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SOME PHARMACOLOGICAL AND CHEMOTHERAPEUTIC PROPERTIES OF NOTATIN

BY

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From the Biological Laboratories of Boots Pure Drug Co., Ltd., Nottingham

(Received April 12, 1946)

This paper includes descriptions of work carried out by colleagues in other member firms of the Therapeutic Research Corporation; such work is referred to under the name of the worker responsible; fuller reference to these colleagues is made in the acknowledgment at the end of the paper.

The nature and some of the properties of notatin have been described briefly by Coulthard *et al.* (1942) and Birkinshaw and Raistrick (1943) and in detail by Coulthard *et al.* (1945). The substances "penatin" (Kochalaty, 1942, 1943) and "penicillin B" (Van Bruggen *et al.*, 1943) are similar to, and probably identical with, notatin (Birkinshaw and Raistrick, 1943). Notatin is a flavoprotein enzyme catalysing the oxidation of glucose to gluconic acid by means of atmospheric oxygen, with the production of hydrogen peroxide. It exhibits high antibacterial activity *in vitro* in the presence of glucose, but is inactive in the absence of glucose or in the presence of catalase; the antibacterial activity therefore appears to be due to hydrogen peroxide formed during the oxidation of glucose.

The present paper is an account of some of the pharmacological properties of notatin, and of unsuccessful attempts to demonstrate an antibacterial action *in vivo*.

Acute toxicity and symptoms

The acute toxicity of the purest samples of notatin yet obtained is indicated by the following approximate median lethal doses:

		Mg. per kg. body weight
Mice.	Intraperitoneal	3
	Subcutaneous	4.5
	Intravenous	13
Rabbits.	Subcutaneous	7.5

The oral toxicity is low ; doses up to 300 mg. per kg. have no effect.

The effect of fatal intravenous doses has been observed in dogs, cats, rabbits, rats, and mice. In a cat or dog an intravenous dose of about 30 mg. per kg. rapidly produces severe cyanosis and anoxaemia. The haemoglobin of the blood is converted into a brown pigment which appears to be methaemoglobin ; at the same time the blood becomes more viscous and the clotting time is reduced. Death occurs in a matter of minutes, and cannot be averted by artificial respiration. At autopsy the lungs are grossly oedematous, and in many cases the liver shows marked degeneration. The general picture is similar in rabbits, rats, and mice, with minor differences. On the other hand, intravenous doses which are not immediately fatal appear to have no effect whatever ; this has been observed in cats, rabbits, and rats, with doses so large as to be only just sub-lethal.

The effect of fatal subcutaneous doses is different from that of intravenous doses ; a longer time elapses before death occurs (6 to 24 hours) and there is no observable blood-pigment change. The outstanding symptom in rabbits is a very marked rise in blood sugar, which is described below.

During the early stages of the production of notatin, as the samples obtained became successively purer, it was found that the toxicity became greater as the antibacterial activity *in vitro* became greater, indicating that the high toxicity of notatin is an inherent property of the active material and is not due to a separable impurity. This is confirmed by an observation by Miss Chapman that at least 90% of the toxicity disappeared when the antibacterial activity was destroyed by mild hydrolysis ; the stimulant action on smooth muscle and the antidiuretic action in rats were similarly decreased.

Effect on blood pigment

The effect of large intravenous doses on the blood pigment was investigated in the rat and the rabbit, and considerable differences were observed between the two species. In the rat, severe cyanosis was evident immediately the dose was injected, and the blood samples taken at any time after the injection showed the characteristic absorption spectrum of methaemoglobin. In the rabbit, although the blood gradually became darker after injection and was very considerably darker at the time of death, no methaemoglobin was detectable except in animals which had been anaesthetized with ether. The pigment causing darkening of the blood in unanaesthetized rabbits was not identified.

The change to methaemoglobin could be produced in rabbit blood *in vitro*, provided glucose was added and heparin was used as the anticoagulant. Methaemoglobin was not produced in the absence of added glucose, and not in any case if oxalate was present. Lysed washed red cells were affected in the same way as whole blood.

The fact that the only rabbits in which methaemoglobin formation was observed *in vivo* were etherized animals may be linked with the finding that a high glucose concentration is necessary *in vitro*, since the etherization of a rabbit raises the blood sugar.

Effect on carbohydrate metabolism

The effect of notatin on the reducing substances in blood was investigated in rabbits ; all estimations were done by the Hagedorn-Jensen method.

After subcutaneous injection of a dose in the region of the median lethal dose, either the blood sugar rose gradually, sometimes reaching very high levels, and the animal eventually died, or else there was no observable effect whatever, the blood sugar remaining normal and the animal appearing to be quite unaffected in any way. The larger doses produced the former response, while the smaller doses were without effect, but it was observed more than once that a dose which caused a rise in blood sugar and subsequent death in one animal would have no effect in another animal. The borderline dose was about 7.5 mg./kg. of the purest preparations.

In those animals in which a blood sugar rise occurred, death usually took place from 20 to 30 hours after injection, the blood sugar having risen to a maximum level of 300 to 500 mg./100 ml. ; one or two animals died later than 30 hours. Only two animals survived after a rise of blood sugar, and in both cases the maximum blood sugar level reached was about 250 mg./100 ml. ; in no case did death occur without a blood sugar rise preceding it.

In several animals insulin was injected subcutaneously when the blood sugar was at its maximum, and the latter then fell rapidly: unfortunately this phenomenon was difficult to observe, as the rabbit seemed unable to withstand the combined action of the two drugs, and invariably died soon after the injection of insulin. In a typical experiment the dose given was 7.5 mg./kg., and the blood sugar, initially 120 mg./100 ml., rose gradually until it was 300 mg./100 ml. 24 hours later: at this point 1.6 units of insulin were injected subcutaneously, and the blood sugar fell rapidly, reaching 72 mg./100 ml. 5 hours later; 15 hours after this it had risen again to 150 mg./100 ml., and death occurred a little later.

Glycosuria was observed in some of the animals with high blood sugars, and the liver glycogen and body temperature fell as the blood sugar rose, as shown by the experiment recorded in Table I.

TABLE I

EFFECT ON BLOOD SUGAR AND LIVER GLYCOGEN OF RABBITS

Treatment	Rabbit No.	Blood sugar in mg./100 ml.			Rectal temperature 9 hrs. after injection ° F.	Liver glycogen 9½ hrs. after injection Per cent.
		Initial	4½ hrs. after injection	9 hrs. after injection		
Notatin 15 mg./kg. subcutaneously	1	115	273	455	94·0	0·26
	2	125	255	450	98·4	1·72
	3	126	204	368	97·8	1·36
Controls	4	136	125	191	—	3·86
	5	137	124	137	102·8	5·40
	6	119	103	112	101·6	4·80

After intravenous injection of notatin into unanaesthetized rabbits, a small fall in blood sugar usually occurred: no considerable rise was ever observed.

