drug.

No inhibition of the activity of the *m*-sulphonamide by MAB could be demonstrated, but high concentrations of PAB had a slight action in this respect.

The Effect of Drugs on Cultures already Grown

The effect of sulphathiazole on parasites already grown in tissue culture was also investigated. For these experiments cultures of spleen containing exoerythrocytic forms were taken after incubation for 7–14 days at 37° C. in drug-free medium, at which time they showed vigorous growth of cells and parasites.

TABLE III

THE EFFECT OF SULPHATHIAZOLE ON EXOERYTHROCYTIC FORMS OF *P. Gallinaceum* ALREADY GROWING IN TISSUE CULTURE

Expt.	Age of culture when drug added Days	Conc. of sulphathiazole mg./100 ml.	Conc. of PAB mg./100 ml.	Days of exposure to the drug 1	3	4	5	6	7
a	9	10·0		+	--		--		
	8	5·0		+	+	+	?--		--
		2·0			+		? -		?--
		1·0		+	+	+	?--		? -
		0·5		+	+	+	?--		--
		0·0		++	++	++	++		+
b	11	5·0			+	+		--	--
		1·0			+	+		--	-
		5·0	20·0		+	+		+	±
			10·0		+			+	±
			5·0		+	+		+	+
		0·0	0·0		++	++		+	+

++ Heavy parasite growth. + Some parasite growth.
± No parasites seen microscopically but chicks infected.
? A few disintegrating parasites seen. Chicks not infected.

The fluid phase was removed and replaced with fresh medium containing varying concentrations of drug. Slips were removed, fixed, stained, and examined after 1, 3, 4, 5, 6, and 7 days' incubation at 37° C. in contact with the drug.

The protocols of a typical experiment are given in Table IIIa. It will be seen that the parasites were all dead after 5 days' exposure to all concentrations of sulphathiazole tried.

Table IV shows percentage counts of parasites in different stages of development from cultures in contact with 0–10 mg./100 ml. of sulphathiazole for 1–5 days. The increasing scarcity of parasites in the cultures with increasing time of exposure is shown by the drop in the total number of parasites counted

TABLE IV

THE EFFECT OF SULPHATHIAZOLE ON DIFFERENT STAGES OF PARASITES GROWING IN TISSUE CULTURE

Sulphathiazole mg./100 ml.	Time of exposure to drug Days	Percentage numbers of different forms of parasite				Total number of parasites counted
		Schizonts 8 nuclei	Schizonts 3–8 nuclei	Schizonts 1–2 nuclei	Groups of Merozoites	
10·0	1	5·4	15	79	0	186
	2	1·58	19	80	0	63
	4	100	0	0	0	16
5·0	1	31·5	11·6	57	0	156
	3	20·5	51	23·5	0	131
	4	61	39	0	0	46
2·0	3	0·54	41·5	58	0	186
	5	36·5	63·2	0	0	30
1·0	1	39	18·6	41	1·5	194
	3	3·5	88	2·9	0	173
	4	100	0	0	0	31
0·0	−2*	33	45	24	0	95
	0†	8·45	3·9	86·5	1·3	154
	3	35·5	29·5	31·7	3·7	164
	5	11·1	59·5	27·7	1·58	126

*Two days before addition of sulphathiazole.

†Immediately before addition of sulphathiazole.

in cultures after 5 days' exposure as compared with the total after one day's exposure and with that in the control flasks. Approximately the same length of time was spent in examining the sample from each flask. It will also be seen that the number of small forms became progressively smaller on successive days of exposure to the drug until on the fourth day they had disappeared altogether, while abundant small forms and merozoites were still found in the control flasks. Sulphathiazole apparently has a gradual effect on the parasites, reducing their power of division.

The larger schizonts still remaining after 3 days' exposure showed definite pathological changes. The chromatin coalesced into lumps, the parasite envelope became indistinct and the cytoplasm became vacuolated and lost its power to take up stain. In later stages the chromatin appeared as indistinct collections of small granules (Fig. 1). These changes were associated with a loss of the ability of the parasites to infect chicks.

The effect of PAB on drug activity was also investigated in this connection, the inhibitor being added at the same time as the drug. Inhibition of drug

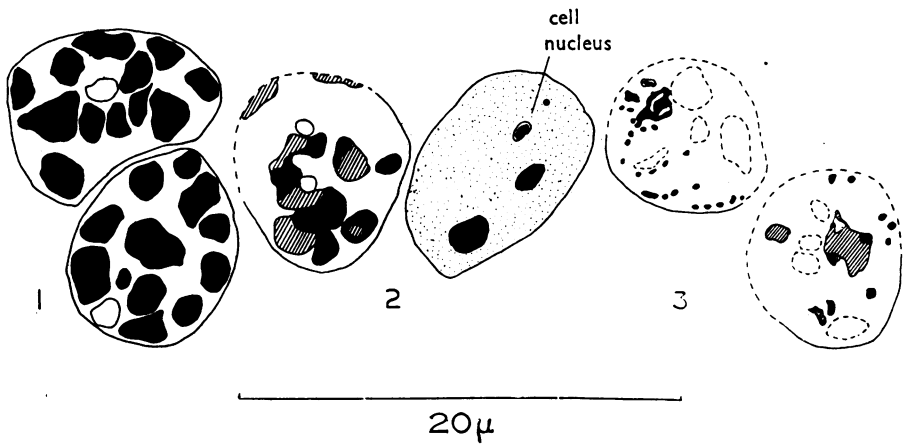


FIG. 1.—Drawn by I.M.T. (magnification as shown by scale).

1. Parasites before treatment with drug.

2. Parasites after exposure for 1 day to sulphathiazole (10 mg./100 ml). Chromatin coalescing into lumps.

3. Parasites after exposure for 5 days to sulphathiazole (10 mg./100 ml.). Chromatin in small granules. Envelope vague, vacuolated cytoplasm.

activity was not so marked as in the experiments given in Table II, the parasites being reduced in numbers compared with those in the control flasks, and also showing pathological changes. However, they maintained their ability to infect chicks for a longer period than those parasites in contact with sulphathiazole alone, showing that some inhibition of drug activity had taken place.

The protocols of an experiment of this type are given in Table IIIb.

DISCUSSION

A method for the *in vitro* testing of drugs against the tissue phase of *P. gallinaceum* is described and the results obtained on several compounds tested by this method are given. The technique is probably too elaborate to be used in a routine screening test, but it might be employed with advantage in the further investigation of promising compounds singled out by the *in vivo* screening tests

at present in force. The method is limited by the fact that many compounds are very toxic to macrophages in tissue culture, and in these cases it is often difficult to detect any direct effect of the drug on parasite growth as distinct from the effect on the growth of the parasites due to the poor survival of the cells which form their substrate. The solubility of the compounds to be tested is another limiting factor.

The antiparasitic activity of sulphathiazole is inhibited by PAB. The equivalence of one molecule of the inhibitor to 270 molecules of the drug cannot be regarded as a true quantitative relationship, because the medium in which the reaction took place contained many natural sulphonamide inhibitors, such as serum, plasma, tissue extracts, etc.

Previous work on this relationship carried out with bacteria shows considerable variation in the sulphathiazole/PAB ratio. Rose and Fox (1942), using *B. coli* growing in a synthetic medium, found one molecule of PAB to be equivalent to 4 molecules of sulphathiazole, while Landy and Wyeno (1941), using a staphylococcus growing in a heart infusion broth, found that one molecule of the inhibitor neutralized 2,000 molecules of the drug. Our figure of 1–270 falls between these two extremes.

Inhibition of the antiparasitic activity of *m*-aminobenzenesulphonamidopyrimidine by MAB in a prophylactic test in chicks had been reported by Dr. S. Brackett (private communication). It was therefore expected that this reaction could be demonstrated in tissue culture. No inhibition was in fact obtained with this combination of reagents, but PAB in a high concentration had a slight effect on the activity of the *meta*-sulphonamide. We have also been unable to confirm this finding of Brackett's in experiments on chicks.

Our experiments in which sulphathiazole was placed in contact with exoerythrocytic forms already grown in tissue culture showed that the drug probably reduced the power of division of the parasites. A similar effect was noted in the case of erythrocytic forms of *P. gallinaceum* in chicks treated with sulphadiazine by Brackett, Waletzky, and Baker (1945).

SUMMARY

(1) The effect of drugs on the exoerythrocytic forms of *P. gallinaceum* has been investigated using an *in vitro* technique. Explants of chick spleen infected with tissue forms of the parasite were grown in Carrel flasks in contact with a solution of the drug in a nutrient medium, and the effect of the drug on parasite and cell growth was observed microscopically.

(2) The following compounds inhibited the growth of the parasite and were comparatively non-toxic to the macrophages: sulphathiazole, sulphadiazine, *m*-aminobenzenesulphonamidopyrimidine, streptothricin, streptomycin, and *p*-anisylguanidine nitrate. Quinine showed a slight antiparasitic activity but was toxic to macrophages. Mepacrine, stilbamidine, M.4430 (N_1-*p*-chlorophenyl-N_5-methyl*iso*propylbiguanide), sontoquin, pamaquin, and paludrine were very toxic

to the cells and were inactive in the highest concentrations tolerated by the macrophages.

(3) The antiparasitic activity of sulphathiazole could be inhibited by *p*-aminobenzoic acid, one molecule of the inhibitor corresponding to 270 molecules of the drug. No inhibition of the *m*-sulphonamide, *m*-aminobenzenesulphonamidopyrimidine, by *m*-aminobenzoic acid could be demonstrated.

(4) When parasites, already grown in tissue culture, were exposed to sulphathiazole, the drug appeared to reduce the power of division of the parasites. After 4 days' exposure to the drug no small forms were found and the larger schizonts became progressively more degenerate ; after more than 4 days' exposure the parasites lost the ability to infect chicks.

(5) The tissue culture technique may not be suitable for large-scale routine drug screening tests, but would be useful for the further investigation of promising compounds.

Grateful acknowledgements are due to Dr. F. Hawking for help and advice, and to Miss R. J. Berson and to Miss P. Davey for technical assistance.

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EXPERIMENTAL DIABETES: THE EFFECT OF LIGATION OF THE PANCREATIC DUCT UPON THE ACTION OF ALLOXAN IN RABBITS*

BY

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It is known from the work of Shaw Dunn and his collaborators [(1943 (*a*) and (*b*), 1944) and Duffy (1945)], Bailey and Bailey (1943) and others that it is possible, by the intravenous administration of alloxan, to produce in rabbits a selective necrosis of the islets of Langerhans and a condition indistinct from diabetes mellitus. We have studied the effect of alloxan administration in rabbits at various times after ligation of the main pancreatic duct. When sufficient time is allowed to elapse after the operation to permit the acinar tissue to degenerate, alloxan, subsequently administered, produces at most slight changes in the surviving islet cells and no diabetes. This result cannot yet be conclusively explained, but strongly suggests, among other possibilities, that the acinar tissue of the pancreas is in some way involved in the action of alloxan upon the islets.

EXPERIMENTAL

The rabbits used in our experiments were adult males and females of various breeds. They were bedded on hay in wire-mesh cages and maintained on a standard composite diet (Thomson, 1936) supplemented with cabbage. They were starved for 24 hours before operation or alloxan administration. Blood sugar was determined by the method of Hagedorn and Jensen on 0.2 ml. samples of blood withdrawn from an ear vein. Alloxan was in all cases given in 4 per cent solution in physiological saline, by slow intravenous injection into a marginal ear vein.

The whole pancreas was taken for histological examination, fixed in Zenker-formol and embedded in paraffin. Sections were stained with (*a*) haematoxylin-eosin, (*b*) Wilder's

* A preliminary communication on this subject was given by one of us (A.L.W.) at the meeting of the British Pharmacological Society, July, 1945.

silver impregnation method, (*c*) a modification of Mallory's aniline blue method for the differentiation of α and β cells in the islets of Langerhans, and occasionally with chlorazol black for elastic fibres.

For the characterization of the changes in the pancreas produced by the various procedures to which rabbits were submitted, certain features of the normal rabbit pancreas are pertinent. In haematoxylin-eosin (H.E.) preparations the islets are extremely numerous and stand out in the lobules as pale staining round or oval bodies against the darker staining acinar tissue (Plate I, Fig. 1). Under higher magnifications they are seen to be composed of anastomosing cords of cells, the outlines of the individual cells being rather obscure (Plate I, Fig. 2). The cytoplasm is pale, but even in the H.E. preparations it is often possible to distinguish between certain cells at the periphery stained faintly eosinophilic and more numerous centrally placed cells in which the cytoplasm is stained a sombre greyish blue. The nuclei of both α and β cells are round or oval, show an even powdered distribution of chromatin particles, and contain a minute nucleolus. A few fibroblasts may be present in the islet tissue and occasionally red blood corpuscles are seen in minute sinusoidal channels. With silver impregnation methods the pancreas is shown to have a scanty and loose inter-lobular stroma while the acini are mapped out by virtue of an enclosing intra-lobular argyrophil reticulum (Plate I, Fig. 3). The latter also forms a compact basket-work around the islets but not in the sense of a capsule ; only very few fibrils penetrate into the centre. With Mallory's method the α cells, present mainly at the periphery of islets, have orange to red granular cytoplasm, while the β cells contain granules stained a much fainter colour and predominate in the centre. Mitoses are rarely observed in the islet cells.

The effects of alloxan in the unoperated rabbit

In the unoperated rabbit an adequate dose of alloxan produces a characteristic sequence of changes in the blood sugar concentration. These effects were described in detail by Shaw Dunn (*loci cit.*) and others, in other animals as well as rabbits, and our observations in this direction add nothing new.

A typical result is shown in Fig. 1 which represents the blood sugar concentration of rabbit W.5 (1.9 kg.) before and after a single intravenous dose of 200 mg./kg. of alloxan.

A sharp initial rise was followed within an hour or two by a fall, during which the animal passed into hypoglycaemic convulsions and coma. Death at this stage was averted by the repeated administration of glucose and there finally ensued a chronic state of hyperglycaemia resembling that of diabetes mellitus.

The pathological effects on the pancreas are briefly as follows. Within a few hours of the injection, minor degenerative changes appear in the islet cells—obscurity in the staining of the cytoplasm and nuclear hyperchromatism, dislocation of cells from those adjacent and the formation of small spaces—limited mainly, if not solely, to the β cells of some isolated islets (Plate I, Fig. 4). Later

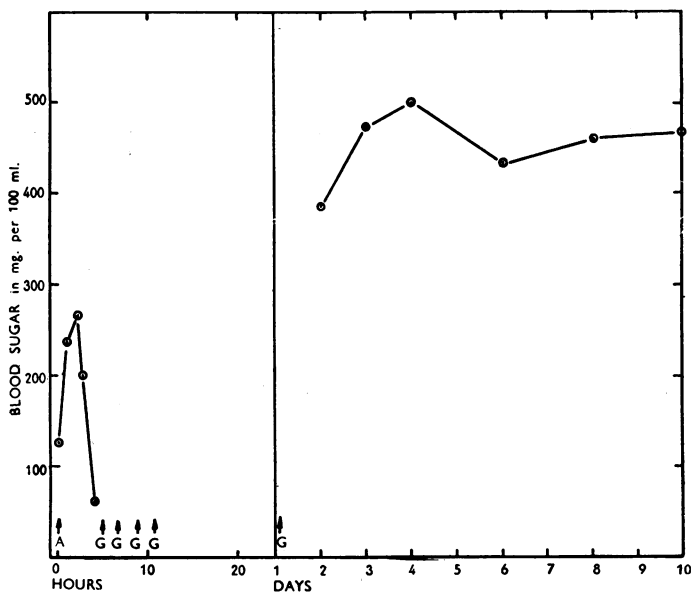

FIG. 1.—Blood sugar changes in unoperated rabbit W.5, given alloxan, 200 mg./kg., i.v., at A. Glucose, 2.5 g. in 50 per cent aqueous solution, given i.v., at G. Rabbit killed after 10 days.

definite coagulative necrosis usually develops (Plate I, Fig. 5). Few islets escape damage and some of the α cells may become involved, so that finally in certain cases it becomes difficult to find normal islet tissue in a number of sections of pancreas. The rate at which these changes occur and their final extent vary from animal to animal, even when the same dose of alloxan is given to all.

In our experiments a single dose of 100 mg. per kg. alloxan was given to each of four rabbits, and a dose of 200 mg. per kg. to a further ten. Thirteen responded with hyperglycaemia followed by a fall in blood sugar to levels at which convulsions supervened. The highest values recorded during the initial hyperglycaemic phase ranged from 155 to 464 mg./100 ml. Three of the animals were given glucose during the hypoglycaemic phase and subsequently developed diabetes with blood sugar levels varying between 350 and 600 mg./100 ml. These three animals were killed at 5, 6 and 10 days respectively after the administration of alloxan and the pancreas removed for histological examination. The pancreas was taken from six of the others at death. In each case definite degenerative changes were found in the islet cells. Four animals died overnight and autolysis was too marked to permit of detailed observations.

In this series only one animal—one which had received the higher dose of alloxan—failed to exhibit marked changes in the blood sugar concentration. This animal was killed seven days after dosing when it had a blood sugar of 115 mg./100 ml. No significant lesions were found in the pancreas. Other workers have encountered the occasional animal which fails to respond to alloxan.

The effect of alloxan at various times after ligation of the pancreatic duct

Young rabbits, 1 to 1.5 kg. in weight, were anaesthetized with ether and a two to three inch laparotomy incision made in the midline from a point about one inch caudal to the xiphoid process. The pancreatic duct was located and a single silk ligature tied around it as near as possible to its point of entry into the duodenum. (In the rabbit the pancreas consists of lobules scattered in the mesentery ; a large duct enters the intestine below the bile duct, while the smaller duct is so atrophied that it is almost impermeable.) The abdominal muscles and skin were closed separately. . Recovery was uneventful and the animals remained in good health and gained in weight. The changes in the pancreas in two rabbits killed at 30 and 56 days respectively after the operation are briefly described below.