Chronic toxicity

Rat growth tests carried out on five groups of five litter-mates of about 35 g. weight showed that daily subcutaneous doses up to the largest tolerated dose (0.8 mg./kg.), given for 14 days, had no effect on the growth rate.

A number of adult rabbits were given a daily subcutaneous dose of 2.5 mg./kg. for 14 days: there was no effect on the red cell count, haemoglobin or blood urea, but there was a marked granulocytosis which generally reached a maximum between the 5th and 8th days of dosage and then gradually returned to normal in spite of continuing daily doses. Rats given daily subcutaneous doses of 2 mg./kg. for 20 days showed a similar granulocytosis.

The toxicity to leucocytes was examined by Mr. Freeman, using a modification of the method of Thrower and Valentine (1943). Dilutions of notatin were prepared in normal citrated human blood and a staphylococcal suspension added. After incubation for 1 hour at 37° C., films were prepared and Gram-stained. The bacteria ingested into each of 25 phagocytes were counted, and a mean figure calculated. In the controls this was 21.4 cocci per phagocyte, and in notatin solution (1 in 200) 20.9 cocci per phagocyte. This concentration of notatin therefore did not inhibit phagocytosis.

Dr. Ungar has observed that in tissue cultures of chick embryo heart, notatin added to the nutrient plasma slightly inhibited the growth of fibroblasts in dilutions from 1 in 10,000 to 1 in 1,000, but some growth still occurred at a dilution of 1 in 250.

Local action

It was noticed throughout the experimental work that subcutaneous injections of notatin gave rise to oedematous swelling and pronounced tenderness at the site of injection. This was confirmed in *ad hoc* experiments on rabbits and rats; post-mortem examination showed widespread oedema, adhesions, and subcutaneous haemorrhage.

The action of notatin in uninfected wounds was observed in rats. The animals were anaesthetized and a small incision made in the skin of one leg: the underlying muscle was snipped with pointed scissors to a depth of 2 or 3 mm. and the skin incision was ligatured, all under sterile conditions. The opposite leg was treated similarly, but notatin was introduced before ligation. In most animals, approximately 0.5 mg. powdered notatin was introduced (about 2.5 mg. per kg. body weight), but in a few animals a solution was used (0.1 ml. of 0.2 per cent; equivalent to 1.0 mg. per kg. body weight). In three of the 12 rats used the foot distal to the notatin-treated wound became grossly swollen and oedematous 4 to 6 days later, the use of and sensation in the limb being temporarily lost. The swelling began to subside in 10 to 12 days and the limb eventually returned to normal in 2 of the 3 animals (the third was killed for examination). Of these animals, two had been treated with notatin powder and one with a solution. In the remaining rats little or no swelling occurred and no difference in degree of healing was apparent between the treated and control wounds. No systemic effects were observed in any of the animals.

The action of notatin in an infected wound was observed in a single rabbit. Wounds were made in each leg as before and both were heavily, and as far as possible equally, infected with *Staphylococcus aureus* (Mrs. Fox): one wound was then treated with 2 mg. powdered notatin. On the 4th day the notatin-treated leg was oedematous. On the 7th day the treated wound was deeply necrosed and very inflamed, while the untreated wound appeared to be healing well. On the 14th day both wounds were discharging pus, but the treated wound was clearly in a worse state than the untreated one. On the 24th day the

animal was killed and both wounds opened; the untreated one contained some pus, but appeared to be healing, while the treated wound contained much pus, showed considerable necrosis of skin and muscle, and was surrounded by swollen and inflamed tissue.

Inactivation of notatin in the body

Notatin loses its antibacterial action in the body. Large doses have been given to rabbits, both intravenously and subcutaneously, and samples of blood removed at varying intervals: in no case has any antibacterial activity been detectable in these samples when tested *in vitro*. Also the antibacterial activity *in vitro* is inhibited in the presence of whole blood, defibrinated blood or serum; this observation has been confirmed by Mr. Freeman. Heat treatment of serum destroys its power of inactivation.

Effect on diuresis

Two groups of eight male rats were given known volumes of water by stomach tube, after being deprived of food and water overnight. The animals in one group received a subcutaneous injection of notatin (0.3 mg./kg.) at the time of administration of the water, and the urine from both groups was collected for 30 hours. A week later the experiment was repeated with the same rats, the two groups being crossed over. The results were expressed as a mean percentage of the total water given which had been excreted as urine at various times after dosing, all sixteen animals being included in both treated and control groups. The results are given in Table II.

TABLE II

EFFECT ON DIURESIS IN RATS

Time after giving water and notatin	Percentage of total water excreted	
	Controls	Notatin treated
2 hours	51	40
4 hours	69	53
7 hours	72	54
22 hours	99	62
30 hours	107	66

Similar experiments were carried out with varying doses of notatin, and they indicated that single doses of 0.1 mg./kg. and upwards have a powerful anti-diuretic action, resulting in the retention in the body of a considerable portion of the water administered for a period of at least 30 hours.

Effect on smooth muscle

Notatin causes contraction of rabbit intestine and virgin guinea-pig uterus *in vitro*; concentrations of 1 in 40,000 down to 1 in 200,000 cause fairly powerful

contractions of the latter muscle; this observation has been confirmed by Miss Chapman. Dr. Wien has shown that notatin produces vasoconstriction in the perfused rabbit ear, in a concentration of 1 in 1,000.

Antibacterial activity in vivo

Tests of antibacterial activity *in vivo* were carried out in male mice of about 20 g. weight. A suspension of the organism was injected intraperitoneally, and this was followed immediately by an injection of notatin: in some cases this was the only dose of notatin, and in others it was the first of a series of doses at regular intervals.

Experiments were carried out with three organisms lethal to mice: *Streptococcus haemolyticus* (Richards), *Staphylococcus aureus* (C.N. 59), and a virulent strain of salmonella isolated from laboratory mice during an epidemic.

Notatin was administered in a variety of ways: subcutaneously, intravenously, and intraperitoneally; as a single dose, and as a series of doses at regular intervals; in doses up to 1.0 mg. per kg. body weight, the maximum tolerated dose; and in some cases with simultaneous injection of glucose. In most of the experiments penicillin or sulphonamides were used as positive control treatments.

The results obtained showed clearly that notatin had no therapeutic activity in these experiments. In view of the negative results, detailed protocols of the experiments are not given. Mr. Standfast also carried out an extensive series of experiments on mice infected with a strain of haemolytic streptococcus, with similar negative results.

Some experiments were also carried out with a strain of *Staphylococcus aureus* (Mrs. Fox) which is not lethal to mice; the usual criterion of therapeutic action—i.e., survival of animals which would otherwise have died—could not, therefore, be used. It was found that if a suitable infecting dose (of the order of 100 million organisms) was given by intraperitoneal injection, a number of small abscesses developed within a few days in the peritoneal cavity, and could be enumerated on autopsy, and an attempt was made to use the number of abscesses as a criterion of therapeutic effect.

A number of mice were given equal infecting doses, after which some groups were given subcutaneous injections of notatin or another drug, either as a single dose or as a series of doses spread over 24 hours, and other groups were left untreated to serve as controls. At a definite interval after injection (usually 72 hours) the mice were killed and autopsied, and the abscesses visible within the peritoneal cavity enumerated, no account being taken of variation in size. The counts varied from 0 to 9 abscesses per mouse.

The results were difficult to interpret. While the animals receiving the various drug treatments (notatin, penicillin, or sulphathiazole) generally showed lower abscess counts than the untreated controls, and the difference was often statistically significant, yet the degree of lowering was erratic and bore little relation to either the drug given or the amount. A given dose level of a given drug would produce a large and significant lowering of the count in one experiment, and have no effect in the next, while a larger or smaller dose of the same drug would behave in the opposite manner.

On the whole notatin caused a greater reduction of the abscess count than did penicillin, in contradistinction to the relative therapeutic activities in the other

in vivo tests (including those using *Staphylococcus aureus* C.N. 59) in all of which penicillin was effective while notatin was inactive. Certain aspects of the technique were investigated as possible sources of erratic variation in the counts, e.g., settling of the bacterial suspensions, and fatigue of the operator during the counting of the abscesses, but the erratic results could not be explained on these lines.

Some twenty experiments were performed in all; for reasons of space, the results cannot be given *in extenso,* but as the method has not been described previously, so far as we are aware, the protocol of one experiment is given in Table III. In this experiment both the mice treated with notatin and those treated with penicillin showed significantly lower counts than the controls.

TABLE III

EFFECT ON INTRAPERITONEAL ABSCESSES IN MICE

Organism. *Staphylococcus aureus* (Mrs. Fox).
Infection. 380 million organisms per mouse.
Treatment. Total dose of 0·1 mg. per kg. notatin, or 1,000 Oxford units per kg. penicillin, divided into two equal doses at 0 and 4 hours after injection.
Counted. 75 hours after infection.

Treatment	No. of Mice	Individual counts of intraperitoneal abscesses	Mean count
Controls	10	6, 6, 9, 6, 6, 5, 3, 6, 4, 6	5·7
Notatin	10	1, 0, 3, 2, 4, 4, 5, 4, 3, 4	3·0
Penicillin	10	5, 5, 5, 4, 5, 2, 4, 4, 2, 1	3·7

Discussion

It has been shown that notatin is highly toxic to animals. When given intravenously it interferes with the oxygen-carrying function of the blood by converting haemoglobin to methaemoglobin, and also causes gross oedema of the lungs. The two conditions proceed simultaneously, and death by asphyxia occurs rapidly. In the rat the blood pigment formed has been identified as methaemoglobin and this has also been found in the etherized rabbit; in the unanaesthetized rabbit the blood darkens, and loses the power of taking up oxygen, but the pigment formed does not appear to be methaemoglobin.

Notatin converts the haemoglobin of rabbit's blood to methaemoglobin *in vitro* only if excess glucose is added. It therefore seems probable that the formation of methaemoglobin is linked with the oxidation of glucose to gluconic acid by atmospheric oxygen, a process which is known to be catalysed by notatin; a reaction of the following kind may occur:

$$C_5H_{11}O_5CHO + Hb + O_2 \longrightarrow C_5H_{11}O_5COOH + HbO$$

Large subcutaneous doses of notatin cause death after several hours. There are no symptoms of asphyxia and the haemoglobin is unaffected, but the blood

sugar (at least in rabbits) rises considerably, at the expense of the liver glycogen. The toxicity is about three times greater when given subcutaneously than it is when given intravenously; together with the difference in symptoms, this leads to the conclusion that the mode of death is different in the two cases. The cause of death after subcutaneous injection is not clear, although it appears to be linked with the rise in blood sugar. It was not found possible to produce the symptoms of intravenous administration by giving notatin subcutaneously, however large the dose.

Sub-lethal subcutaneous doses given daily for a long period have no apparent systemic toxic effect. Subcutaneous injection or local application produce marked tissue damage, including oedema, haemorrhage, and necrosis. The only marked systemic effect of non-lethal doses which has been observed is a powerful anti-diuretic action; small subcutaneous doses cause retention of water in the body for 30 hours or more. The antidiuretic action and characteristic severe oedema produced by notatin suggest that it is a powerful capillary poison.

No definite evidence has been obtained of any antibacterial activity *in vivo*; mice were not protected from lethal doses of streptococci, staphylococci, or salmonella by notatin given either intravenously or subcutaneously. Since the antibacterial action of notatin *in vitro* in the presence of glucose is due to the hydrogen peroxide liberated, it is easy to understand its inactivity *in vivo*, as any hydrogen peroxide formed would be immediately destroyed by the catalase universally present in the blood and tissues.

The significance of the staphylococcal abscess experiments is doubtful. In many cases notatin reduced the number of intraperitoneal abscesses formed after injection of a non-lethal strain of staphylococcus in mice; other drugs of known antibacterial efficiency (penicillin and sulphathiazole) also produced this effect. There was, however, no relationship between the size of dose and degree of effect, and the relative efficiencies of the three drugs and of various doses of them ran quite contrary to their known relative efficiencies in normal *in vivo* experiments in which survival is the criterion. The abscess experiments cannot therefore be accepted as unequivocal evidence of antibacterial activity.

SUMMARY

1. The purest samples of notatin yet obtained have the following acute median lethal doses:

		Mg. per kg. body weight
Mice.	Intraperitoneal	3
	Subcutaneous	4.5
	Intravenous	13
Rabbits.	Subcutaneous	7.5

The oral toxicity is low; doses up to 300 mg. per kg. have no effect.

2. Fatal intravenous doses of notatin cause rapid death by anoxia due to pulmonary oedema and loss of the oxygen-carrying capacity of the blood; in some species severe methaemoglobinaemia occurs.

3. Fatal subcutaneous doses of notatin cause death several hours later: the cause of death is unknown, but death is associated with a progressive and marked rise in blood sugar (at least in the rabbit).

4. Local application of notatin causes marked tissue damage. Severe oedema, haemorrhage, and necrosis occur.

5. Single small subcutaneous doses of notatin cause retention of water in the body for periods of 30 hours or more. Notatin appears to act as a powerful capillary poison.