Rabbit W.23, killed 30 days after ligation of the duct.

The gland was markedly pale and very much reduced in size. Little more remained than a fibrous cord of tissue surrounding the dilated main duct proximal to the ligature. Histologically the lobules were atrophied and so reduced in size that in any one low power field the remnants of about six were visible (Plate I, Fig. 6). The normal inter-lobular tissue showed a gross increase in collagenous fibres in which persisting normal inter-lobular ducts were present. The acinar tissue proper had undergone extreme atrophy ; the acini showed dilated lumina with a low cubical type of epithelium and occasionally contained hyaline material. Throughout the fibrosed gland the islets, both intra- and inter-lobular in position, stood out clearly as irregular, round or oval masses of cells in which both α and β types could be distinguished (Plate II, Fig. 7).* In silver preparations these islets were seen to be enclosed in a thick felt work of argyrophil reticulum (Plate II, Fig. 8).

Rabbit W.38, killed 56 days after ligation of the duct.

More advanced changes were found in the pancreas of the same nature as those described above ; sclerosis was much more severe ; islet tissue was normal.

(*a*) *Short term ligation. Effect of alloxan.* Two rabbits, W.55 and W.59, were each given a single dose of 200 mg./kg. alloxan on the day after ligation of the duct. The blood sugar changes were indistinguishable from those in unoperated animals treated with alloxan.

One rabbit, W.55, died in hypoglycaemic convulsions overnight, the other was kept alive during the hypoglycaemic phase by repeated injections of glucose and subsequently developed a persistent hyperglycaemia. When killed four days after the administration of alloxan, it had a blood sugar concentration of 552 mg./ 100 ml. Pathological examination of the pancreas showed the lobules of the pancreas to be greatly shrunken, so that in any one low power field as many as about eight could be seen (Plate II, Fig. 9). The acinar tissue showed the initial

* These changes were exactly similar to those described in the early original work of Schultze (1900), Ssobolew (1902), and McCallum (1909).

changes of the same nature as that described for rabbit W.23, i.e., dilation of ductules due to retention stasis, but no gross fibrosis of the inter-lobular stroma. Islets were difficult to find, and many must have disappeared entirely. Those remaining (Plate II, Fig. 10) were shrunken and showed changes such as have been described for unoperated animals treated with alloxan.

(*b*) *Long term ligation.* A number of rabbits were treated with alloxan at longer times, ranging from 23 to 58 days, after ligation of the duct; the effects were very different from those in unoperated rabbits and are described below.

Rabbit W.26. Alloxan, 100 mg./kg., given 30 days after duct ligation.

No significant change in the blood sugar concentration occurred within the first seven hours of dosing or at twenty-four hours (Fig. 2), and at this point, therefore, a further dose of 200 mg./kg. was given. No significant change occurred subsequently, and when the animal was killed eight days later the blood sugar was still normal.

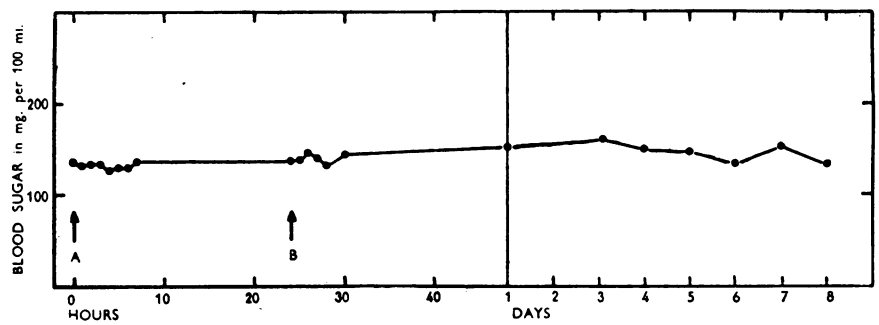

FIG. 2.—Blood sugar changes in rabbit W.26, pancreatic duct ligated 30 days before the administration of alloxan, 100 mg./kg., i.v., at A. Alloxan 200 mg./kg. i.v. given at B. Rabbit killed at 10 days.

The pancreas showed a severe sclerosis such as has been described for rabbits W.23 and W.38. The islets were easy to find and prominent amidst grossly thickened collagenous stroma or as solid cellular bodies in atrophic parenchyma (Plate II, Fig. 11). Normal α and β cells were present.

Rabbits W.27, W.36 and W.37. Alloxan, 200 mg. per kg., given 34, 53 and 51 days respectively after duct ligation. Killed four days later. For blood sugar changes see Fig. 3.

In *rabbit W.27* severe pancreatic sclerosis was found. The islets were prominent in the fibrosed stroma and along the grossly thickened wall of the main duct, and most of the atrophic lobules showed one or two. Both α and β cells could be clearly distinguished and showed no evidence of degeneration.

In *rabbit W.36* very advanced pancreatic sclerosis was found. Scattered

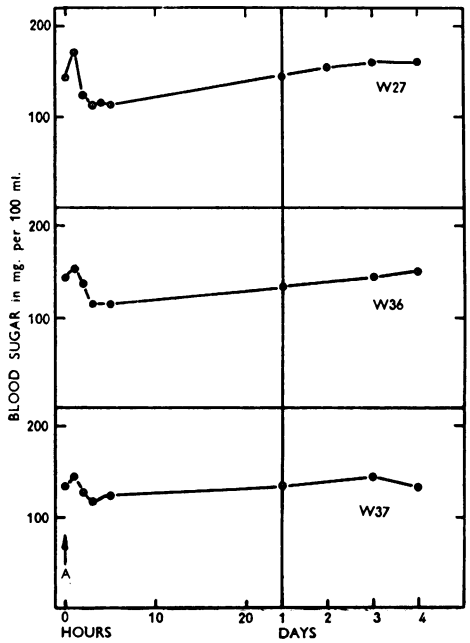

FIG. 3.—Blood sugar changes in rabbits W.27, W.36 and W.37, pancreatic duct ligated 34, 53 and 51 days, respectively, before the administration of alloxan 200 mg./kg. i.v., at A. Rabbits killed 4 days later.

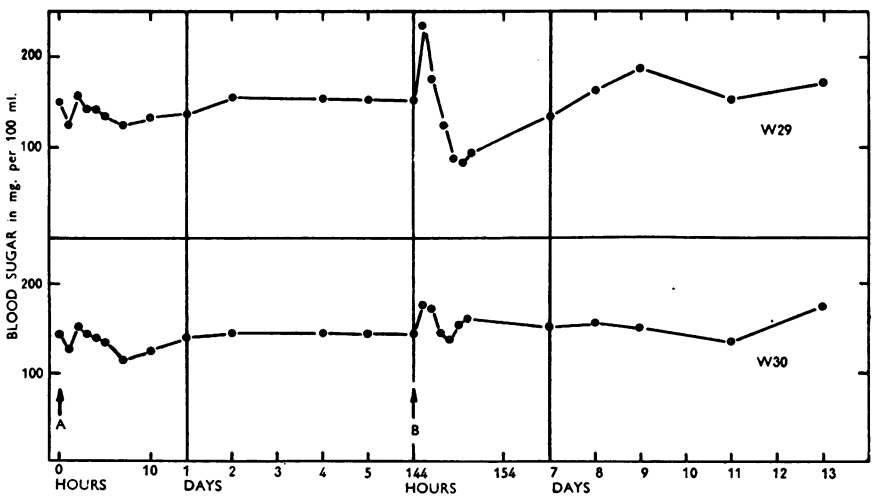

FIG. 4.—Blood sugar changes in rabbits W.29 and W.30, pancreatic duct ligated 31 and 23 days, respectively, before the administration of alloxan 200 mg./kg., i.v., at A. Alloxan 300 mg./kg., i.v., given at B, 6 days later. Rabbits killed 13 days after the first injection.

throughout the abundant collagenous tissue normal islets stood out as small and large, round or irregular compact masses of cells (Plate II, Fig. 12).

In *rabbit W.37* the appearance of the pancreas was almost identical with that in rabbit W.36.

Rabbits W.29 and W.30. Alloxan, 200 mg./kg., given 31 and 23 days respectively after duct ligation.

Blood sugar changes up to six days after dosing were slight (Fig. 4) and at this point the animals were each given a further dose of 300 mg. per kg. The blood sugar changes thereafter were of the same general nature as those produced by alloxan in unoperated animals but were less pronounced and there was no marked persistent hyperglycaemia. The animals were killed seven days after the second injection.

Advanced pancreatic sclerosis was found in both cases. Almost as much normal islet tissue was present as remnants of atrophied parenchyma. Peri-ductal islet tissue was particularly prominent along the course of the main duct.

Rabbits W.40 and W.73. Alloxan, 400 mg. per kg., given 58 and 39 days respectively after ligation of the duct. For blood sugar changes see Fig. 5.

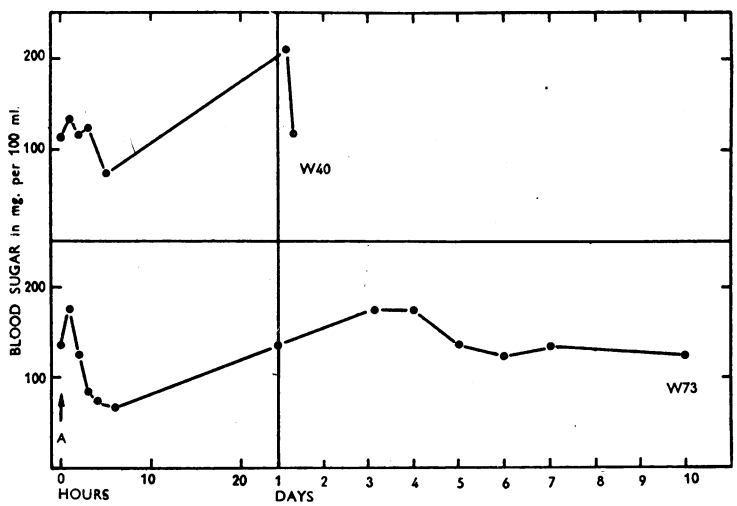

FIG. 5.—Blood sugar changes in rabbits W.40 and W.73, pancreatic duct ligated 58 and 39 days, respectively, before the administration of alloxan 400 mg./kg., i.v. at A. Rabbit W.40 died after 24 hours, rabbit W.73 was killed 10 days after the injection.

Rabbit W.40 had a blood sugar concentration of 210 mg. per cent on the morning following the day of injection. It died at 4 p.m. on the same day. The blood sugar concentration determined on blood taken from the heart immediately after death was 118 mg./100 ml. Post-mortem examination revealed hydrothorax, hydropericardium and pulmonary oedema. Histologically the lungs showed areas of alveolar haemorrhage and oedema, and the kidneys a diffuse,

patchy condition of tubular necrosis, particularly affecting the proximal convoluted tubules, with haemoglobin casts in the lower parts of the nephron. Pancreatic sclerosis was more advanced than that seen in any other animal. The gland had shrunk to a very small fibrous mass in which were ducts with cystic dilatations. There was calcification of the stroma, of the intima and media of the blood vessels and of some parts of the walls of the persisting ducts. There was a moderate degree of lymphocytic infiltration. Normal islet tissue was abundant and prominent. The death of this animal cannot be attributed to damage of the pancreas. (The renal lesion in this animal is similar to that described by Dunn *et al.* (1943b) as a nephrotoxic effect of alloxan, and, as they stated then, is probably due to the concentration of the agent in the tubules in process of excretion. This, however, has no direct significance as far as the main object of this paper is concerned.)

Rabbit W.73 was killed ten days after being given alloxan. The pancreas was similar to that of rabbit W.40, but there was neither calcification nor lymphocytic infiltration. Islet tissue did not appear to be so prominent in this animal, and in a few islets minor degrees of cellular damage were observed.

Discussion

The results described above leave no doubt that ligation of the main pancreatic duct in the rabbit leads in time to almost complete resistance to alloxan in so far as its action upon the islet cells, with the consequent production of diabetes, is concerned.

The operation leads to atrophy and replacement fibrosis of the pancreatic parenchyma proper while the islets remain but slightly affected, even after very long periods. With the development of sclerosis they become enclosed in a thick felt-work of argyrophil reticulum and appear perhaps somewhat more compact than in the normal animal, but most of their cells remain normal in appearance. These changes are exactly similar to those occurring in the so-called chronic interstitial pancreatitis in man, which commonly results from occlusion of the duct by calculi (see Warren, 1930). The fact that the fasting blood sugar remained within but slightly elevated limits after ligation of the duct is further evidence for the persistence of healthy functioning islet tissue. The mean fasting level of 16 normal animals of our stock was 118.4 mg./100 ml., with extremes of 105 and 130, while that of the eight animals in our experiments in which the duct had been ligated for times ranging from 23 to 58 days was 136.1 mg./100 ml., with extremes of 110 and 149.

In rabbits in which the duct had been ligated for 23 or more days, alloxan in doses up to 400 mg./kg. produced in most cases comparatively slight fluctuations in the blood sugar level. In no case were hypoglycaemic convulsions or a final persistent hyperglycaemia observed. In no case, moreover, was marked damage to the islet cells encountered. (The possibility of alloxan having caused damage to some islets and of their subsequent regeneration cannot, of course, be excluded.)

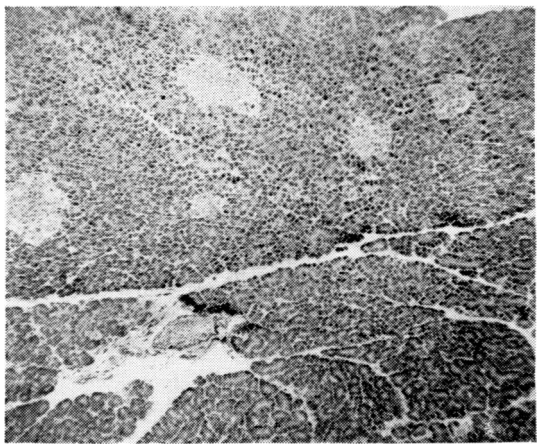

FIG. 1.—Pancreas, normal rabbit. Showing six pale staining islets standing out against the darker stained parenchyma.
15.46Q.– H.E.×75.

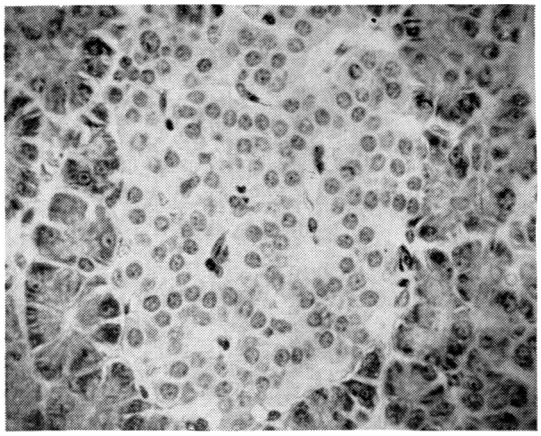

FIG. 2.—Pancreas, normal rabbit. Islets showing anastomosing cords of cells, cytoplasmic outlines obscured, round nuclei with even powdered distribution of chromatin. Minute vascular channels. Field from Fig. 1.
H.E.×400.

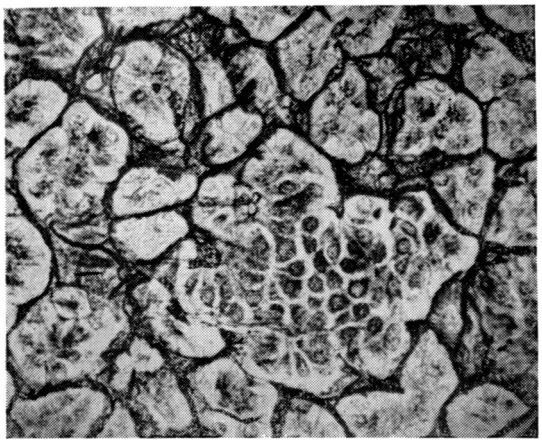

FIG. 3.—Pancreas, normal rabbit. Showing distribution of collagenous fibres and reticulum. The islets contain no reticulum.
Wilder's method and van Gieson×400.

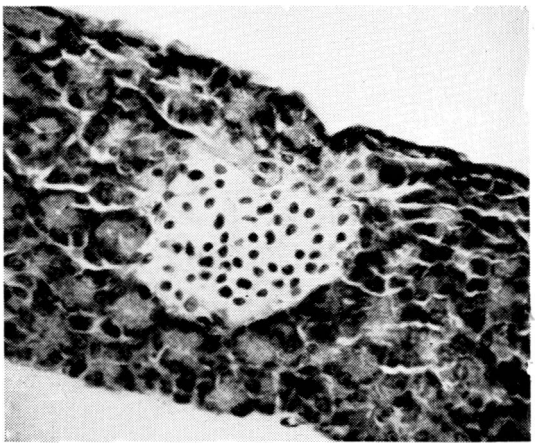

FIG. 4.—Pancreas, rabbit. Effects of alloxan. Rabbit killed after 7 hours. Shows minor degenerative changes and hyperchromatism of the β cells of the islet. Rabbi W.34
15.46E– H.E.×400.