6. Notatin causes contraction of isolated smooth muscle, and produces vasoconstriction in the perfused rabbit ear.

7. Notatin does not exhibit antibacterial activity *in vitro* in the presence of blood or serum.

8. Notatin does not protect mice from lethal doses of streptococci, staphylococci, or salmonella.

9. Notatin sometimes reduces the number of intraperitoneal abscesses formed after intraperitoneal injection of a non-lethal strain of *Staphylococcus aureus* in mice, but it is doubtful whether this indicates any antibacterial action.

The work described in this paper was carried out as part of a programme of the Therapeutic Research Corporation of Great Britain, Ltd., to which acknowledgments are given. The authors wish to acknowledge the co-operation received from the other member firms of the Corporation, and particularly to thank Miss C. J. Chapman and Mr. A. B. Standfast of The British Drug Houses, Ltd., Dr. J. Ungar of Glaxo Laboratories, Ltd., Mr. W. A. Freeman and Dr. R. Wien of May & Baker, Ltd., and Mr. R. Thorp of the Wellcome Physiological Research Laboratories, for permitting the incorporation of their results in this paper.

REFERENCES

Birkinshaw, J. H., and Raistrick, H. (1943). *J. biol. Chem.*, **148**, 459.
Coulthard, C. E., Michaelis, I., Short, W. F., Sykes, G., Skrimshire, G. E. H., Standfast, A. F. B., Birkinshaw, J. H., and Raistrick, H. (1942). *Nature, Lond.*, **150**, 634.
Coulthard, C. E., Michaelis, I., Short, W. F., Sykes, G., Skrimshire, G. E. H., Standfast, A. F. B., Birkinshaw, J. H., and Raistrick, H. (1945). *Biochem. J.*, **39**, 24.
Kochalaty, W. (1942). *J. Bact.*, **44**, 143.
Kochalaty, W. (1943). *Science*, **97**, 186.
Thrower, W. R., and Valentine, F. C. O. (1943). *Lancet*, **1**, 133.
Van Bruggen, J. T., Reithel, F. J., Cain, C. K., Katzman, P. A., Doisy, E. A., Muir, R. D., Roberts, E. C., Gaby, W. L., Homan, D. M., and Jones, L. R. (1943). *J. biol. Chem.*, **148**, 363.

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CHANGES IN *CIS-* AND *TRANS-* STILBAMIDINE SOLUTIONS ON EXPOSURE TO LIGHT

BY

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The changes which *trans-* 4:4′-diamidinostilbene (stilbamidine) undergoes in aqueous solutions exposed to light have recently received much attention. Fulton and Yorke (1942) demonstrated experimentally the greatly increased toxicity of exposed solutions for mice. Barber, Slack, and Wien (1943) concluded that the main product of change was 4:4′-diamidinophenylbenzylcarbinol produced by the addition of water to the ethylenic double bond in the parent substance. The saturation of this double bond was confirmed spectrographically by Goodwin (1943). Henry (1943) stated that one or both amidine groups were hydrolysed by prolonged exposure to diffuse daylight with accompanying formation of ammonium chloride in solution. He believed that a dimer of the parent substance was formed as a principal primary product as well as *cis-* stilbamidine.

We have investigated the changes which *cis-* and *trans-* stilbamidine undergo on exposure to light in solution by biological as well as spectrographic and chemical methods. We are indebted to Dr. H. J. Barber of May and Baker, Ltd., for a gift of *cis-* stilbamidine and also for the purified product of irradiation of the *trans-* form.

EXPERIMENTAL

Biological

The toxicities of fresh and exposed solutions of *cis-* and *trans-* forms in distilled water were determined in mice by intraperitoneal injection. The *cis-* form was used as sulphate dissolved in the cold to give a 0.2 per cent solution. The *trans-* sulphate is almost insoluble, and solutions of the dihydrochloride were used. Similar solutions were tested after being kept in the dark. Therapeutic experiments with these solutions were also carried out in mice infected with *T. rhodesiense*. The results obtained are recorded in Tables I and II. It will be seen that the *cis-* form, in freshly prepared solutions, is more toxic and less active therapeutically than the *trans-* isomer. Both are stable when stored in solution in the dark (confirmed spectrographically). When exposed to light the *cis-* form in the above concentration became less toxic for mice and more active

therapeutically, which is the reverse of what takes place with the *trans*- form. The reason for these changes is discussed below. It was noted that a 0.2 per cent solution of the *cis*- form began to deposit crystals on the sides of the flask within an hour after exposure, whereas a 0.05 per cent solution did not give rise to any deposit however long exposed. In the animal tests the stronger solution was administered after making a homogeneous suspension by shaking. The examination of the supernatant solution and crystalline deposit is described in the next section.

TABLE I

TOXIC EFFECTS OF FRESH AND EXPOSED SOLUTIONS OF *CIS*- AND *TRANS*- STILBAMIDINE IN MICE

D = Died in less than 1 hour after injection.
P = Died within a few days of injection.
S = Survived during observation period of two weeks or longer.

Drug	Nature of solution	Effect of doses (mg./20 g. mouse, intraperitoneally)		
		1·0	0·5	0·25
Cis- stilbamidine sulphate (contains 72·9% of the base)	Fresh	6D/6	4D / 2P /10* 4S /	5S/5
	7 days in dark	5D/5	8D / 2S /10	5S/5
	14 days in dark	3D/3	6D / 4S /10	5S/5
	7 days in sunlight	8D / 1P /10 1S /	1P / 14S/ 15	5S/5
	14 days in sunlight	5D/5	1D / 9S /10	5S/5
Trans- stilbamidine dihydrochloride (contains 78·3% of the base)	Fresh	1P / 9S/ 10	10S/10	—
	7 days in sunlight	—	5D/5	5S/5*

* Doses not well tolerated by surviving animals.

Chemical and spectrographic

Most spectrographic data were obtained with a Beckman photoelectric spectrophotometer (Cary and Beckman, 1941), but at the start of the investigation photographic records were obtained with a Hilger E3 spectrograph used in conjunction with a rotating sector photometer and iron-nickel arc.

It was found that the absorption spectra of *cis*- and *trans*- stilbamidine (Fig. 1) bore the same relationship to one another as those of the parent stilbenes

(Smakula and Wasserman, 1931), thus indicating the purity of the specimens employed. In the *cis-* compound the ϵ value is reduced and the position of λ max. is shifted to shorter wavelengths (*cis-* ϵ max. 14,000, λ max. 299 mμ; *trans-* ϵ max. 37,800, λ max. 329 mμ).

TABLE II

RESULTS OF TREATMENT OF *T. RHODESIENSE* INFECTIONS IN MICE WITH SOLUTIONS OF *CIS-* AND *TRANS-* STILBAMIDINE

D = Died within an hour of receiving drug.
C = Cured.
R = Blood free from trypanosomes but a relapse occurred.
N = Blood never free from trypanosomes.