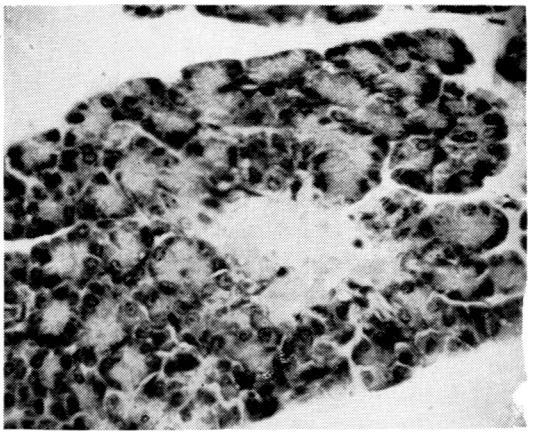

FIG. 5.—Pancreas, rabbit. Effect of alloxan. Showir definite coagulative necrosis of islet. Rabbit W.35.
15.46G.– H.E.×400

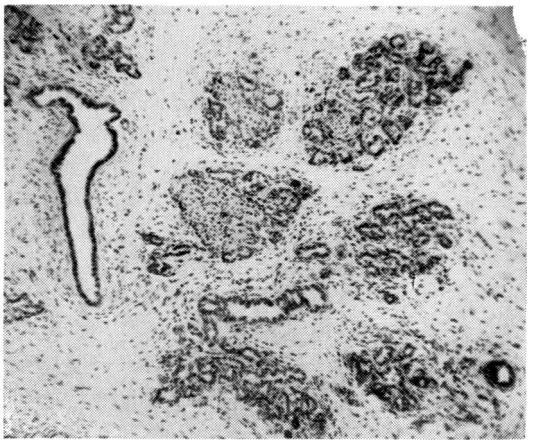

FIG. 6.—Pancreas, rabbit. Duct ligated, animal killed after 30 days. Showing atrophic lobules, dilatation of persisting acini and replacement fibrosis. Normal islet tissue. Rabbit W.23.
15.46A.– H.E.×75.

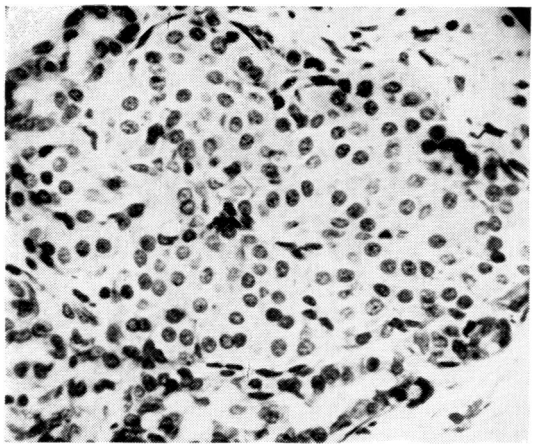

FIG. 7.—Pancreas, rabbit, from same animal as Fig. 6. Islet showing normal appearance.
H.E. × 400.

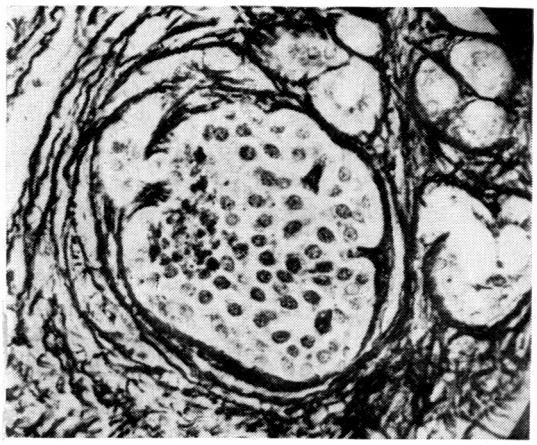

FIG. 8.—Pancreas, rabbit, from same animal as Figs. 6. nd 7. Showing fibrosis and the thick felt work of fibrils round islet.
Wilder's method and van Gieson × 400.

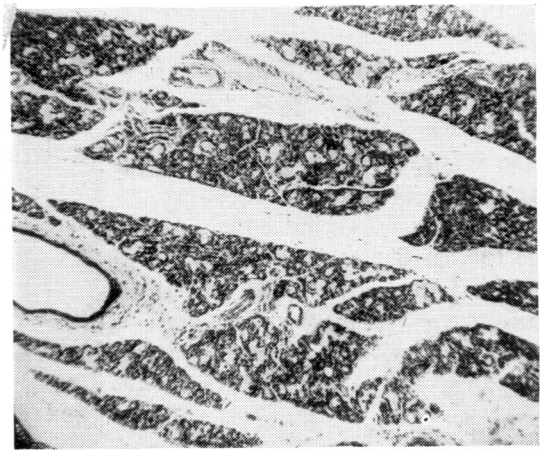

FIG. 9.—Pancreas, rabbit. Effect of alloxan injected shortly after iigation (24 hours) of duct. Atrophic lobules, beginning of fibrotic changes. Rabbit W.59.
15.46H.—H.E. × 75.

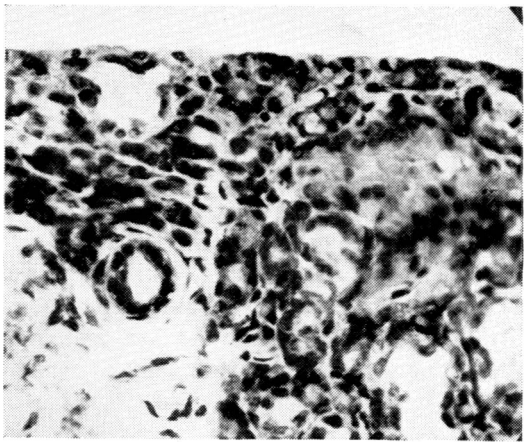

FIG. 10.—Pancreas, rabbit, from same animal as Fig. 9. Showing islet under high magnification with necrotic changes. H.E. × 400.

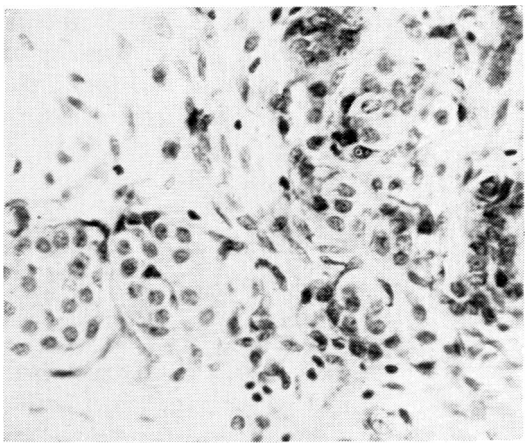

FIG. 11.—Pancreas, rabbit. Effect of alloxan after prolonged period of ligation of duct (30 days). Showing condition of pancreatic sclerosis but normal islets. Rabbit W.26. 15.46.—H.E. × 400.

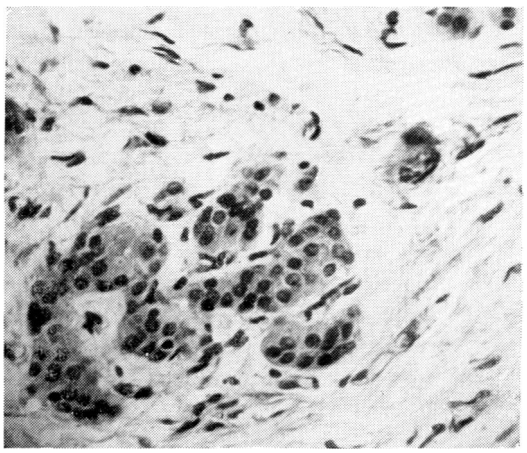

FIG. 12.—Pancreas. rabbit. Effect of alloxan after prolonged ligation of duct (53 days). Very advanced pancreatic scierosis. Normal islets. Rabbit W.36.
15.46K.—H.E. × 400.

Since resistance to alloxan develops *pari passu* with atrophy and replacement fibrosis in the acinar tissue it seems reasonable to conclude that it is a direct result of these changes.

The nature of the mechanism involved is not clear. It may be simply physical —explanations of this kind have been given traditionally, though without much real evidence to support them, for somewhat analogous phenomena. The fibrosis which develops in the duct-ligated gland may produce such a degree of ischaemia that alloxan subsequently administered cannot reach the islet cells in sufficient concentration to produce its necrotic effect before it is fixed or destroyed in other tissues. It is known that alloxan disappears very rapidly from the blood stream. Leech and Bailey (1945) were able to demonstrate the compound in the blood for only five minutes after intravenous injection and Gomori and Goldner (1945) showed that in the dog its toxic activity towards the islet cells was limited to the few minutes immediately after administration. They clamped off a portion of the pancreas during and for periods of from one to six minutes after intravenous injection of alloxan and found that this procedure protected the clamped off portion of the gland from damage, while in the unclamped parts necrosis developed in the β-cells of the islets. This physical explanation is perhaps further supported by our observation that massive doses of alloxan produced some damage in the surviving islet cells of one duct-ligated rabbit. The fact remains, however, that in all our experimental animals the vascular supply to the pancreas was sufficient to prevent general ischaemic necrosis of the sclerotic organ.

If we reject this explanation we can only conclude from our results that the acinar tissue is in some way involved in the action of alloxan upon the islets. The precise nature of any such involvement remains undetermined.

It may be (*a*) that alloxan facilitates the access of trypsinogen in an activated form to the islet cells and that the enzyme is immediately responsible for their necrosis, or (*b*) that it is not alloxan itself but some product of its interaction with the acinar secretion that causes damage to the islets.

The participation of trypsin appears to be excluded by observations of Rich and Duff (1936). They found that trypsin, trypsinogen, or crude pancreatic extracts injected into the main pancreatic duct of the dog produced at first mainly vascular damage—necrosis of vessel walls and haemorrhage—and ultimately a condition similar to acute haemorrhagic pancreatitis in man, with extensive general damage to the organ. They showed also that bile, injected similarly in quite small amounts, was sufficient to cause rupture of the terminal ductules and acini and liberate into the inter-acinar tissue some substance in sufficient quantity to cause similar lesions. There was no evidence that any of these procedures resulted in selective damage to islet tissue. If alloxan did in fact activate trypsinogen and in some way facilitate its access to the islet cells one would expect it to produce widespread pancreatic necrosis and haemorrhage—effects, that is, similar to those observed by Rich and Duff. The selective nature of the damage produced by alloxan would thus appear to exclude the possibility

of its acting in this way. It does not exclude the possibility that, as our findings strongly suggest, it is alloxan in combination with actively functioning acinar tissue which is responsible for necrosis of the islets and the consequent development of a diabetic syndrome. This, if confirmed, might have an important bearing upon the pathogenesis of spontaneous diabetes in man.

SUMMARY

1. The observation of Shaw Dunn *et al.* (1943) and others of the production of diabetes in the rabbit by alloxan has been confirmed.

2. In rabbits in which the main pancreatic duct has been ligated for 30 to 60 days, pancreatic sclerosis with persistence of normal islet tissue is found.

3. In such animals alloxan has little or no pathological effect upon the islet tissue and does not produce diabetes.

4. Possible mechanisms for this effect are discussed.

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ACTIONS OF CYANATE

BY

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The pharmacological actions of cyanate, except some recently described by one of us (Schütz 1945, 1946*a*), do not appear to have been previously investigated. This paper describes some of the general effects.

Preparations.—Commercial preparations of sodium or potassium cyanate usually contain varying amounts of cyanide. No such contamination is present if cyanate is prepared from urea in one of the known ways (Haller, 1886*a* and *b*; Emich, 1889). Only preparations made from urea were used in the present investigation.

Ammonium cyanate was prepared from pure sodium cyanate (previously obtained from urea) through the silver salt. Silver cyanate, precipitated by mixing solutions of silver nitrate and sodium cyanate, was washed with water and acetone; a suspension of the dry salt in an aqueous solution of one equivalent of ammonium chloride was shaken for 1 hour; silver cyanate has a very low solubility and the exchange into ammonium cyanate takes place slowly, leaving behind solid silver chloride. After 45–60 minutes the exchange is complete ($AgCNO + NH_4Cl = NH_4CNO + AgCl$), and after centrifuging, the solution of ammonium cyanate was used within the next half hour. Solutions of ammonium cyanate are known to undergo the isomeric change into urea at an appreciable rate, even at room temperature. Solutions of sodium cyanate are, in comparison, much more stable, and were freshly made up each day.

General effects in rats.—Doses of 5–15 mg./100 g. sodium cyanate, injected intramuscularly, produced marked drowsiness after 10–15 minutes. If undisturbed the rats slept almost continuously, but during the sleep they could always be easily awakened. If suddenly aroused they sometimes exhibited a very short state of excitement, when they jumped once or twice, but they soon settled down again to sleep and complete immobility.

With higher doses (20 mg./100 g.) some secretion from the eyes and nose was observed. With small doses (5–10 mg.), which still produced marked drowsiness, no such increased secretion was observed. Even after very small doses (3–6 mg./100 g.) the tendency to keep the eyes closed for some time was evident.

A graphical record of the activity of rats was made by a device which registers the movements of two suspended cages (Schütz, 1946*b*). Six rats were given cyanate and were placed in one cage and six similar controls, which were given water instead of cyanate, in another. A typical record is shown in Fig. 1.

The minimum effective dose which influenced the motility of rats (120–150 g.) was found to be 2–3 mg./100 g. These doses produce a short but definite drowsiness. The effect of such small doses could, however, only be detected when the injections were given shortly before or during the normal time of wakefulness of the rat, i.e., after sunset, when the controls were invariably at the height of activity. In day time, when rats have their natural sleep periods, higher doses were required in order to produce a detectable difference; a significant difference could then be obtained after injection of 6–8 mg./100 g.

The drowsiness was marked but full anaesthesia could not be produced.

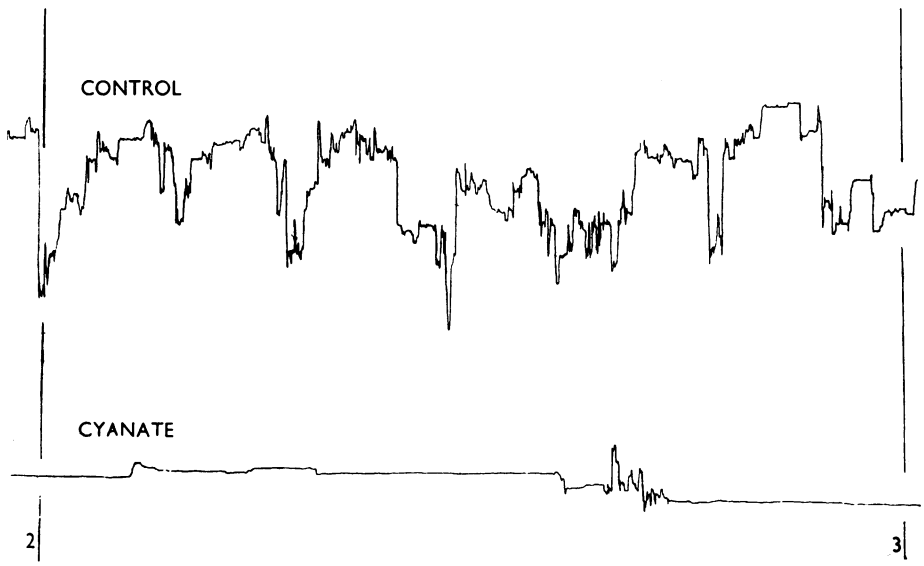

FIG. 1.—Motility record of 2 cages each containing 6 rats. The control (upper) record shows the activity characteristic for the peak period of wakefulness of rats during the early part of the night. The cyanate (lower) record shows the effect of 8 mg./100 g. sodium cyanate, given intramuscularly two hours before. Note the cessation of activity after cyanate, interrupted for a short period. Both records are for the third hour after the injections.

Although deeply asleep, the rats could always be aroused. As can be seen from the motility record shown in Fig. 1, short periods of activity occur spontaneously between periods of profound quiescence. In this point the drowsiness caused by cyanate seems to differ essentially from the effect of any of the usual narcotic drugs, although the drowsiness lasts several hours after a dose of 10 mg./100 g.

The threshold towards some stimuli did not seem to be much raised after small doses (5 mg./100 g.), a sharp hiss or pinching of the tail usually eliciting the same reaction in the experimental and the control group.

Ammonium cyanate had apparently the same effect as sodium cyanate; an accurate comparison was difficult since the former is rapidly transformed into urea even at room temperature. In three experiments the drowsiness appeared

perhaps even more marked, though shorter in duration, after ammonium cyanate than after sodium cyanate.

The drowsiness wore off completely, according to the dose given, e.g., 5–6 hours after 10 mg./100 g. intramuscularly, 8–9 hours after 15 mg./100 g. After that period, and especially when higher doses were given, it was regularly noticed that the rats had not only recovered completely from the profound drowsiness, but also that they exhibited a certain increase in activity, although this could not be called a state of excitement. The rats which had received a large dose of cyanate the day before could always be recognized on the next day by their greater liveliness.

Acute toxicity.—Although the actions of sodium cyanate are not strictly comparable with those of phenobarbitone, the L.D. 50 of each was determined simultaneously for female albino rats (100–150 g.) of the same kind.

For sodium cyanate this was found to be 31 (± 5) mg./100 g. body weight, for phenobarbitone, also injected intramuscularly, 19 (± 6) mg./100 g. The figures ($\pm$ s.d.) were obtained from a graphical estimate according to Gaddum's method (1933), using a group of 12 rats at three dose levels. The L.D. 50, expressed in gram molecules is thus for sodium cyanate 4.76×10^{-4}/100 g., for phenobarbitone 8×10^{-4}/100 g.

Terminal convulsion.—If the lethal dose was in the region of 60–150 mg./100 g., death ensued with a terminal convulsion which was characteristic for cyanate. Non-lethal doses never caused convulsions in rats. The drowsiness gradually increased, the animals became apparently very weak and lay flat on the abdomen, without much support from the legs. The eyes were tightly shut and there were a few occasional movements, usually a short wiping of the nose and eyes with the forelegs. At the end of this period, a short convulsion was always seen. The animal very rarely recovered, but if it did, another convulsion occurred after an interval which might be several minutes, or as long as half an hour. No rat was seen to recover from this second convulsion.

Two stages of the convulsions could clearly be distinguished. The first stage started with the rat suddenly turning round and throwing itself either on its back or side. The head was maximally bent down and forwards, the nose and mouth being closely pressed against the breast. The eyes were tightly shut. From them and the mouth a small quantity of liquid was sometimes expelled. The lips were extremely retracted, producing a curious "grin-like" expression of the mouth, while the jaw seemed to be clenched. The ears were laid back. There was arrest of respiration. The forelegs were straight, extended, and maximally adducted to the abdomen. The paws were tightly bunched. The hindlegs were maximally stretched, the toes extended and fully splayed. Faeces were often expelled and, more rarely, urine. This stage only lasted 5–10 seconds. During the second stage many of the characteristics of the first one were altered in such a way that the ultimate death posture resulted. During this stage, clonic movements of apparently all the muscles occurred, while the head, ears, forelegs, and toes returned slowly to a more normal position, but the maximal extension of the hindlegs remained. During this stage, one could hear a deep inspiration. Sometimes the animal lay without any visible respiration, resuming its respiration gradually, but dying soon afterwards in another similar convulsion.

Diuretic action.—Intramuscular injections of both sodium and ammonium cyanate were followed by a marked diuresis (Schütz, 1946*a*). This effect was

evident with rats and rabbits, even if no excess water was given previously. Typical results are shown in Fig. 2. For these experiments the rats were fully hydrated. They were given food and water up to the time of the injections. It can be seen that in the doses given, cyanate was at least as potent in rats as a number of other well-known diuretics. In order to investigate whether the diuretic effect of injected cyanate could be ascribed to ammonia, which may be formed from cyanate in the body, an equivalent amount of ammonia was injected and found to be ineffective. For the same reason the action of urea, cyanide, and thiocyanate was recorded with similar animals.

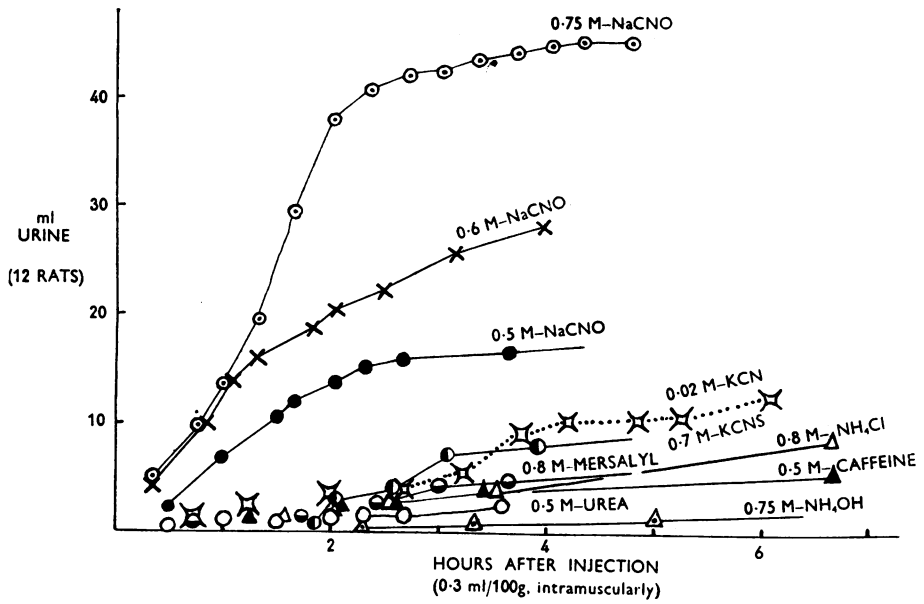

FIG. 2.—Diuretic action of cyanate. Doses 0.3 ml./100 g. No excess water was given previously.

From the evidence submitted in Fig. 2 it can be concluded that the diuretic action must be due to the CNO group, and not to an eventual breakdown or transformation product. Ammonia was quite inactive; the diuretic potency of urea was found to be approximately a thirtieth of that of cyanate. Cyanide could not, for obvious reasons, be given in the same doses. Fig. 2 shows the diuretic effect of a dose of KCN which is approximately 50 per cent of a dose found to be lethal in 10 rats.

Blood sugar.—Intravenous injection of sodium cyanate in rabbits was followed by a similar, but less obvious, drowsiness to that observed in rats. A small and transient rise of the blood sugar was recorded with doses from 5 mg./100 g. upwards. Typical results are shown in Table I.

TABLE I

SMALL AND TRANSIENT RISE OF BLOOD SUGAR IN RABBITS AFTER INTRAVENOUS INJECTION OF SODIUM CYANATE.

Dose, in mg./100 g. body weight	Blood sugar in mg./100 ml. at stated intervals of time after the injection					
	30′	60′	90′	120′	150′	24 hr.
5 mg.NaCNO	105	145	160	148	120	112
9 mg.NaCNO	112	165	170	140	115	110
Saline	95	112	106	95	95	95
Saline	95	110	102	100	105	105

Even with the highest tolerated doses only a transient rise was observed. Also after daily intramuscular injections of 6 mg./100 g. over a period of 10 days the blood sugar was found to be within normal limits 24 hours after the last injection (80–90 mg./100 ml., in 6 rabbits, not starved). The observed levels were always rather low, probably owing to the much reduced intake of food mentioned below.

Body temperature.—Intravenous and intramuscular injections in rabbits were followed by a significant fall in body temperature. The rabbits were starved 12 hours before the injections. The effect was taken as the lowest rectal temperature recorded within 3 hours of the injection. The mean value in 6 rabbits receiving saline on three occasions was $38.9° \pm 0.2$ (S.D. mean) while that of 6 rabbits receiving sodium cyanate in three experiments was 37.7 ± 0.3. There was an interval of 3–4 days between the experiments. The drug thus caused a clearly significant mean fall of temperature of $1.2° \pm 0.36$ ($P < 0.1$) compared with the controls. At 24 hours after the last injection the mean temperature of 6 rabbits receiving sodium cyanate in two experiments was 38.8 ± 0.34 and that of 6 control rabbits receiving saline by the same route in two experiments was 38.7 ± 0.31. The temperature was thus in the normal range and not significantly different from that of the control rabbits.

Effects of repeated injections.—While the effects of a single injection of even a large dose of cyanate (10 mg./100 g.) wore off completely, smaller doses repeated daily soon caused a continuous drowsiness in rats and rabbits, only interrupted for eating and drinking. This was very marked from the third or fourth injection onwards. Similarly, the effect of smaller doses seemed to increase. Of 24 rats which were given 6 mg./100 g. daily two died within 2 weeks. Soon after the injections were stopped no abnormality in the behaviour of the rats could be observed. No withdrawal symptoms were noted. During the period of the injections the rats rarely moved at all, except to take food and drink. They lost weight and ate less, but drank more and had periods of diarrhoea. At the end

of this period their body weight was on average 34 per cent below that of the controls, which were given daily injections of saline.

Daily intramuscular injections of 10 mg./100 g. in rabbits produced an increasing drowsiness and apathy. They took very little food, although when aroused they reacted very similarly to the controls. They lost weight rapidly and appeared so emaciated that from the fourth injection the dose was reduced to 7.5 mg./100 g. and from the sixth injection to 5 mg./100 g. Eight rabbits were injected in this way for 18 days. One died on the seventh day. The others recovered completely and soon gained weight. Their blood sugar was within normal limits.

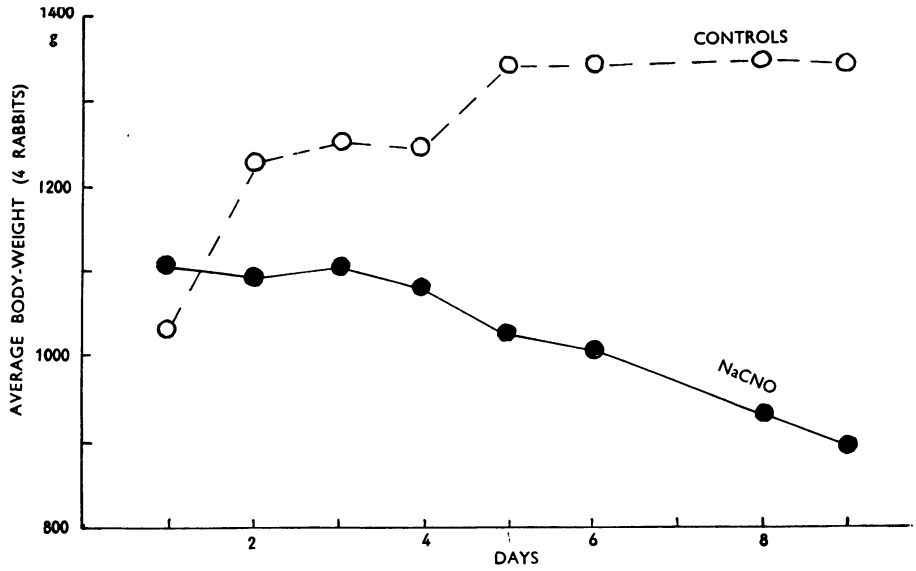

FIG. 3.—Fall of average body weight of 4 rabbits during a period of daily injections of sodium cyanate. Average weight of 4 saline-treated controls is also shown.

The average weight of two groups of 4 rabbits is shown in Fig. 3. One group received daily intramuscular injections of sodium cyanate (10 mg./100 g.) and the control group received the same volume of saline.

The faeces of both rats and rabbits usually became very moist in consistency. This moistness, however, often disappeared, although the dose was not changed. Sometimes the faeces were found to be much paler in colour than those of the controls. This symptom, which was not regularly seen, disappeared without any change in the daily dosage.

Rabbits and guinea-pigs which were injected with doses causing death after 6–8 days showed an almost empty intestinal tract. No formed faeces were found and there was hardly any visible fat in those rabbits which received the injections for at least one week. The heart was always found distended and full of blood. A definite hyperaemia was observed in the lower pelvic region. No other obvious abnormality was found. The absorption spectrum of the blood of these animals showed no difference from that of normals as observed with the Hartridge reversion spectroscope.

Although no determination of the L.D. 50 in rabbits and guinea-pigs was attempted, the few data obtained with these animals suggested that the toxicity of sodium cyanate given intramuscularly was of the same order of magnitude for rabbits, guinea-pigs, and rats. On repeated injections rabbits seemed to be more sensitive than rats.

Comparison with cyanide.—Although injected cyanate is probably transformed into urea in the body, the possibility could not be excluded *a priori* that cyanate might be reduced to cyanide in the body. After repeated injections of cyanate into three rabbits, with a lethal dose as the last injection, no trace of cyanide could be found in their blood and collected organs. The same method of detection (Feigl, 1943) gave a strongly positive test for cyanide in the blood and organs of a rabbit which received a minimum lethal dose of cyanide. When the actions of cyanide were compared with those of cyanate in similar rats a number of differences were observed. After injection of cyanide, rats became dull and lifeless and moved very little, but they did not appear to have the long and deep sleep which was produced by medium doses of cyanate. The cyanide rats usually had their eyes wide open and the drowsiness never lasted longer than ½–1 hour. A further striking difference between cyanate and cyanide was the terminal convulsion which was produced after the former drug, and the death posture regularly found after it. Cyanide, as well as cyanate, had a marked diuretic action. Compared with its toxicity, cyanide was, however, much less active than cyanate.

Discussion

The main actions of cyanate, as observed in the whole animal, which are reported in this paper, were compared with those which can be produced by ammonia, urea, cyanide, and thiocyanate. All these comparisons, as well as the identical action obtained with pure preparations of sodium and ammonium cyanate made from urea, support the view that the actions are due to the CNO group and not to any of the above-mentioned substances into which injected cyanate might conceivably be transformed in the body.

Summary

1. The general effects of injected sodium and ammonium cyanate on the intact animal were studied.

2. Cyanate produces pronounced drowsiness and sleep in rats, but not full anaesthesia.

3. Lethal doses produce a characteristic terminal convulsion and death posture in rats.

4. Intramuscular injections of cyanate produce a marked diuresis and diarrhoea in rats and rabbits.

5. There is a small rise of blood sugar and a small fall of body temperature after the injection of cyanate into rabbits. Both changes are transient.

6. Repeated daily injections of cyanate lead to a rapid fall in body weight. Blood sugar and body temperature remain within normal limits.

7. It is concluded that the actions are due to the CNO group and not to any substance into which injected cyanate may conceivably be transformed in the body (urea, ammonia, cyanide, or thiocyanate).

We are greatly indebted to Mr. Garfield Thomas for having determined the blood sugar levels mentioned in this paper and to Dr. R. F. A. Dean and Professor A. C. Frazer for help given in the presentation of our results. Thanks are also due to Glaxo Laboratories for presenting us with a specially prepared sample of pure sodium cyanate, and to John Tatlow for technical assistance.

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THE ACTION OF MERSALYL, CALOMEL AND THEOPHYLLINE SODIUM ACETATE ON THE KIDNEY OF THE RAT

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It has been shown that the creatinine clearance in man (Blumgart, Gilligan, Levy, Brown, and Volk, 1934) or dogs (Davenport, Fulton, Van Auken and Parsons, 1934; Walker, Schmidt, Elsom, and Johnston, 1937) is not increased by mercurial compounds; nor did thermostromuhr measurements in anaesthetized dogs reveal any constant changes of the renal blood flow when mercurial diuretics were injected (Walker *et al.*, 1937). It was, therefore, concluded that the mercurial compounds exert their diuretic effect by reducing the tubular water reabsorption. Objections could be raised against the interpretation of some of these experiments: (1) the creatinine clearance can no longer be regarded as the expression of the glomerular filtration rate in man (Shannon, 1935); (2) anaesthetics or surgical measures like those necessary for thermostromuhr measurements are likely to have a disturbing effect on renal function (Smith, 1943). It was, therefore, decided to reinvestigate the renal action of mercurial compounds in unanaesthetized intact animals by means of simultaneous inulin and diodone clearance estimations. The albino rat was chosen because it had previously been shown (Dicker and Heller, 1945) that renal function in this species resembles that of dog and man.

METHODS

Experimental animals.—Male adult albino rats of an average weight of 176.5 g. were used throughout. The animals were kept on a standard diet (vitamin A test diet, U.S.P., XI revised 1937, with the addition of cod liver oil and of tocopherol) for some time before the experiments were done.

Experimental procedure.—The experimental procedures for the determination of simultaneous inulin and diodone clearances in rats have been described in a previous paper

(Dicker and Heller, 1945). Inulin in plasma and urine was determined by the method of Smith, Goldring, and Chasis (1938), and diodone iodine in plasma and urine by that of Alpert (1941). Chloride in plasma was estimated by Whitehorn's method (1921); chloride in urine by that of Volhard-Arnold. Chlorides were expressed in terms of NaCl. Inulin (Kerfoot & Co.) and Per-Abrodil (Bayer Products, Ltd.) were used.

Diuretic drugs used.—(1) Mersalyl (Burroughs, Wellcome & Co., London) is described by the firm of makers as "a complex mercurial compound containing about 40% of the metal in a non-ionizable form." It contains also 5% of theophylline sodium acetate.

(2) Mercurious chloride (calomel). The preparation used was a 0.14% suspension of mercurous chloride in adeps lanae and ol. arachidis.

(3) Theophylline sodium acetate.

Mode of administration of the diuretics.—Mersalyl and calomel were injected intramuscularly. Theophylline sodium acetate was administered either by stomach tube or by intramuscular injection.

Methods for quantitative comparison of diuretic activity.—During the period of experimentation, the animals were kept in individual glass metabolism cages, as previously described (Dicker and Heller, 1945). In the experiments with mersalyl, calomel, and theophylline sodium acetate, the rats were given 5% of their body weight of tap water by stomach tube. The action of these drugs on the rate of urine flow was compared with that of non-injected control rats which received the same amount of water. The volume of urine was measured at intervals of 30 minutes, during a total period of 3 hours, and expressed as percentage of the volume of water given.

Evaluation of clearance estimations.—The nomenclature and method of calculation of glomerular filtration rate (GFR), effective renal plasma flow (RPF) and total tubular excretory mass (Tm_D) follow substantially those adopted in the publications of Smith (1943) and his associates. The rate of tubular reabsorption of chloride (T_{Cl}) was calculated as follows: $T_{Cl}=(P_{Cl}\times C_{In})-(U_{Cl}\times V)$ where P_{Cl} = concentration of plasma chloride in mg./100 ml., C_{In} = inulin clearance in ml./100 g./min., U_{Cl} = concentration of urinary chloride in mg./100 ml., and V = urine flow in ml./min. The rate of the tubular water reabsorption (T_W) was calculated as $T_W=C_{In}-V$. In order to permit the comparison of T_W and T_{Cl} values obtained at different values of GFR, T_W and T_{Cl} were regularly expressed as the percentage of water and chloride filtered.

Statistical treatment of results.—Fisher's "t" test was applied for estimation of the significance of the difference of means. "Small sample" methods were used for populations less than 15. The correlation coefficient "r" and the interclass coefficient "z" were calculated according to Mainland (1938). The probability (P) for "t" or "r" was obtained from Fisher and Yates' (1943) tables.

RESULTS

1. Normal rats

To obtain a basis of comparison for the results of the experiments with diuretic compounds, values for the different renal functions in normal control rats have first to be given.