Drug	Nature of solution	Effect of doses (mg./20 g. mouse, intraperitoneally)					
		0·5	0·25	0·1	0·05	0·025	0·01
Cis- stilbamidine sulphate	Fresh	2D / 1R /4 1C/	3R / 4 1C/	4R/4	3R/3	3N/3	4N/4
	7 days in sunlight	5C/5	5C/5	4C/4	2R / 5 3C/	8R / 9 1C/	2N / 5 3R/
	14 days in sunlight	—	—	5C/5	4R / 5 1C/	2N / 2R /5 1C/	3N / 5 2R/
Trans- stilbamidine dihydrochloride	Fresh	4C/4	4C/4	4C/4	3C/3	3C/3	2R / 4 2C/

In view of the biological changes noted in the exposed *cis-* solutions the solid deposit was separated by centrifugation and examined separately from the supernatant liquid. The absorption spectrum of the latter was identical with the irradiation product of *trans-* stilbamidine (Fig. 1), and its toxicity for mice was of the same order. The solid which had separated was identified as *trans-* stilbamidine sulphate by its insolubility in water and other solvents, by its fluorescence, solubility in hydrochloric acid and characteristic absorption spectrum in that medium, as well as by analysis (Found: C, 52.40; H, 4.99; N, 15.42; S, 8.41. Calc. for $C_{16}H_{16}N_4H_2SO_4$: C, 53.0; H, 4.98; N, 15.47; S, 8.84 per cent). The fact that the solid *trans-* sulphate is produced from a 0.2 per cent irradiated solution of the *cis-* isomer gives an explanation of the changes which occur in biological properties (Tables I and II). The therapeutically more active *trans-* form which is deposited as solid remains unaffected by light (Fulton and Goodwin, 1945), whereas the relatively small amount of *trans-* form remaining in solution is converted to the saturated product as shown by spectrographic evidence.

In more dilute solutions (0.05 per cent) of the *cis-* compound no precipitation occurred on irradiation, and an examination of the absorption spectra of such solutions irradiated for varying periods of time showed that small changes took place within half an hour from the start of exposure. Even after 72 hours, however, no further change could be detected. The new absorption curve corresponded with that obtained by a combination representing 89 per cent of the *cis-* and 11 per

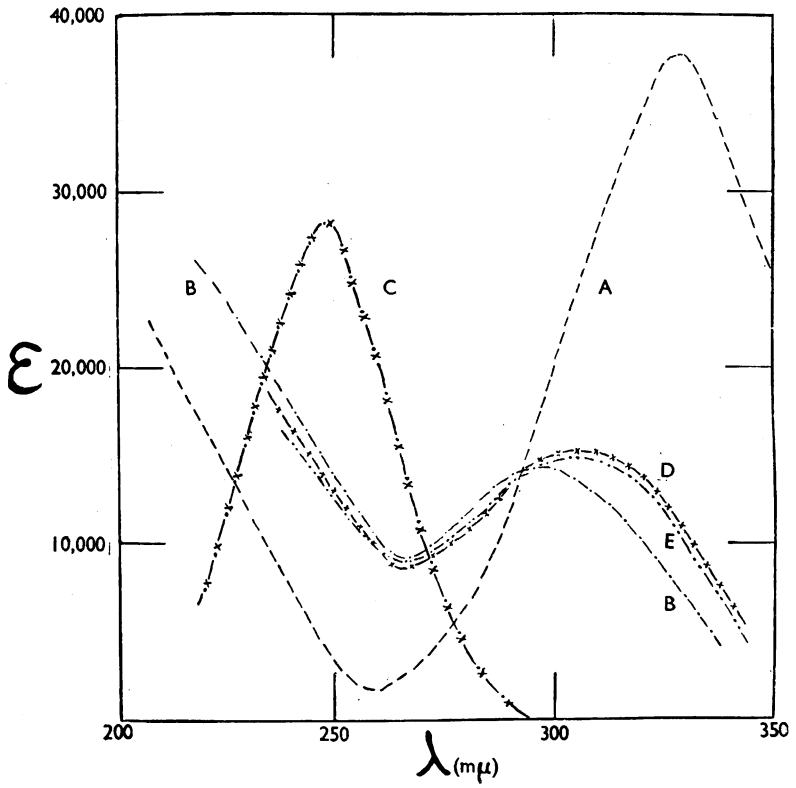

FIG. 1.—Absorption spectra of *cis-* and *trans-* stilbamidine. A - - - *trans-* stilbamidine, fresh ; B -·-·- *cis-* stilbamidine, fresh ; C -·x-·x product of irradiation of *trans-* stilbamidine ; D - x - x - *cis-* stilbamidine (0.01 per cent) exposed to U.V. light ; E -·-·-·- computed curve for a 9 : 1 mixture of *cis-* and *trans-* stilbamidine.

cent of the *trans-* isomers. It was difficult to state definitely that the saturated product was completely absent in the solutions. A computed curve for 85 per cent *cis-*, 10 per cent *trans-* and up to 5 per cent of the saturated compound was not definitely distinguishable from the 89/11 per cent *cis- trans-* curve. The correct conclusion to be drawn is that if the saturated product was present in solution it was in a concentration of less than 5 per cent of the dissolved substances.

Pure *trans-* solutions undergo photochemical change so quickly with production of the saturated compound that any tendency to *cis- trans-* equilibrium is not observed. This fact, combined with the results already considered, points to the conclusion that *cis- trans-* rotation is the first photochemical change and that it is the *trans-* compound which undergoes the change involving saturation of the double bond.

Why dilute solutions of the *cis-* form give rise to a relatively stable equilibrium with the *trans-* isomer without the latter being converted to the saturated substance in any appreciable amount, with resultant disturbance of such equilibrium, cannot be easily answered. Repeated experiments have, however, given similar results. Here, no precipitation of the *trans-* sulphate occurs as in the case of the stronger solution. If it be assumed that the *trans-* form is stabilized in some way, the results obtained on irradiation of concentrated and dilute solutions of the *cis-* compound are not necessarily contradictory. The strong *cis-* solution could proceed to equilibrium with formation of 11 per cent of the *trans-* compound when the solubility product of the *trans-* sulphate would be exceeded ; precipitation of the latter would disturb the equilibrium. If no stabilization of the *trans-* form occurred, the solubility product of its sulphate would not be exceeded on account of the formation of the saturated form.