Normal rats which received 5% of their body weight of water excreted in 180 min. $81.5\pm2.66\%$ (S.E. of mean of 108 observations) of the amount of water given (Fig. 1); expressed in terms of ml./100 g. body weight/min. they excreted

during these three hours an average of 0.023 ± 0.0004 ml./100 g./min. of urine, with a mean sodium chloride concentration of 0.8 ± 0.08 mg./100 g./min.

The renal function tests during such a water diuresis showed that the glomerular filtration rate (GFR), the renal plasma flow (RPF), the filtration fraction (FF), the total tubular excretory mass (Tm_D), and the rate of tubular chloride reabsorption (T_C) remained practically unchanged for urine flows ranging from 0.0017 to 0.1030 ml./100 g./min. The mean GFR amounted to 0.35 ± 0.005 (134) ml./100 g./min. and the mean RPF to 2.22 ± 0.006 (28) ml./100 g./min.; the mean FF was 0.17 ± 0.003 (28), the mean $Tm_D = 0.13 \pm 0.002$ (84) mg./100 g./min., and the mean $T_{Cl} = 97.1 \pm 0.25\%$ (41). Figures in parentheses indicate the number of observations.

In contrast to the stability of these different functions, the rate of tubular water reabsorption (T_W) showed a decrease closely correlated with the increase of the rate of urine flow ($r = -0.99$, S.E. $= \pm 0.089$, $P < 0.001$ (134)).

It can therefore be concluded that a water diuresis in a normal rat is solely effected by changes in the rate of tubular water reabsorption.

II. Renal effects of mersalyl

Two series of experiments were performed; in the first, rats were injected with a low, non-diuretic amount of mersalyl (0.0006 mM./100 g.); in the second, the animals were injected with 0.0027 mM./100 g. mersalyl. This last dose has been shown to exert the optimum diuretic effect in rats (Lipschitz, Hadidian, and Kerpcsar, 1943).

(1) Effect of 0.0006 mM./100 g. mersalyl. After injection with mersalyl, the rats were divided into three groups: (i) those which received 5% of their body weight of water after one hour; (ii) those which were given water 2 hours after the injection; (iii) those to which the standard dose of water was given after 10 hours. The rate of urine flow of each group was measured during 3 hours following the administration of water and the quantity excreted expressed in percentage of the water given. No significant difference could be noted between the rate of urinary excretion in any of these rats and that of normal controls which had received the same standard amount of water.

To ascertain whether a non-diuretic amount of mersalyl had any effect on the kidneys at all, inulin and diodone clearance determinations were carried out in the three groups of rats, inulin and diodone being injected about 1 hour before water was given. The results were comparable in the three groups and can be considered together (Table I). The mean value for GFR, RPF, and FF were significantly increased. (t for the respective values was found to be: 11.43, $P < 0.001$; 7.50, $P < 0.001$; and 6.89, $P < 0.001$.)

Tubular activity in these animals as gauged by the rate of chloride reabsorption and the maximum rate of transfer of diodone did not differ significantly from

that of the controls (Table I); (t for the respective values was found to be 1.58, P>0.1; and 0.36, P>0.7.) The rate of tubular water reabsorption was, as in control rats, significantly correlated with the rate of urine flow (r = −0.94; S.E. = ±0.258, P<0.001.)

TABLE I

RENAL EFFECTS OF MERSALYL, CALOMEL, THEOPHYLLINE SODIUM ACETATE AND A HYPOTONIC NACL SOLUTION

The figures are mean results with their standard error. r ± S.E. = correlation coefficient between the value indicated and the urine flow, and standard error of r. Number of experiments in parentheses.

	GFR (Inulin Clearance) ml./100 g./min.		RPF (Diodone Clearance) ml./100 g./min.		Tm_D (mg.I./100 g./min.) M±S.E.	T_{Cl} (as % of chloride filtered) M.±S.E.	T_w (as % of water filtered) r±S.E.
	M.±S.E.	r.±S.E.	M.±S.E.	r.±S.E.			
Control rats (134)	0·35 ±0·005	—	2·22 ±0·006	—	0·13 ±0·002	97·1 ±0·25	−0·99 ±0·089
Mersalyl: 0·0006 mM./100 g. (18)	1·11 ±0·075	—	3·62 ±0·190	—	0·13 ±0·008	97·8 ±0·39	−0·94 ±0·258
Mersalyl: 0·0027 mM./100 g. (20)	1·02 ±0·067	+0·79 ±0·223	3·51 ±0·486	+0·86 ±0·392	0·035 ±0·0098	98·4 ±0·28	−0·97 ±0·223
Calomel: 0·00027 mM./100 g. (18)	1·04 ±0·176	+0·75 ±0·360	3·42 ±0·578	—	0·13 ±0·011	99·5 ±0·07	−0·54 ±0·360
Theophylline: 0·01 mM./100 g. (19)	1·05 ±0·109	+0·72 ±0·330	4·15 ±0·342	+0·71 ±0·313	0·13 ±0·083	98·6 ±0·29	−0·68 ±0·302
NaCl: 85 mM./100 g. (22)	1·08 ±0·035	—	2·72 ±0·154	—	0·14 ±0·009	98·2 ±0·18	−0·90 ±0·210

It can therefore be assumed that the renal effect of a non-diuretic dose of mersalyl is mainly characterized by a vascular reaction, without any appreciable changes in the tubular activity.

(2) *Effect of 0.0027 mM./100 g. mersalyl.* Three groups of well hydrated rats were injected with 0.0027 mM./100 g. mersalyl. The standard amount of water was given 2, 4, and 10 hours after the administration of the diuretic. The urinary excretion was measured for 3 hours, and expressed as percentage of the water given. No diuretic action could be noticed in the two first groups, i.e., in rats which received 5% of their body weight of water 2 and 4 hours after the

injection of mersalyl. But a clear diuretic effect could be observed in the rats to which the standard amount of water was given 10 hours after the mercurial compound. In that group the mean average excretion of urine observed during 3 hours was 0.029 ± 0.0041 (18) ml./100 g./min. (t with control rats = 2.46,

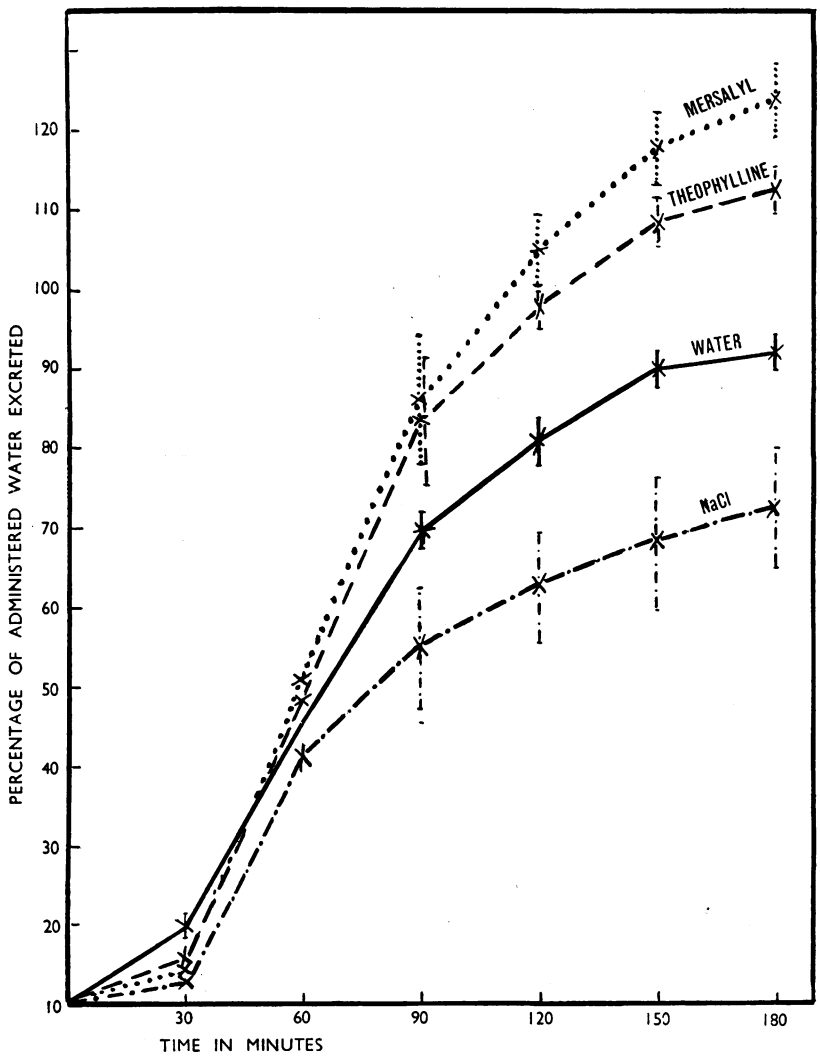

FIG. 1.—Effect of diuretics on the water diuresis of rats. All rats received 5% of their body weight of water by stomach tube. ×————×: controls (water only). ×·····×: rats injected with 0.0027 mM./100 g. mersalyl. ×— — — —×: rats injected with 0.01 mM./100 g. theophylline sodium acetate. ×—·—·—×: rats which received 5.0 ml./100 g. of an 85 mM. NaCl solution by stomach tube. The vertical lines indicate the standard error.

$P<0.02$) with an average sodium chloride concentration of 4.5 ± 0.31 mg./100 g./min. ($t=11.34$, $P<0.001$). Fig. 1 shows the amount of urine excreted during that period, expressed in percentage of the dose of water given ; after 180 minutes the " mersalyl rats " excreted $114.0 \pm 5.55\%$ (18) compared with $81.5 \pm 2.66\%$ ($t=5.66$, $P<0.001$) in the controls. It should be pointed out that in no instance had the mercurial any diuretic effect when water was not supplied to the animals, a fact which agrees with the findings of Fourneau and Melville (1931) and of Sollman and Schreiber (1936).

To determine the renal mechanism responsible for the enhanced diuresis observed in the last group, inulin and diodone were injected about 1 hour before the water was given, i.e., 9 hours after the administration of 0.0027 mM./100 g. mersalyl. Clearance determinations were carried out in the usual way. Most of these rats were found to have sugar in their urine and glucose clearances were therefore estimated, whenever possible, simultaneously with the others. It should be noted that, in nearly all these experiments, an appreciable amount of albumin could be detected.

The mean value for GFR in this series was much the same as that observed after the injection of 0.0006 mM./100 g. mersalyl (mean GFR $=1.02 \pm 0.067$ (20), $t=0.86$, $P>0.4$), but in contrast to the latter was found to be correlated with the urine flow ($r=+0.79$, S.E. $= \pm 0.223$, $P<0.001$) and with the renal plasma flow ($r=+0.83$, S.E. $= \pm 0.267$, $P<0.001$).

The increase of GFR could be explained by a change in the permeability of the glomerular vessels (albuminuria) were it not for the fact that the rate of glomerular filtration is significantly correlated with both the urine flow and the renal plasma flow. It seems, therefore, more likely that the mercurial compound produced, directly or indirectly, an increase in the blood supply, which, in turn, would increase the rate of glomerular filtration. It may in any case be assumed that the enhanced diuresis observed in rats injected with 0.0027 mM./100 g. mersalyl is to some extent a function of the increased rate at which water is filtered through the glomeruli.

The reabsorption of chloride was also found to be enhanced. The values for T_{Cl} were not correlated with the increased GFR or the urine flow. The mean value for T_{Cl} was found to be $98.4 \pm 0.28\%$ (20), which is significantly higher than that observed in control rats ($t=3.54$, $P<0.001$). It can therefore be concluded that in " mersalyl rats " chloride is reabsorbed at a higher rate than in normal animals. But how can this fact be reconciled with the increased concentration of urinary chloride? When the mean plasma chloride concentration was compared with that observed in control rats, it was found that, whereas the mean plasma chloride for normal rats was 356.2 ± 18.40 (41) mg./100 ml., the mean plasma chloride in rats injected with 0.0027 mM./100 g. mersalyl was 430.5 ± 8.68 (20) mg./100 ml., which is significantly different ($t=3.45$, $P<0.001$). This fact agrees with the findings of Crawford and McIntosh (1925), who showed that

after injection of novasurol there was a transfer of chlorides from tissues to plasma. If more chloride is filtered because the plasma chloride concentration is higher or because the glomerular filtration rate is increased, or because both these factors apply, more chloride should be excreted, unless the excess of chloride filtered is reabsorbed by the tubules.

It seems likely that mersalyl has some depressor effect on tubular activity. This is suggested by the markedly decreased Tm_D (mean $Tm_D = 0.035 \pm 0.0098$ mg./100 g./min., as compared with 0.13 ± 0.002 in control rats; $t = 9.74$, $P < 0.001$) and by the fact that glucose was found in the urine of these rats. The objection may be made that changes in the rate of filtration and hence in the amount of glucose delivered to the tubules may produce glycuresis (Shannon, 1938) without any decrease in the capacity for glucose reabsorption. However, glycuresis after mersalyl has been observed at low as well as at high glomerular filtration rates, i.e., in circumstances in which the amount of sugar delivered to the tubules was very low in one instance and very high in another.

III. Renal effects of mercurous chloride

Various doses of mercurous chloride (calomel) were tried. The dose of 0.0027 mM./100 g., approximately equivalent to the mercury content of the higher dose of mersalyl used, proved to be toxic; so was the dose of 0.00135 mM./100 g. calomel, though to a lesser degree. Rats injected with these doses remained in a state of anuria for several hours and developed severe oedema, with large amounts of free fluid in the abdominal cavity. The scanty urine finally passed was regularly mixed with blood. After several trials the dose of 0.00027 mM./100 g. rat was adopted. This dose has been shown to be highly diuretic in rabbits (Fourneau and Melville, 1931).

(a) *Effect of injections of calomel (0.00027 mM./100 g. rat) on the diuresis.* The standard amount of water was given by stomach tube 2, 3, or 10 hours after the injection of calomel; the urine was measured every 30 minutes during 3 hours after water administration. The amounts of urine excreted were expressed, as usual, in percentage of the amount of water given. In none of these groups did calomel exert a diuretic effect; the total quantity of urine excreted in 3 hours was $82.8 \pm 2.47\%$ (48) of the standard amount of water given, a figure which is statistically comparable with that obtained in control rats which had received the same amount of water ($t = 0.36$, $P > 0.7$), but the urine excreted during these 3 hours contained a much higher concentration of sodium chloride than that of normal animals: 1.7 ± 0.30 mg./100 g./min., as compared with 0.8 ± 0.08 mg./100 g./min. ($t = 2.93$, $P < 0.01$), but it contained significantly less than that of "mersalyl rats" ($t = 6.43$, $P < 0.001$).

(b) *Renal effects of 0.00027 mM./100 g. calomel.* The glomerular filtration rate of rats injected with 0.00027 mM./100 g. of calomel was increased (mean

GFR = 1.04 ± 0.176 (17) ml./100 g./min.) and correlated with the rate of urine flow (r = +0.75, S.E. = ±0.360, P<0.001). The renal plasma flow was also increased (mean RPF = 3.42 ± 0.578 (7) ml./100 g./min.), but not correlated with the urine flow.

Concerning the tubular activity, it will be seen (Table I) that the tubular excretory mass (Tm_D) compared with that of control rats was not affected (mean Tm_D = 0.13 ± 0.011 (10) mg./100 g./min. ; t = 0.24, P>0.9), but that the tubular rate of chloride reabsorption was significantly increased (mean T_{Cl} = 99.5 ± 0.07% (17) ; t = 3.11, P<0.001) and comparable to that of rats injected with 0.0027 mM./100 g. mersalyl (t = 1.42, P>0.2). It will also be noted that, as in rats injected with mersalyl, the plasma chloride concentration was significantly higher than that of control rats (t = 2.76, P<0.001). The increased rate of chloride reabsorption was accompanied by an increase in the rate of tubular water reabsorption and the correlation coefficient between T_W and rate of urine flow lost its significance (r = −0.54, S.E. = ±0.360). In other words, the rate of water reabsorption, expressed as percentage of the water filtered (T_W), remained practically the same at any rate of urine flow, a finding which is in sharp contrast to that described for normal rats.

It has been shown that, in contrast to mersalyl, calomel did not exert any diuretic effect. The mercurial compounds had the same effect on the glomerular filtration rate in both series, and the correlation coefficients between GFR and rate of urine flow were almost the same (mean z = 0.08, S.E. = ±0.595). The diuretic effect of mersalyl and the non-diuretic action of calomel were thus due to the difference between the mean rates of tubular water reabsorption.

The fact that the plasma chloride concentration was found to be higher in rats injected with mersalyl or with calomel than in control rats, and the fact that the rate of chloride filtration was also higher, raises the question whether the renal effect of these mercuric compounds is not partly the result of an increase in the osmotic pressure of the fluid passing through the glomeruli.

To answer this question, rats were given 5% of their body weight of 85 mM. NaCl solution by stomach tube and the diuresis and renal function were investigated in the usual way.

The mean diuresis of these rats is recorded in Fig. 1 and shows a decrease in the urine flow. The results of the clearance tests showed normal values for the total tubular excretory mass (mean Tm_D = 0.143 ± 0.0093 mg./100 g./min.), but values of T_{Cl} of the same order as those observed in rats injected with 0.0027 mM./100 g. mersalyl (mean T_{Cl} = 98.2 ± 0.18 ; t = 0.46, P>0.6). The mean plasma chloride concentration was 486.4 ± 7.87 mg./100 ml. ; it was thus slightly higher than that of the rats injected with mersalyl. The increase observed in the glomerular filtration rate (mean GFR = 1.08 ± 0.035 (22) ml./100 g./min.) was partly produced by an increase in the filtration fraction (mean FF = 0.41 ± 0.032) and partly by an increase in the renal plasma flow (mean RPF = 2.72

± 0.154 ml./100 g./min.), but GFR was not correlated either with the rate of urine flow or with the rate of tubular water reabsorption. When compared with the mean GFR in rats injected with mersalyl, the glomerular filtration rate proved to be of the same magnitude ($t=0.79$, $P>0.4$). It may therefore be assumed that the chloride concentration of the glomerular filtrate in the rats which received a saline solution (85 mM. NaCl) was much the same as that of the glomerular filtrate in rats injected with mersalyl. However, far from having a pronounced diuretic effect, like that of mersalyl, an 85 mM. NaCl solution lowered the rate of diuresis.