It is very probable that the formation of the " saturated " product by irradiating aqueous solutions of *trans-* stilbamidine is due to collisions between activated *trans-* molecules. The high absorption coefficient of this compound indicates that a large proportion of the molecules present in an irradiated solution may be activated. In a solution in which the *trans-* isomer is the only solute, the chance of collisions between activated molecules will thus be high, permitting rapid formation of the saturated compound. In a dilute solution containing a mixture of 89 per cent *cis-* and 11 per cent *trans-* stilbamidine an activated *trans-* molecule is much more likely to collide with a *cis-* molecule than with another *trans-* molecule. Deactivation consequent upon such collisions may well be the explanation of the " stabilizing " of the *trans-* compound in such a solution resulting in an apparent equilibrium. In a concentrated *cis-* solution the formation of *trans-* proceeds almost to completion owing to its removal from solution as the insoluble sulphate. Towards the end of the photochemical reaction small amounts of both isomers probably remain in solution, but now the ratio of *cis-* to *trans-* is much less than in the dilute solution discussed above. Chances of collisions between activated *trans-* molecules with formation of the saturated compound are much greater ; small amounts of this substance were in fact detected (Table III).

Trans- stilbamidine base in *iso*butyl alcohol distilled over calcium metal had the same absorption spectrum in fresh preparations as an aqueous solution of the salt. Both solutions underwent the same changes on exposure to light, the former much more slowly than the latter. In view of the slight solubility of the

base in *iso*butyl alcohol and the small amount exposed it was difficult to exclude the rôle which traces of water may have played in formation of the saturated compound. The same change into the saturated product occurred in the absence of oxygen when stilbamidine in boiled distilled water in a sealed tube filled with nitrogen gas was exposed to light. The formation of the carbinol involves the production of an asymmetric centre, but, as we expected, under the conditions of experiment, both *cis-* and *trans-* solutions after exposure remained optically inactive.

Table III shows the amount of each substance present following irradiation of a 0.2 per cent solution of *cis-* stilbamidine sulphate under different conditions.

TABLE III

PRODUCTS OF IRRADIATION OF 10 MG. *CIS-* STILBAMIDINE IN 0·2 PER CENT SOLUTION

	Time of irradiation			
	7 days sunlight	7 days sunlight	12 hrs. u.v. lamp	14 days sunlight
Cis- form unchanged ..	Not estimated		1·1 mg.	0·9 mg.
Saturated compound ..	3·6 mg.	2·8 mg.	2·4 „	1·5 „
Trans- form as solid ..	4·3 „	6·4 „	5·4 „	6·4 „
Percentage recovery	[79]	[92]	89	87

Contrary to the findings of Henry (1943) we have not been able to detect the presence of ammonia in exposed solutions in any significant amount. Our method consisted in aspirating these solutions into Nessler's reagent both before and after alkalinization with potassium carbonate. Only the minutest traces were detected and we therefore concluded that under our conditions of experiment hydrolysis to amide did not occur, or only to a negligible degree, and in support of this fact precipitation of solid from exposed solutions of *trans-* stilbamidine was never detected. These conclusions were confirmed by spectrographic data. Thus, the irradiated product, whatever its constitution, has an ε value much higher than that of diphenyl ethane (amidine groups absent) and suggests that these groups remain intact after irradiation. Further, irradiated *trans-* stilbamidine gives an absorption spectrum with molecular extinction coefficient identical with that of the purified compound isolated from the irradiated solution which shows that complete conversion to the latter occurs. In irradiation experiments with the *cis-* compound only 80–90 per cent of the original material could be accounted for, but as very small quantities were used it is probable that the manipulations involved accounted for the loss. The measurement of ε would not allow us to distinguish between the parent substance and a dimer, if such were formed, with amidine groups intact. However,

from an examination of the position of the absorption bands and toxicities for mice of *p*-tolamidine (λ max. 241 mμ: 4 mg. tolerated per 20 g. mouse i.p.); 4:4′-diamidinodiphenylethane (λ max. 245 mμ: 1.0 mg. tolerated) and the irradiated product (λ max. 251 mμ: 0.1–0.2 mg. tolerated) it appears that a cleavage or simple reduction of stilbamidine at the ethylenic double bond has not taken place.

Summary

An examination has been made by biological and spectrographic methods of the changes which occur in aqueous solutions of *cis*- and *trans*- stilbamidine when exposed to light. From experimental data we conclude that the change *cis*→*trans* occurs previous to formation of the saturated product of irradiation. No evidence of the reverse *trans*→*cis* change was obtained. Under our experimental conditions no conversion of the parent substances to amides occurred. Some observations have been made on the nature of the irradiation product formed.

References

Barber, H. J., Slack, R., and Wien, R. (1943). *Nature, Lond.*, **151**, 107.
Cary, H. H., and Beckman, A. O. (1941). *J. opt. Soc. Amer.*, **31**, 682.
Fulton, J. D., and Goodwin, T. W. (1945). *J. Pharmacol.*, **84**, 42.
Fulton, J. D., and Yorke, W. (1942). *Ann. trop. Med. Parasit.*, **36**, 134.
Goodwin, T. W. (1943). *Ann. trop. Med. Parasit.*, **37**, 59.
Henry, A. J. (1943). *Nature, Lond.*, **152**, 691.
Smakula, A., and Wasserman, A. (1931). *Z. phys. Chem.*, **A155**, 353.

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EXPERIMENTAL DIPHTHEROID INFECTIONS OF THE RABBIT'S EYE AND THEIR TREATMENT

BY

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In the course of investigations of trauma produced by various toxic agents in the rabbit's eye, it was found that infection usually occurred. The organism most frequently recovered was a diphtheroid of the Hofmann group, and evidence was obtained that the infection with this organism materially contributed to the severity of the ensuing lesions.

It became of interest to determine: (1) the methods by which infective lesions of the eye could be produced by this organism, and (2) the effect of various antiseptic and chemotherapeutic agents upon this infection.

MATERIALS

Bacteriology of the organism

The organism used in the present investigation (Ho.R.) is a Gram-positive bacillus which shows perfect uniformity in films from cultures and appears as a rod, sometimes so short that it might be mistaken for a coccus of a slightly ovoid shape. In the Albert stain (modification of Neisser's method) many metachromatic granules are seen, but their size and arrangement are much less regular than those in the diphtheria bacillus. Films made from the purulent corneal lesion always show the organism in a definite bacillary form, sometimes even considerably elongated. The growth of the organism on blood agar is rapid and luxuriant; large, round, elevated colonies of moist, smooth surface and a definite white or slightly yellow colour are formed; this cultural appearance is almost identical with that of staphylococcus aureus, from which it could hardly be differentiated without further study. The growth of Ho.R. on ordinary agar is equally abundant but is apt to be unreliable; very small inocula may fail to grow at all. The growth in ordinary broth is poor and slightly granular. Suspensions in saline, if made from blood agar, show a similar granular appearance; if made from ordinary agar, however, a perfectly homogenous suspension can be obtained.

No attempt was made to relate the organism to any of the animal-pathogenic diphtheroids; but in one test on mice it was found that intraperitoneal injection of half a loopful was lethal within 24 hours with the production of bacteraemia, while 1/100th of this dose produced no effect.

It is worth mentioning that several times in the course of our experiments the organisms isolated from the infected eyes had been transformed into a variant with the features of *C. xerosis*, which grows in characteristically small colonies.

Therapeutic substances

The following substances were used:

(1) Penicillin: a solution of 500 units per ml.

(2) Sodium sulphacetamide: 30 g./100 ml., used in combination with a detergent.

(3) Marfanil (*p*-aminomethyl-benzene sulphonamide): 10 and 30 g./100 ml. Solutions prepared by dissolving marfanil hydrochloride in saline and neutralizing to *p*H 7.0.

(4) V335 (*p*-methylsulphonyl benzylamine): 10 and 30 g./100 ml. The hydrochloride was dissolved in saline and neutralized to *p*H 7.0.

(5) V187 (*p*-methylsulphonyl benzamidine): 10 g./100 ml. The hydrochloride was dissolved in saline, giving a solution of *p*H 6.0, which was not neutralized, as the free amidine base is liable to be precipitated.

V335 and V187 are new chemotherapeutic substances described by Evans, Fuller, and Walker (1944).

(6) Proflavine: 0.1 g./100 ml. A buffered solution of *p*H 6.3 was made up from isoflav (Boots).

(7) In two experiments a detergent, 0.1 g./100 ml. duponal (sodium lauryl sulphate), was added to the solutions of sodium sulphacetamide and marfanil.

METHODS

In vitro *tests of drugs on Ho.R.*

For testing bacteriostatic and bactericidal efficacy in one and the same operation, the following simple technique was devised. Small test tubes were used, receiving 1 ml. of the drug solution in serial aqueous dilutions. To each dilution and a saline control was added a drop of a dense suspension of Ho.R. (50–60,000 million per ml.) so that the number of organisms in each tube was about 1,500 million. The tubes were kept at room temperature (about 18° C.), shaken from time to time, and tested at intervals by inoculation on blood agar plates. This inoculation was done by a method which made possible the determination of both bacteriostatic and bactericidal effects. The plates were thoroughly dried before inoculation and divided into 6–8 horizontal strips, each strip being reserved for the testing of one tube. A loopful was taken from the bottom part of each test tube without shaking and seeded at one end of an agar strip on a circular area 10–15 mm. in diameter (primary inoculation area). The drop was allowed to soak into the medium, while being spread over this small circle, within 5–10 seconds. When all the primary areas were inoculated in this way, the organisms deposited on each of them were spread over the remaining part of the agar strip: the loop was stroked several times over the whole of the primary area and then, without retouching it, stroked in as many parallel lines as possible over the so far untouched length of the strip (secondary inoculation area). It is obvious that the primary areas contain the organisms together with the drug, though in a concentration which is probably well below that in the corresponding test tube, owing to the diffusion of the fluid into the agar. The secondary areas on the other hand contain the organisms freed from the drug, which has seeped away before the bacterial inoculum has been picked up from the dry surface of the primary inoculation area. The correctness of this assumption was borne out by the appearance of the plates after 24 hours' incubation: a bacteriostatic effect was shown by absence of growth in the primary area and abundant growth in the secondary area; absence of growth in both zones indicated a bactericidal effect in the test tube, whereas equally full growth in both areas proved the lack of any bacteriostatic or bactericidal effect.

In vitro tests with some of the drugs on Ho.R. gave the following results: Penicillin was bacteriostatic in the smallest concentration used, viz., 28 units per ml.; at 226 units per ml. it was strongly but not completely bactericidal in

24, but not in 4 hours; at 113 units per ml. it was not bactericidal in 24 hours. Sulphacetamide was bacteriostatic at 0.75 g./100 ml., but not bactericidal at 30 g./100 ml. even after 48 hours. Marfanil had no bacteriostatic or bactericidal effect whatever at a concentration of 1 g./100 ml.; at 10 g./100 ml. it was completely bacteriostatic and killed the organisms in 48, but not in 24 hours. Proflavine had no bacteriostatic effect at all, but within 24 hours, though not in 4 hours, it killed the organisms at 0.4 g./100 ml. and still showed at 0.1 g./100 ml. a marked though incomplete bactericidal effect.

Animal Experiments

(1) *Intracorneal injections.*—Intracorneal injections were performed by a method previously described (Robson and Scott, 1943). When a suspension containing 25 million organisms per ml. was used, an extensive abscess of the cornea developed in 48 hours, ending in perforation. With a suspension containing fewer organisms the severity of the lesions showed great variation in different animals; occasionally a severe lesion resulted from injection of a suspension containing so few as 2,500 organisms per ml., while in other animals the injection of a suspension containing 2.5 million per ml. produced only a mild lesion. Consequently, this method was considered unsatisfactory for the investigation of therapeutic substances.

(2) *Inoculation of the denuded cornea.*—In these experiments the epithelium was removed from an area about 6×6 mm. in the centre of the cornea. In the absence of deliberate infection the denuded area becomes epithelialized within 48 hours. Inoculation of the conjunctival sac with a few drops of a suspension containing 2,500 million organisms per ml. was always followed by infection of the denuded area, which delayed epithelialization. In some animals severe ulcers developed, and in others only a mild infiltrative process, healing within a week. When this suspension was diluted 100 times there was no appreciable delay in the process of healing. This method also was considered unsuitable for therapeutic investigations.

(3) *Deep scarification.*—The method found suitable for testing drugs was as follows. The corneal epithelium was removed from an area 6×6 mm. and the subjacent substantia lightly scarified with a discission needle. Inoculation of the conjunctival sac with the diphtheroid suspension (2,500 million per ml.) was always followed by the development of a severe and progressive infection running a fairly typical course. In 24 hours the area staining with fluoresceine was more extensive than that originally denuded, and the cornea in this area was very hazy. In two to three days the lesion became a corneal abscess which spread and, in the more severe cases, involved most of the cornea by the end of the first week; in many cases perforation occurred. The accompanying conjunctivitis was always severe, with profuse discharge, and membrane formation on the palpebral conjunctiva was common.

(4) *Treatment.*—In all animal experiments lesions were produced in both eyes, one eye being treated and the other used as control. At each treatment three drops of the drug solution were instilled into one eye, whilst the control eye received an equal amount of normal saline. Treatment was started one or six hours after the production of the lesion and continued at hourly intervals for 8–11 hours on the first day, for 13 hours on the second day, and for 8 hours on subsequent days. The duration of treatment was from 7 to 10 days.