Comparison of the coefficient of correlation between T_W and rate of urine flow in rats which received 5% of their body weight of an 85 mM. NaCl solution with that of rats injected with mersalyl, will show that $r=-0.90$, $P<0.001$, in the former, but that $r=-0.97$, $P<0.001$, in the latter. A statistical comparison revealed a significant difference (mean $z=1.78$, S.E. $=\pm 0.166$) between these values. It is thus evident that the plasma chloride concentration and the glomerular filtration rate in rats which received a hypotonic NaCl solution was increased to much the same degree as in rats injected with mersalyl. However, the former reabsorbed more water per unit time than the latter. It would seem therefore that the increase in the diuresis observed in rats injected with mersalyl cannot be explained by the increased amount of chloride filtered, but must be explained by some other action of the mercurial compound.

IV. Renal effects of theophylline sodium acetate

There is a widespread opinion that xanthine derivatives induce hyperaemia in the mammalian kidney (Cushny, 1917; Richards and Plant, 1922; Janssen and Rein, 1928; Ellinger, 1929; Verney and Winton, 1930; Walker, Schmidt, Elsom, and Johnston, 1937). However, Chasis, Ranges, Goldring, and Smith (1938) examined the action of theophylline on man by means of simultaneous inulin and diodone clearances and concluded that theophylline and caffeine consistently reduced the blood flow through the normal human kidney, but increased the glomerular filtration rate and the filtration fraction.

To investigate the diuretic action of theophylline in rats the drug was injected subcutaneously or given by mouth, as suggested by Lipschitz *et al.* (1943). These investigators have shown that within certain limits there is a linear relationship between log. dose of theophylline and log. action in the rat, but that an overdose produces a decrease in the diuretic effect. They established 0.07 and 0.10 mM./kg. as the optimum diuretic dose for the rat. A dose of 0.01 mM./100 g. theophylline was, therefore, chosen and given by mouth in the present series.

The diuretic effect of 0.01 mM./100 g. rat given in 5% of body weight of water by stomach tube was first investigated. In two groups of rats the urinary output was measured for 3 hours and 10 hours respectively. The rats whose

diuresis had been measured during the longer period received a second dose of water 10 hours after the first to ascertain whether the diuretic effect of the theophylline had ceased.

In the first group, theophylline exerted a marked diuretic effect: the total amount of urine excreted in 3 hours exceeding the quantity of water given by nearly 5% (Fig. 1). When comparing the total urinary chloride excretion in this group with that of control rats, it was found that rats which had received a theophylline solution excreted 2.2 ± 0.23 mg. NaCl/100 g./min., the control rats excreting only an average of 0.8 ± 0.08 mg. NaCl/100 g./min. ($t = 5.78$, $P < 0.001$).

In the second group, i.e., in the rats whose urine was collected for 10 hours, a comparison with control rats showed that "theophylline rats" excreted 0.012 ± 0.0011 (24) ml./100 g./min. with a mean chloride content of 2.1 ± 0.61 mg./100 g./min. NaCl, while in the control rats the urine excretion during 10 hours amounted to 0.009 ± 0.001 ml./100 g./min. and a mean chloride content of 1.4 ± 0.36 mg./100 g./min. The mean quantity of urine excreted by the rats which received theophylline was significantly higher than in control rats: $t = 2.65$, $P < 0.05 > 0.02$, but the difference between the mean chloride concentrations was not significant: $t = 0.97$, $P > 0.4$. However, if after 10 hours "theophylline rats" and controls were given 5% of their body weight of water, the water diuresis was found to be the same in the two groups ($t = 0.04$, $P > 0.9$), the chloride excretion being also comparable in the two groups ($t = 0.63$, $P > 0.5$).

It can therefore be concluded that theophylline sodium acetate given by mouth, simultaneously with 5.0 ml./100 g. water, produces a marked increase in the urine flow and in the chloride excretion. Compared with a dose of 0.0027 mM./100 g. mersalyl, these effects of theophylline were of comparatively short duration; after 10 hours, and possibly sooner, the water diuresis and the chloride excretion had returned to normal values.

To investigate the renal mechanism of the xanthine diuresis, rats were given theophylline either by stomach tube or by subcutaneous injections simultaneously with 5.0 ml./100 g. of water. Inulin and diodone clearances were carried out in the usual way.

The main features of the results of the clearances determinations were: (a) an increase in both glomerular filtration rate (mean GFR $= 1.05 \pm 0.108$ (24) ml./100 g./min.) and renal plasma flow (mean RPF $= 4.15 \pm 0.342$ ml./100 g./min.); (b) a significant correlation between GFR and rate of urine flow ($r = +0.72$, S.E. $= \pm 0.330$, $P < 0.001$); (c) a significant correlation between RPF and GFR ($r = +0.68$, S.E. $= \pm 0.313$, $P < 0.001$) and between RPF and rate of urine flow ($r = +0.71$, S.E. $= \pm 0.313$, $P < 0.001$). The difference between these three coefficients of correlation is not significant, as can be shown by transforming the values of "r" into the interclass coefficient "z."

The total tubular excretory mass (Tm_D), throughout the whole range of

urine flow remained unchanged and was of normal magnitude (mean $Tm_D = 0.13 \pm 0.083$ (14) ml./100 g./min.). However, an increase of the filtration fraction was noted (mean $FF = 0.28 \pm 0.035$, compared with 0.17 ± 0.002 in control rats; $t = 3.29$, $P < 0.001$), which suggests a slight constriction of the glomerular efferent vessels.

The data in Table I show that in theophylline diuresis the rate of tubular water reabsorption, as expressed in percentage of the glomerular filtration rate, was not as closely correlated with the rate of urine flow as in control rats (coefficient of correlation between T_W and the rate of urine flow: $r = -0.68$, S.E. $= \pm 0.302$, $P < 0.001$, compared with $r = -0.99$ in control rats).

T_{Cl} remained practically constant at both low and high rates of urine flow encountered in these experiments (mean $T_{Cl} = 98.6 \pm 0.29\%$). It follows that, compared with the rate of tubular chloride reabsorption in control rats, theophylline increased the rate at which chloride was reabsorbed ($t = 3.99$, $P < 0.001$).

A comparison of the renal mechanism of diuresis in "theophylline rats" and in controls leads to the conclusion that the rate of glomerular filtration takes an active part in the high rate at which urine is formed, the rate of tubular water and chloride reabsorption being partly compensated by the enhanced rate at which water and chloride are filtered.

Discussion

A common feature in all the experiments with diuretics reported was the increase in the rate of glomerular filtration. But, while in some series this increase was not related to the urine flow, in others it was significantly correlated with the urine flow.

An increase in glomerular filtration rate of constant magnitude at all levels of urine flow was found in rats injected with a non-diuretic dose of mersalyl (0.0006 mM./100 g.) and in rats which received 5.0 ml./100 g. of an 85 mM. NaCl solution. There was no increased diuresis in these series: the "mersalyl rats" excreted about 80% of the standard amount of water in 3 hours, and the "NaCl animals" only 63%. However, the mean values for GFR in both series were comparable; the difference in the volume of urine excreted during the period of 3 hours must therefore have been due to the difference in the rate at which water was reabsorbed. It can therefore be concluded that, with a glomerular filtration rate not correlated with the urine flow, the regulation of the urinary volume will depend on variations of the rate of water reabsorption, even when GFR is nearly three times as high as in normal rats.

This finding should be compared with those experiments in which the rate of glomerular filtration was correlated with the urine flow, i.e., in rats injected with

0.0027 mM./100 g. mersalyl, with 0.01 mM./100 g. theophylline sodium acetate, or with 0.00027 mM./100 g. calomel. In these rats the rates of glomerular filtration were practically the same at the same rate of urine flow, and statistical analysis showed that the correlation coefficients between GFR and rate of urine flow of the three series were not significantly different and were, therefore, comparable. It could further be shown that variations in urine flow in these series were the result of variations in two functions: variations in the glomerular filtration rate (GFR) and variation in tubular water reabsorption (T_W), instead of that in one (T_W) only, as in the rats with a constant GFR. It may be asked: do variations in the glomerular filtration rate necessarily have a diuretic effect? It has been shown that calomel was not diuretic, but that both theophylline and mersalyl had a diuretic effect. However, Table I shows that the values for the glomerular filtration rates were much the same in all three series. It follows that the diuretic effect of theophylline and of 0.0027 mM./100 g. mersalyl and the absence of a diuretic effect of calomel must have been produced by changes in the rate of water reabsorption (T_W). If this is so, it should be possible to show that less water had been reabsorbed in rats injected with 0.0027 mM./100 g. mersalyl than in rats which received theophylline, and that more water had been reabsorbed in rats injected with calomel. That this occurs may be seen from a comparison of the three coefficients of correlation between rate of water reabsorption and rate of urine flow (for the mersalyl series, $r = -0.97$; for the theophylline series, $r = -0.68$; and for the calomel series, $r = -0.54$). It can thus be concluded that in rats injected with calomel no diuretic effect is exerted because the rate of tubular water reabsorption is increased to such an extent that it counterbalances the correlated increase in the rate of filtration. On the other hand, in rats injected with 0.0027 mM./100 g. mersalyl or with theophylline, the rate of tubular water reabsorption increased to a lesser degree, and insufficiently to counterbalance the increased rate at which water was filtered.

It follows that the classical explanation that the diuretics used produce an enhanced diuresis by reducing the tubular water reabsorption applies also to the rat—provided that the rate of tubular water reabsorption obtained in experiments on rats is not compared with the rate of tubular water reabsorption of the controls. All the drugs investigated produced an *increase* in the rate of tubular water reabsorption, as expressed in percentage of glomerular filtration rate, if compared with that observed in a normal water diuresis in control rats. However, it would clearly be a mistake to compare a diuresis experiment in which only one variable (T_W) is involved with one in which two variables (T_W and GFR) operate, i.e., experiments in which an increase of urine flow is determined by two different mechanisms.

Considering the increases in GFR and RPF, it remains to be seen how far the increase in chloride excretion observed in some of the series should be related

with the rate of tubular chloride reabsorption (T_{Cl}). A significant increase of T_{Cl} was noted after a diuretic dose of mersalyl and after the injection of calomel and theophylline: theophylline produced a greater chloride excretion than calomel, but the highest chloride concentrations were met after the injection of 0.0027 mM./100 g. mersalyl. Two factors were involved in the increased excretion of chloride: (a) an increase in the plasma chloride concentration which occurred in rats injected with calomel or with a diuretic dose of mersalyl; (b) the rate at which the chlorides were reabsorbed. The rates of filtration were much the same in the three series, and it is therefore easy to understand why theophylline, which did not produce an increase of the plasma chloride level, produced a lower chloride excretion than 0.0027 mM./100 g. mersalyl. After the injection of the higher dose of mersalyl or of calomel, plasma chlorides and rate of glomerular filtration were raised to approximately the same extent, and the amounts of chloride filtered were therefore comparable. It could thus be expected—and this was actually found in the calomel experiments—that a higher rate of tubular chloride reabsorption would result in a lower concentration of chloride in the urine.

It might be objected that expressing tubular water and chloride reabsorption as percentage of water and chloride filtered (T_W and T_{Cl}) is a misleading way of describing these renal functions, and that absolute amounts of chlorides or water should be given in preference (Barclay and Cooke, 1944). But it would be an obvious mistake to divorce the quantity of chloride reabsorbed from that which is made available by filtration, and to deny the fact expressed by Shannon (1942) that "the burden presented to the tubules is quantitatively determined by composition and rate of formation of glomerular filtrate." Expressing the rate of water and chloride reabsorption as absolute amounts may be a permissible simplification when the GFR remains constant. However, it has been shown (Chasis, Ranges, Goldring, and Smith, 1938; Shannon, 1936) that this does not always apply even in man or the dog.

How do the results on rats compare with those obtained on isolated kidneys, or on dogs or man? Schmitz (1932), Blumgart, Gilligan, Levy, Brown, and Volk (1934), Davenport, Fulton, Van Auken, and Parsons (1934), Walker, Schmidt, Elsom, and Johnston (1937) found, by means of creatinine and urea clearance determinations in anaesthetized animals and in man, that salyrgan, a proprietary brand of mersalyl, did not increase the glomerular filtration rate, and that the renal plasma flow, as measured with a thermostromuhr, was not affected in a constant manner. In contrast to this, it has been shown that a diuretic dose of mersalyl in rats produces an increase in both glomerular filtration rate and renal plasma flow when estimated by simultaneous inulin and diodone clearances. From the findings in man and in the dog it was furthermore concluded that the mercurials act on the kidney by reducing the tubular water reabsorption. It could be shown that this also applies to rats, provided that the rate of water reabsorption in

rats injected with a non-diuretic dose of a mercurial " diuretic " is chosen as basis of comparison, and not that of control animals. It would seem, therefore, that, while in higher mammals the diuretic action of mersalyl is achieved by changes in one renal function only, i.e., in tubular water reabsorption, the diuretic action in the rat is more involved and is produced by changes in both the rate of glomerular filtration and the rate of water reabsorption.

It has been demonstrated that these renal effects of mersalyl on rats are the result of the mercurial constituent only, and not of the theophylline content of this preparation. Renal effects of mersalyl have been observed 10 hours after the injection, i.e., at a time when any diuretic action of theophylline had long subsided.

No indication of a central action of theophylline at the doses given was observed ; and its renal effects were much the same as those observed by Verney and Winton (1930) in the heart-lung-kidney preparation, and those found by Smith and collaborators (1938) in man. These authors, who used inulin clearances, observed that theophylline produced an increase of the glomerular filtration rate which was correlated with the urine flow. Another analogy with their findings consists in the absence of a diuresis without previous hydration. It can therefore be concluded that the mechanism of the diuretic effect of theophylline sodium acetate is comparable in species as widely different as man, the dog, and the rat, but that a mercurial compound like mersalyl produces a diuretic action in rats by the combination of changes in the rate of glomerular filtration and of tubular water reabsorption.

Summary

1. The injection of 0.0006 mM./100 g. mersalyl had no diuretic effect in rats, but it increased the glomerular filtration rate.

2. A marked diuretic effect was obtained with 0.0027 mM./100 g. mersalyl. This diuretic effect was obtained about 10 hours after the intramuscular injection of the mercurial compound and only in well-hydrated rats.

3. Ten hours after the administration of 0.0027 mM./100 g. mersalyl the glomerular filtration rate and the renal plasma flow were increased and significantly correlated with the urine flow.

4. Injections of 0.00027 mM./100 g., 0.00135 mM./100 g., and of 0.0027 mM./100 g. calomel failed to exert any diuretic effect. Rats injected with the two latter doses remained almost completely anuric for several hours.

5. The injection of 0.00027 mM./100 g. calomel produced an increase of glomerular filtration rate which was significantly correlated with the urine flow. The renal plasma flow was also increased, but not correlated with the urine flow.

6. The injection of a dose of 0.01 mM./100 g. theophylline sodium acetate in well-hydrated rats exerted a marked diuretic effect. Both glomerular filtration rate and renal plasma flow were increased and significantly correlated with the urine flow.

7. After injection of (a) a non-diuretic dose of 0.0006 mM./100 g. mersalyl, (b) a diuretic dose of 0.0027 mM./100 g. mersalyl, (c) a non-diuretic dose of 0.00027 mM./100 g. calomel, and (d) a diuretic dose of 0.01 mM./100 g. theophylline sodium acetate, the tubular water reabsorption (expressed as percentage of the glomerular filtration $= T_W$) was increased when compared with that of control rats. However, a comparison of the rate of tubular water reabsorption in rats injected with a diuretic dose of 0.0027 mM./100 g. mersalyl or of 0.01 mM./100 g. theophylline, with that in rats injected with a non-diuretic dose of a mercurial compound, showed that the diuretic effect was accompanied by a reduction in the rate of tubular water reabsorption. It would seem, therefore, that in rats the diuretic effect of mersalyl and of theophylline sodium acetate was produced by changes in the rate of both the glomerular filtration and tubular water reabsorption.

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METHOD FOR THE ESTIMATION OF BARBITURIC AND THIOBARBITURIC ACIDS IN BIOLOGICAL MATERIALS

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In preliminary work on the fate and distribution of barbiturates in the animal body several known methods of determining them were tried (Levvy, 1940; Delmonico, 1939; Anderson and Essex, 1943), but none was completely satisfactory. With these methods the recoveries of known amounts of barbiturates added to samples of blood and tissues were low.

A method has been developed for the estimation of barbituric acids based on Koppanyi's colour reaction (1934) and for the estimation of thiobarbituric acids based on Cowan's colour reaction (1939). The main feature of this method is the purification of the extracts containing barbituric and thiobarbituric acids by chromatography, which has the additional advantage of permitting the determination of both types of compounds when present together in the same sample.

The method involves the following stages: (i) extraction of the drugs from the biological material; (ii) purification of the extracts by chromatography; and (iii) determination of the drug content in the eluates.

REAGENTS

1. Peroxide-free ether. Ether (technical) was treated overnight with ferrous sulphate, washed with water, dried with anhydrous calcium chloride and distilled over sodium.
2. 10 per cent (w/v) solution of sodium dihydrogen phosphate.
3. Crystalline sodium dihydrogen phosphate.
4. Anhydrous sodium sulphate.
5. Chloroform free from alcohol: chloroform (B.P.) was washed with water and then with a saturated solution of calcium chloride. After drying with anhydrous calcium chloride it was distilled and kept in a dark bottle.

6. Methanol A.R. quality.

7. Benzene A.R. quality.

8. Activated alumina: 1,200 g. activated alumina, "grade O," supplied by Messrs. Peter Spence, Manchester, were boiled for 2 hours with 1,800 ml. 10 per cent (v/v) acetic acid. The alumina was filtered and the excess of acetic acid removed by washing with at least 20 l. of hot distilled water. The alumina was dried and reactivated by heating until the temperature reached 360° C.; it was then partly deactivated by adding water (2.5 per cent w/v).

9. For the estimation of barbituric acids: (*a*) 1 per cent (w/v) cobalt acetate in methanol and (*b*) 5 per cent (v/v) *iso*propylamine in methanol.

10. For the estimation of thiobarbituric acids: (*a*) a saturated solution of anhydrous copper sulphate in methanol and (*b*) 10 per cent (v/v) diethylamine in methanol.

PROCEDURE

EXTRACTION—*Blood.*—10–20 ml. volumes of oxalated blood are mixed with equal volumes of water and of the sodium dihydrogen phosphate solution and extracted with ether in a continuous extractor (at 45–50° C.) for 8–10 hours. The ether extract is evaporated to dryness.

Urine.—The total or an aliquot of the urine is acidified with conc. HCl to pH 5. It is then extracted with ether in a continuous extractor (at 45–50° C.) for 8–10 hours and the extract evaporated to dryness.

Tissues.—Ether extracts of tissues are difficult to purify by chromatography, but the following method of extraction has been used with success.

Samples of about 10–20 g. of tissues are ground in a mortar with sand and then mixed with solid sodium dihydrogen phosphate (1 g. for every 10 g. of tissue) and allowed to stand for 5–10 minutes. Anhydrous sodium sulphate (20 g. for every 10 g. of tissue) is then added slowly, with continuous grinding, to give a fine homogeneous powder. The whole is transferred to a desiccator and left over anhydrous calcium chloride for one hour. The dry powder is extracted for 2–3 hours with 50 ml. benzene in a well-stoppered 100 ml. conical flask. The benzene extract is then filtered and the residue and flask washed three times with about 10–15 ml. of benzene. The filtrate and washings are pooled and concentrated to about 5 ml. in a distillation flask at 50° C. under reduced pressure. This method of extraction can also be applied to blood.

PURIFICATION OF THE EXTRACTS AND SEPARATION OF BARBITURIC FROM THIOBARBITURIC ACIDS

This is based on the work of Kondo (1937), who separated barbitone from phenazone by chromatography on alumina columns.

Urine and blood.—The residues left by evaporation of the ether extracts are dissolved in 5 ml. chloroform and dried by shaking with about 1–2 g. anhydrous sodium sulphate. The solutions are then chromatographed on alumina columns (3/8″ × 4″). The chloroform solutions are filtered directly on to the columns; the

flask and filter are washed three times with 5 ml. of chloroform and the washings poured on to the column. The column is then washed with chloroform until the eluates are free from pigment.

Tissues.—The benzene tissue extracts are passed through alumina columns, with slight suction. The flasks are washed three times with benzene and the washings added to the column. The column is then washed with benzene until the eluates are free from pigment and finally with 20 ml. chloroform.

The chloroform and benzene eluates are discarded. If the extracts contain more than 2 mg. of a thiobarbituric acid, the latter can be seen under ultraviolet light as a dark band at the top of the column.

Separation.—Thiobarbituric acids are recovered from the columns by elution with 50 ml. 2 per cent methanol in chloroform (v/v). Barbituric acids are not eluted by methanol and chloroform in this proportion, but they can be recovered by further elution with 50 ml. 10 per cent methanol in chloroform (v/v). These eluates are kept for estimation.

The separation of thiobarbituric acids from barbituric acids is complete and the recoveries of both fractions are almost theoretical. Mixtures containing 0.25–0.5 mg. thiophenobarbitone and 0.25–1 mg. phenobarbitone were added to alumina columns, and the average recoveries were: thiobarbituric acid 102 per cent and barbituric acid 98 per cent.

ESTIMATION.—The eluates from the columns are evaporated to dryness in distillation flasks under reduced pressure at 40–50° C. The residues are dissolved in chloroform and their barbituric or thiobarbituric acid content estimated by the following reactions.

Thiobarbituric acids.—Thiobarbituric acids are estimated by a modification of the reaction demonstrated by S. L. Cowan at the Physiological Society in 1939. An aliquot of the final chloroform solution is taken in a test tube and for every 2 ml., 0.2 ml. of the diethylamine solution and 0.5 ml. of the copper sulphate solution are added in that order. A green coloration develops at once which is stable for about two hours. The samples are compared in a colorimeter, photoelectric or otherwise, with a series of similarly treated standard solutions of the thiobarbituric acid to be estimated, containing from 0.03–0.5 mg./ml. These are prepared by diluting a fresh solution containing 0.5 mg./ml. of the thiobarbituric acid in chloroform.

Extracts of tissues, such as brain and liver, give a slight blank with the copper reaction for thiobarbituric acids. This can be as high as 1 mg./100 g. of tissue and it is necessary to subtract this blank from the estimations.

The reaction is fairly specific. According to Cowan (personal communication) it is not given by malonic acid, theophylline, theobromine, thiourea, caffeine, guanine, uric acid, urea, creatinine, oxamide, succinic acid, lecithin, cholesterol, cystine or glutathione.

Barbituric acids give a faint bluish colour under the conditions described above. The intensities of the colours given by standard solutions of phenobarbitone and thiophenobarbitone are compared in Fig. 1.

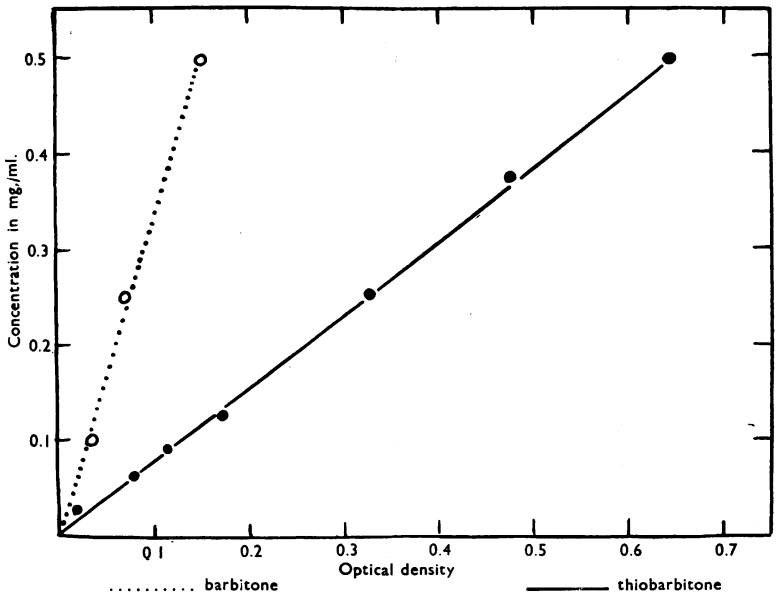

FIG. 1.—Intensity of the colour given by solutions of barbitone and thiobarbitone in Cowan's reaction. Measurements made in the Pulfrich photometer with filter 66, 6/3.5, and 10 mm. cells.

The Pulfrich photometer was used in these estimations. With filter S.66, 6/3.5, maximum transmission at 679 mg. and 1 cm. cells, the reaction is sensitive to concentrations of 0.03 mg./ml., but the sensitivity can be increased to 0.005 mg./ml. if 5 cm. cells are used.

Barbituric acids.—These are estimated by Koppanyi's reaction (1934). For every 2 ml. of the final chloroform solution, 0.6 ml. *iso*propylamine and 0.1 ml. cobalt acetate reagents are added. The reddish colour given by the sample is compared in a colorimeter with a series of similarly treated standards containing 0.1–1.0 mg. of the appropriate barbituric acid per ml. chloroform.

Recovery.—The method was tested in a series of control experiments in which known amounts of sodium kemithal (5-$\Delta^{2:3}$-*cyclo*hexenyl-5-allyl-2-thiobarbituric acid) were added to samples of blood and tissue and treated as described above. The results of these experiments are summarized in Table I.

The recovery of known amounts of barbituric or thiobarbituric acids added to samples of blood and tissues is approximately complete, except when the amount in the 10 ml. sample is less than 0.3 mg., when the recovery may fall below 95 per cent.

TABLE I

RECOVERIES OF 5-$\Delta^{2:3}$-*cyclo*HEXENYL-5-ALLYL-THIOBARBITURIC ACID (KEMITHAL) FROM BLOOD AND TISSUES

Tissue	Tissue in g. Blood in ml.	Na kemithal added mg.	Equivalent to kemithal acid. mg.	Kemithal acid found mg.	Per cent recovery
Blood	10	8.0	7.15	6.5	91
	10	8.0	7.15	6.9	96
	10	8.0	7.15	7.2	103
	10	1.125	1.0	0.98	98
	10	1.125	1.0	1.01	101
	10	1.0	0.89	0.86	97
	10	1.0	0.89	0.86	97
	10	0.5	0.445	0.43	96.5
	10	0.5	0.445	0.42	94.5
	10	0.5	0.445	0.45	101
Liver	10	0.884	0.788	0.69	89
	10	0.884	0.788	0.75	97
	10	1.768	1.576	1.42	91
	10	1.768	1.576	1.47	95
	10	1.125	1.0	0.96	96
	10	1.125	1.0	0.98	98
	10	1.125	1.0	0.97	97
	10	1.125	1.0	0.96	96
Brain	10	1.0	0.89	0.86	97
	10	1.0	0.89	0.91	102
	10	1.0	0.89	0.84	94.5
	10	1.0	0.89	0.94	105
	10	1.0	0.89	0.84	97
	10	2.0	1.78	1.70	95.5
	10	2.0	1.78	1.88	105
	10	2.0	1.78	1.80	101

Average recovery per cent 97.3 $\pm$ 4

SUMMARY

A method for the estimation of barbituric acids and thiobarbituric acids in tissues and animal fluids is described.

The method enables both types of barbiturates to be separated and estimated when they are present together.

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KEMITHAL: A NEW INTRAVENOUS ANAESTHETIC

BY

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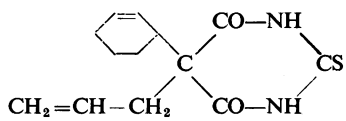
Since the introduction of evipan (now hexobarbitone B.P.) by Weese and Scharpff (1932) and pentothal by Lundy (1935), the use of the ultra-short-acting barbiturates for intravenous general anaesthesia has steadily increased. The literature on this subject has been reviewed by Adams (1944).

An extensive investigation on new thiobarbiturates has led to the discovery of a drug which has pharmacological advantages over those already in use. This



substance, to which the name of kemithal has been given, is 5-$\Delta^{2:3}$-*cyclo*hexenyl-5-allyl-2-thiobarbituric acid. The free acid, m.p. 142° C., is sparingly soluble in water but readily soluble in most organic solvents, e.g., alcohols, ether, chloroform, benzene, etc. Its sodium salt, a pale yellow slightly hygroscopic powder, is readily soluble in water up to 20 per cent. The pH of a 10 per cent (w/v) solution is 10.6 (cf. evipan, pH 11.5; pentothal, pH 10.6). The sodium salt is stable in absence of air; its solutions are stable for 4–5 hours.

This paper describes the pharmacological investigation of the drug. The results of its clinical trials on 4,000 cases have been reported elsewhere (Mackintosh and Scott, 1946; Halton, 1946; Gordon, 1946).

Hypnotic action and toxicity

In this paper hypnosis is defined as that condition in which it was not possible to elicit "body righting reflexes" (Fulton, 1938). The term anaesthesia defines the state during which it is possible to make a cutaneous incision without evoking a response.

Mice.—The median hypnotic dose (HD 50) and the median lethal dose (LD 50) of the three drugs were measured after intravenous, intraperitoneal, and oral administration. At least twelve mice were used at each dose level. For one hour before, and during the first

24 hours of the experiments, the mice were kept in thermostatically controlled cages at 30° C.; they were then transferred to a room maintained at 25° C., and kept under observation for a week. The HD 50 and LD 50 were calculated from the incidence of hypnosis and death by the usual statistical methods. The term therapeutic ratio is used to denote the quotient LD 50/HD 50, and is an approximate indication of the safety margin of the drugs. The times of onset of hypnosis and duration of action were recorded. The drugs were administered as 0.1 per cent (w/v) or 1.0 per cent (w/v) solutions. Intravenous injections were carried out at a constant rate of 0.05 ml. per 5 seconds. The results are summarized in Table I.

TABLE I

HYPNOTIC ACTION AND TOXICITY IN MICE

Administration	Kemithal			Pentothal			Hexobarbitone		
	i.v.	i.p.	oral	i.v.	i.p.	oral	i.v.	i.p.	oral
HD 50 (mg./kg.)	55	100	165	20	42	—	30	47	—
LD 50 (mg./kg.)	390	384	370	80	154	600	190	280	1200
Therapeutic ratio $\frac{LD\ 50}{HD\ 50}$	7.1	3.84	2.25	4	3.65	—	6.3	6	—
Time of onset of hypnosis in min.	at once	3	2–5	at once	3	—	at once	4	—
Duration of action of 2 x HD 50 in min.	30–90	30–45	3hr	30–90	30–60	—	45–60	50–60	—

Monkeys.—The duration of hypnosis produced by the intravenous administration of 5 per cent (w/v) or 10 per cent (w/v) solutions of the drugs in 3 monkeys (*M. rhesus*) was studied. In order to avoid habituation, the monkeys were only injected once a week, and the order of the injections of the different drugs was randomized. A range of doses from 5 mg./kg. to 100 mg./kg. was tried and Table II shows those which produce anaesthesia of 10 and 60 minutes' duration.

Dogs.—The technique was similar to that used in monkeys. Hexobarbitone is not a satisfactory anaesthetic for dogs, and when anaesthesia was obtained it was associated with convulsions and delayed recovery. Thus in one dog 40 mg./kg. hexobarbitone produced hypnosis without loss of the corneal reflex. The recovery, which began 20 minutes after the injection, was only complete after 100 minutes; convulsions occurred throughout this period. A comparison of kemithal and pentothal is given in Table II.

TABLE II

ANAESTHETIC ACTION IN MONKEYS AND DOGS

Animal		Kemithal	Pentothal	Hexobarbitone
Monkeys (*M. rhesus*)	i.v. Dose (mg./kg.) producing 10 min. anaesthesia	22.5	11.5	20
	i.v. Dose (mg./kg.) producing 60 min. anaesthesia	45	30	45
Dogs	i.v. Dose (mg./kg.) producing 10 min. anaesthesia	50	20	—
	i.v. Dose (mg./kg.) producing 60 min. anaesthesia	80	40	—

Respiratory volume

The changes in respiratory volume following kemithal and pentothal were measured in the unanaesthetized rabbit by Gaddum's method (1941), using a specially constructed rubber mask fitting closely to the head of the animal and connected through a set of valves to the recording apparatus. Solutions of the drugs were injected into the marginal vein of the ear, several doses being administered at intervals sufficiently long to allow the respiration to return to normal. The results are shown in Fig. 1, which indicates that a dose of about 2.5 mg./kg.

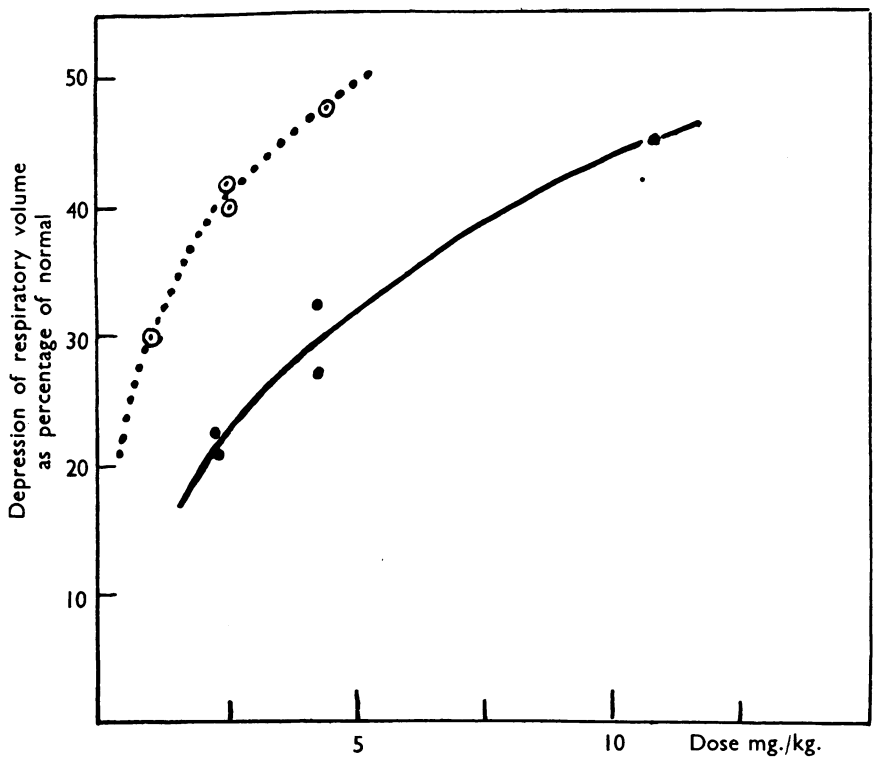


FIG. 1.—Depression of respiratory volume of the unanaesthetized rabbit following i.v. administration of kemithal and pentothal. Line—kemithal. Dots—pentothal.

pentothal produces a depression of respiratory volume equivalent to that produced by 8 mg./kg. kemithal. Experiments on decerebrate cats gave similar results.

Blood pressure

The effects of kemithal and pentothal on the blood pressure of cats, anaesthetized with chloralose or after decerebration, were compared. Solutions of both compounds (10 per cent w/v) were injected into the femoral vein, and the

blood pressure was recorded from the carotid artery, the animals being kept under artificial respiration throughout the experiments. Both compounds produced a fall of blood pressure; 10 mg./kg. kemithal produced approximately the same fall of blood pressure as 5 mg./kg. pentothal.

Rate of inactivation of kemithal in mice

The rate of inactivation of kemithal in mice was measured by the continuous intravenous injection method described by Das and Raventós (1939). The mice received an initial intravenous dose of 80 mg./kg. kemithal followed by a continuous injection in a series of doses, calculated in mg./kg./min. and adjusted by alteration of the concentration of the solution, the rate of the injection being kept constant at 0.02 ml./min. The rate of inactivation was calculated from the dose in mg./kg./min. necessary to maintain the level of anaesthesia produced by the initial injection for a period of 90–120 minutes. The results are summarized in Table III.

TABLE III

RATE OF INACTIVATION OF KEMITHAL IN MICE

Dose mg./kg./min.	Number of mice	Average time of recovery in minutes	Average time of death in minutes	Remarks
0.8	3	5	—	Progressive decrease in depth of anaesthesia.
1.5	3	12	—	Ditto.
2 0	5	90	—	Slight decrease in depth of anaesthesia followed by increase 40–50 min. after the beginning of the continuous injection. This level of anaesthesia is maintained for more than 90 min.
4 0	3	—	45	Progressive increase in depth of anaesthesia followed by death.
5.0	2	—	40	
7.5	3	—	35	
15 0	3	—	22	
20.0	3	—	14	

From these results the rate of inactivation is calculated as follows:

Initial dose = 80 mg./kg.

Dose for maintenance of anaesthesia = 2 mg./kg./min.

Rate of inactivation per minute = $\frac{2}{80}$, i.e., 2.5 per cent of the initial dose.

Blood concentration of kemithal during anaesthesia

The concentration of kemithal in the blood during anaesthesia was studied in rabbits and men. The drug concentration was measured by the method described in the previous paper (Raventós, 1946), which can be applied to any barbituric acid derivative.

Rabbits of 2–3 kg. received large doses of kemithal (100–130 mg./kg.) by slow intravenous injection over a period of 3 to 5 minutes. In some cases temporary arrest of the respiratory movements occurred and the animals were kept under artificial respiration until spontaneous respiratory movements were re-established. Samples of blood were taken from the femoral artery at intervals.

Immediately after administration, the blood concentration of kemithal was 15–18 mg./100 ml., falling to 5–7 mg./100 ml. in 15 minutes. From this point the blood concentration decreased progressively for a further 75 minutes until it reached levels of 2–2.5 mg./100 ml. After this fall of concentration, the disappearance of the drug from the blood became slower and 1.25–1.75 mg./100 ml. of kemithal were found in the blood 3–4 hours after injection. A comparison of the results in one experiment with kemithal with those obtained in one experiment with hexobarbitone is shown in Fig. 2. In rabbits, recovery begins when the blood concentration of kemithal is 2.0–1.5 mg./100 ml.

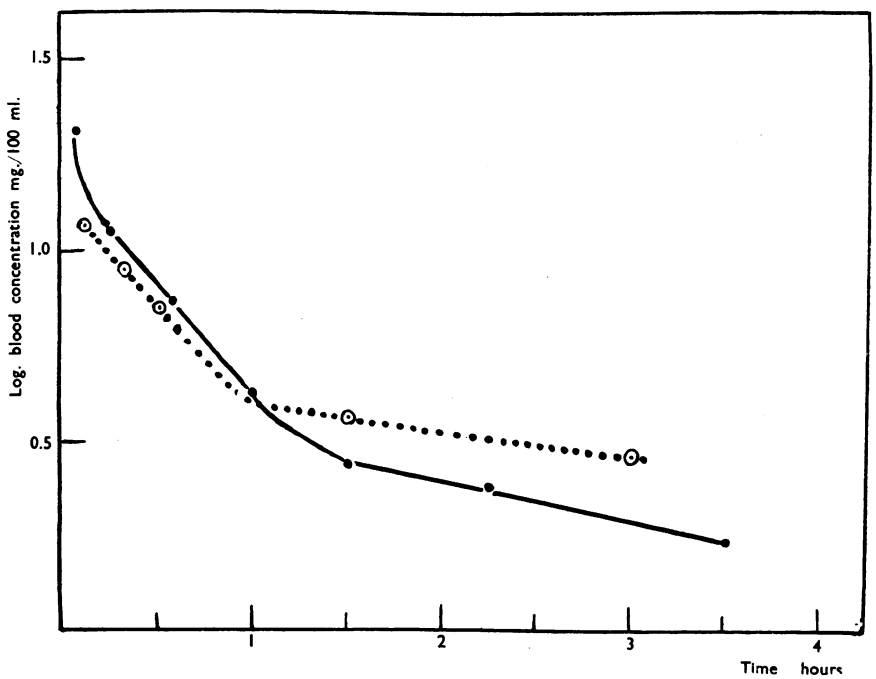


FIG. 2.—Blood concentration of kemithal and hexobarbitone in the rabbit during anaesthesia. Line—kemithal. Dose, 130 mg./kg. i.v. Dots—hexobarbitone. Dose, 130 mg./kg. i.v.

Changes of the concentration of kemithal in the blood of men under anaesthesia were studied in patients under the care of Dr. J. Halton, Liverpool. An initial dose of 1.5 or 2.0 g. kemithal was injected intravenously and anaesthesia was maintained with cyclopropane. Blood samples of at least 20 ml. were taken at intervals from the cubital vein.

When 1.5 g. kemithal were administered, the initial blood concentration was 2.3–3.5 mg./100 ml. Immediately after the administration of 2.0 g. kemithal the blood concentration was 3.5–4.5 mg./100 ml. and it decreased progressively to 1.2–1.9 mg./100 ml. in about 45 minutes. It was not possible to follow the changes in blood concentration after the end of the operation. The fall in blood concentration during the first 45 minutes of one of these experiments is shown in Fig. 3.

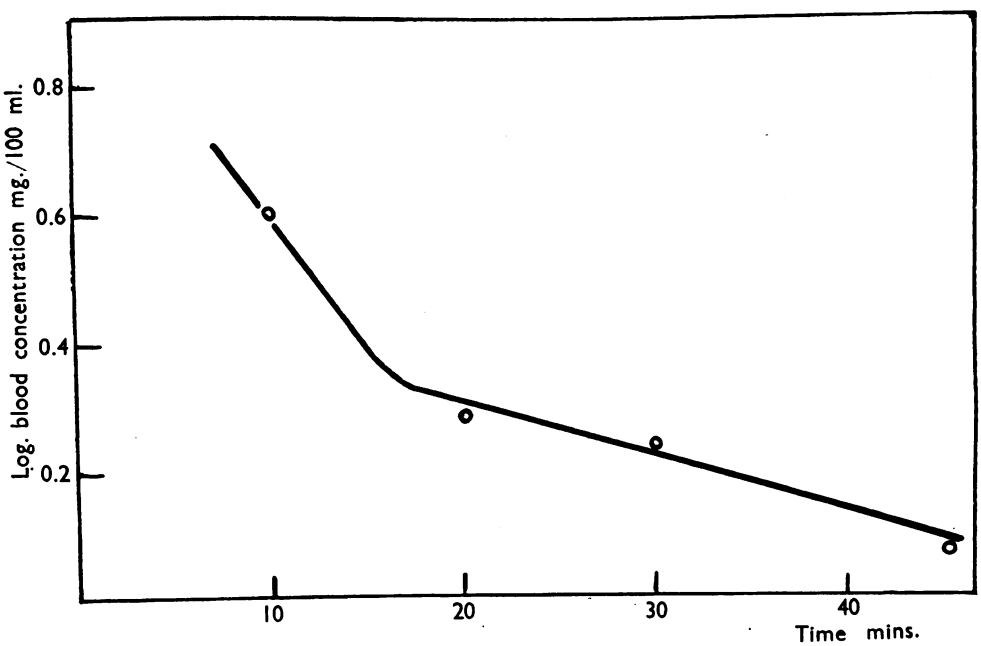


FIG. 3.—Blood concentration of kemithal in a 60-kg. man after a dose of 2.0 g. Anaesthesia maintained with cyclopropane.

Relationship between the blood concentration of kemithal and the level of anaesthesia

The depth of anaesthesia produced in rabbits by a continuous intravenous injection of 3 mg./kg./min. kemithal, a dose large enough to produce an accumulation of the drug, was compared with the blood concentration. The solution was injected at a rate of 0.15 ml./min. Samples of blood were taken from the femoral artery as the anaesthesia deepened, at the levels of light and surgical anaesthesia and at the arrest of respiratory movements. Light anaesthesia is assessed as a level of anaesthesia sufficient for a painless incision of the skin; surgical anaesthesia is a level of anaesthesia sufficient for the opening of the peritoneal cavity with good abdominal relaxation. Similar experiments were carried out with pentothal, injected continuously at a dose of 2 mg./kg./min.

The average blood concentrations of kemithal and pentothal at these levels of anaesthesia are shown in Table IV. In the same table the ratios between these concentrations give a further indication of the safety margin of the compounds.

TABLE IV

THE RELATIONSHIP BETWEEN BLOOD CONCENTRATION AND LEVEL OF ANAESTHESIA

Drug	Number of experiments	Average drug concentration in the blood in mg./100 ml.			Ratios		
		Light anaesthesia A	Full surgical anaesthesia B	Respiratory arrest C	$\frac{C}{A}$	$\frac{C}{B}$	$\frac{B}{A}$
Kemithal	8	3.55	8.82	18.8	5.28	2.13	2.44
Pentothal	7	2.7	4.78	7.42	2.74	1.55	1.77

Excretion

A total of 750 mg. kemithal, divided into three equal doses administered at two-hourly intervals, was injected intravenously into 2–3 kg. rabbits and the urine collected during the three following days. The excretion of thiobarbituric and barbituric acids was estimated for each of the three days.

In these experiments about 15 mg. kemithal were recovered in three days, accounting for only 2 per cent of the administered material. In addition to the recovery of this small amount of unchanged thiobarbiturate, substances giving a positive reaction in the barbituric acid test were excreted to a total of about 20 mg., equivalent to 2.5 per cent of the dose. Nearly all this material was excreted during the first 24 hours following injection, as shown in Table V.

TABLE V

EXCRETION OF KEMITHAL BY RABBITS AFTER 3 × 250 MG. I.V.

Day	Urinary excretion of		Total
	Thiobarbituric acid (kemithal) mg.	Barbituric acid, as 5-$\Delta^{2:3}$-*cyclo*hexenyl-5-allyl-barbituric acid mg.	
1	14.87	16.50	31.37
2	1.17	3.05	4.22
3	0.13	0.79	0.92
Total	16.17	20.34	36.51
Per cent excreted	2.15	2.7	4.85

Man also excretes only a very small proportion of unchanged kemithal. In one of Dr. Halton's cases, receiving 6.0 g. kemithal by drip, only 46 mg. of thiobarbituric acid were recovered from the urine during the first 24 hours after anaesthesia.

Histology

Microscopical sections of tissues of rabbits injected with 50 mg. kemithal daily for two weeks have been examined. At the end of this period the animals were killed and the specimens fixed in formol-saline, embedded in paraffin, and the sections stained with haematoxylin and eosin. The liver, kidney, spleen, lung, intestine, pancreas, suprarenal and heart of these animals showed no pathological changes.

Discussion

As an intravenous anaesthetic in mice, monkeys and dogs, kemithal is about one-half as active, weight for weight, as pentothal, and slightly less active than hexobarbitone. The therapeutic ratio in mice is, however, markedly greater than that of either of the other drugs. The times of onset and maintenance of anaesthesia and the rates of recovery are similar in equiactive doses in all the animals tested, except that hexobarbitone gives anomalous and unsatisfactory results in dogs. The therapeutic ratio of kemithal in mice varies considerably with the method of administration: as shown in Table I, the LD 50 remains approximately the same irrespective of the route of administration, but the HD 50 i.p. is approximately twice, and the HD 50 oral three times, the HD 50 i.v.; whereas with pentothal and hexobarbitone the changes in HD 50 and LD 50 vary *pari passu.*

The favourable intravenous therapeutic ratio of kemithal was confirmed in the experiments on the blood concentration in rabbits at different levels of anaesthesia: it was shown that the ratios of the concentrations at the arrest of respiratory movements to those at both full surgical and light anaesthesia are considerably greater with kemithal than with pentothal (Table IV). Thus kemithal appears to have a greater factor of safety than pentothal whatever the level of anaesthesia. It is difficult to make a precise correlation between the levels of anaesthesia used in the rabbit and the stages of anaesthesia in man as laid down by Guedel (1937), but the level of "light anaesthesia" would correspond approximately to Guedel's first plane of the third stage and "full surgical anaesthesia" to his third plane of the third stage.

The depression of the respiratory volume produced by kemithal, both in rabbits and decerebrate cats, weight for weight, is one-third to one-quarter that of pentothal. As the equiactive dose of kemithal is only about twice that of pentothal, the advantage of kemithal in this respect is clear. The depressant action of kemithal on the blood pressure is similar to that of equiactive doses of pentothal.

In determining the rate of inactivation of kemithal in mice by continuous injection, it was found that about 2 mg./kg./min. was required to maintain the level of anaesthesia induced by 80 mg./kg., i.e., about 2.5 per cent of the initial dose. This rate of inactivation is similar to that obtained by Das and Raventós (1939) for hexobarbitone in mice.

The disappearance of kemithal from the blood of rabbits which have received large intravenous doses falls into three main phases. During the first 10–15 minutes there is a rapid fall in blood concentration, which is clearly associated with the distribution of the drug throughout the body. During the second phase, up to 90 minutes after the injection, the concentration decreases progressively, falling to about one-half of the original concentration in 30–40 minutes, or at a rate of 2–2.5 per cent per minute. This figure is in close agreement with that of the rate of inactivation of kemithal found in mice by the continuous injection method. In the third phase, the fall in blood concentration is very much slower. The cause of this change is not clear; it may be due to the continuous slow removal of the drug from some depot after the main excretion is complete, or it may be caused by the formation of a metabolic product which interferes with the normal process of destruction of the drug. After the administration of hexobarbitone the same changes in rate of disappearance of drug from the blood were found.

In the experiments on the rate of inactivation of kemithal in mice injected with 2 mg./kg./min. (Table III) it was observed that the level of anaesthesia tended to decrease during the first 30–45 minutes of the continuous injection, but later this level increased slightly and remained constant until the end of the experiments. We think this result is perhaps further evidence of the formation of an inhibitory metabolite.

In man the rate of disappearance of kemithal from the blood is about the same as in the rabbit. Kemithal is almost completely destroyed in the body. In rabbits only about 2 per cent of the dose administered can be detected in the urine, in man less than 1 per cent. In addition, the urine of rabbits after administration of kemithal contains substances which give Koppanyi's (1939) test for barbiturates, but not the test for thiobarbiturates, in quantities corresponding to 2–3 per cent of the administered kemithal. This may indicate that the inactivation of kemithal involves the removal of sulphur from the molecule.

SUMMARY

1. As an intravenous anaesthetic in mice kemithal (5-$\Delta^{2:3}$-*cyclo*hexenyl-5-allyl-2-thiobarbituric acid) is about half as potent as pentothal and slightly less active than hexobarbitone. It has a higher therapeutic ratio (LD 50/HD 50) than either of the other two compounds.

2. The duration of action of equiactive anaesthetic doses of the three compounds is about the same.

3. Kemithal in anaesthetic doses depresses the respiratory volume to a less extent than pentothal.

4. The concentrations of kemithal and pentothal in the blood at different levels of anaesthesia and the ratios between these concentrations have been determined. They suggest that with kemithal anaesthesia can be obtained with less danger of respiratory arrest.

5. The rate at which kemithal and hexobarbitone disappear from the blood after administration has been determined in rabbits.

6. Kemithal is almost completely destroyed in the body. The urine of rabbits injected with kemithal contains small amounts of the unchanged material and similar quantities of barbituric acids, leaving about 95 per cent of the administered dose to be accounted for.

7. No histological changes have been found in the tissues of rabbits injected intravenously with 50 mg. kemithal daily for two weeks.

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CONTENTS

PAGE

The Pharmacology of Basic Esters of Thiazole Carboxylic Acids. M. R. CHANCE, P. DIRNHUBER and F. A. ROBINSON 153

The Testing of Drugs against Exoerythrocytic Forms of *P. Gallinaceum* in Tissue Culture. ISABEL M. TONKIN 163

Experimental Diabetes: The Effect of Ligation of the Pancreatic Duct upon the Action of Alloxan in Rabbits. A. L. WALPOLE and J. R. M. INNES 174

Actions of Cyanate. K. M. BIRCH and F. SCHÜTZ 186

The Action of Mersalyl, Calomel and Theophylline Sodium Acetate on the Kidney of the Rat. S. E. DICKER 194

Method for the Estimation of Barbituric and Thiobarbituric Acids in Biological Materials. J. RAVENTÓS 210

Kemithal: A New Intravenous Anaesthetic. H. C. CARRINGTON and J. RAVENTÓS 215

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