Cultures were taken from the conjunctival sacs in the mornings, always before the first treatment.

The results were assessed numerically according to an arbitrary scale:
1 = slight,
3 = severe, and
5 = very severe with perforation.
Intermediate figures were used for lesions of intermediate severity.

RESULTS

These are given in Table I.

Deep scarification (diphtheroid infection)

It will be seen that penicillin, 30 g./100 ml. marfanil, and 30 g./100 ml. V335 all had a very beneficial effect when the treatment was started an hour after infection; at 6 hours the effect was slight but definite.

Marfanil and V335 were appreciably less effective in 10 g./100 ml. solutions, and at 6 hours these weaker solutions had no significant effect even, in the case of marfanil, when combined with the detergent. V187 was without appreciable effect.

TABLE I

TREATMENT OF LESION PRODUCED BY INOCULATION OF SCARIFIED CORNEA WITH DIPHTHEROID

Treatment		Interval between inoculation and first treatment (Hours)	Number of animals	Assessment of final corneal lesion (Mean values on arbitrary scale)		Difference between treated and control eyes
				Treated eyes	Control eyes	
Marfanil	30 g./100 ml.	1	5	1·4	4·0	2·6
	10 ,,	1	3	3·4	5·0	1·6
	30 ,,	6	3	2·5	3·9	1·4
	10 ,,	6	3	4·4	4·7	0·3
	10 ,, + duponal	6	3	4·0	4·5	0·5
V335	30 g./100 ml.	1	3	1·6	5·0	3·4
	10 ,,	1	3	2·8	4·9	2·1
	30 ,,	6	3	3·7	5·0	1·3
	10 ,,	6	3	4·6	4·8	0·2
V187	10 ,,	1	3	4·3	4·7	0·4
Penicillin 500 U/ml.		1	6	2·2	5·0	2·8
		6	3	2·7	4·1	1·4
Proflavine 0·1 g./100 ml.		1	3	3·5	5·0	1·5
Sodium sulphacetamide 30 g./100 ml. + duponal		1	3	4·1	3·8	−0·3

Proflavine (0.1 g./100 ml.) was found to be of definite value in these surface infections, though it had previously been found to be of little or no value in the

treatment of lesions produced by the intracorneal injection of the pneumococcus, staphylococcus aureus, and haemolytic streptococcus (Robson and Scott, 1944). Sodium sulphacetamide (30 g./100 ml.), combined with the detergent, was ineffective; this is in contrast to the considerable bacteriostatic effect of this sulphonamide on Ho.R. when tested *in vitro,* and at present we have no explanation of this discrepancy.

Intracorneal injections

Preliminary experiments with lesions produced by the intracorneal injection of the diphtheroid suggested that marfanil and V335 were without effect. But, since these lesions were technically unsatisfactory, no great significance could be attached to these results. It did seem desirable, however, to determine whether these drugs were in actual fact without value in intracorneal infection in view of their proved effectiveness in superficial lesions. Consequently it was decided to determine their value in intracorneal infection produced by other organisms known to be susceptible to the action of other substances, e.g., penicillin. For this purpose, standard lesions were produced by staphylococcus aureus and pneumococcus by the technique previously described (Robson and Scott, 1943). The results given in Table II show clearly that marfanil and V335 were ineffective.

TABLE II

TREATMENT OF LESIONS PRODUCED BY INTRACORNEAL INJECTION OF ORGANISMS

Interval between inoculation and first treatment = 1 hour.

Injected organism	Treatment	Number of animals	Assessment of final corneal lesion (Mean values on arbitrary scale)		Difference between treated and control eyes
			Treated eyes	Control eyes	
Staph. aureus ..	Marfanil 30 g./100 ml.	4	3·0	3·3	0·3
Pneumococcus (19)		3	2·5	3·0	0·5
Staph. aureus ..	V335 30 g./100 ml.	3	2·9	2·7	−0·2
Staph. aureus ..		4	3·4	3·7	0·3

DISCUSSION

The main aim of this investigation has been to devise an open infected lesion of the rabbit's cornea suitable for the testing of antiseptic and chemotherapeutic substances. The present study has been confined to the local application of drugs.

By the technique described, a lesion of fairly uniform severity was invariably produced. Such a lesion cannot be obtained by the inoculation of the deeply scarified rabbit's cornea with the main organisms pathogenic to the human eye,

such as gonococci, pneumococci, staphylococci, and streptococci. However, the infection of the rabbit's cornea with Ho.R., although this organism may be without much importance in human pathology, provides an experimental lesion, valuable as a counterpart to human disease and suitable for the testing of chemotherapeutic substances. The infection with Pyocyaneus previously studied (Robson and Scott, 1942) produces a severe and reasonably uniform lesion in the rabbit, but is rather unsatisfactory because this organism is insensitive to many drugs.

In the experiments with intracorneal infections only penicillin was effective (Robson and Scott, 1943); marfanil and V335 did not influence the progress of the lesions. This observation is in agreement with the previous findings that marfanil and V335 are of little value in the treatment of systemic infections. It seems likely that these drugs either do not penetrate or are in some way inactivated, but this point requires further investigation.

Summary

1. A technique is described for the production of open infections of the rabbit's cornea, such that drugs have direct access to the lesion. The organism used was a diphtheroid of the Hofmann group; in all cases severe destructive lesions resulted.

2. Early application of penicillin (500 U/ml.), marfanil (30 g./100 ml.) and V335 (*p*-methylsulphonyl benzylamine, 30 g./100 ml.) had a very beneficial effect on the course of the lesions; proflavine (0.1 g./100 ml.) had some effect, but sodium sulphacetamide (30 g./100 ml.) and V187 (*p*-methylsulphonyl benzamidine, 10 g./100 ml.) had no effect.

3. Marfanil and V 335 (30 g./100 ml. solutions) had no effect on lesions produced by the intracorneal injection of staphylococcus aureus and pneumococcus (type 19).

We are greatly indebted to the "W. H. Ross Foundation (Scotland) for the Prevention of Blindness," who have defrayed the expenses of this investigation. British Schering, Ltd., and Messrs. Boots, Ltd., very kindly supplied us with some of the drugs used.

References

Evans, D. G., Fuller, A. T., and Walker, J. (1944). *Lancet*, **2**, 523.
Robson, J. M., and Scott, G. I. (1942). *Brit. med. J.*, **1**, 5.
Robson, J. M., and Scott, G. I. (1943). *Lancet*, **1**, 100; *Brit. J. exp. Path.* **24**, 50.
Robson, J. M., and Scott, A. A. B. (1944). *Lancet*, **2**, 29.

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THE TOXICITY OF ALKYL-BIS(β-CHLOROETHYL)-AMINES AND OF THE PRODUCTS OF THEIR REACTION WITH WATER

BY

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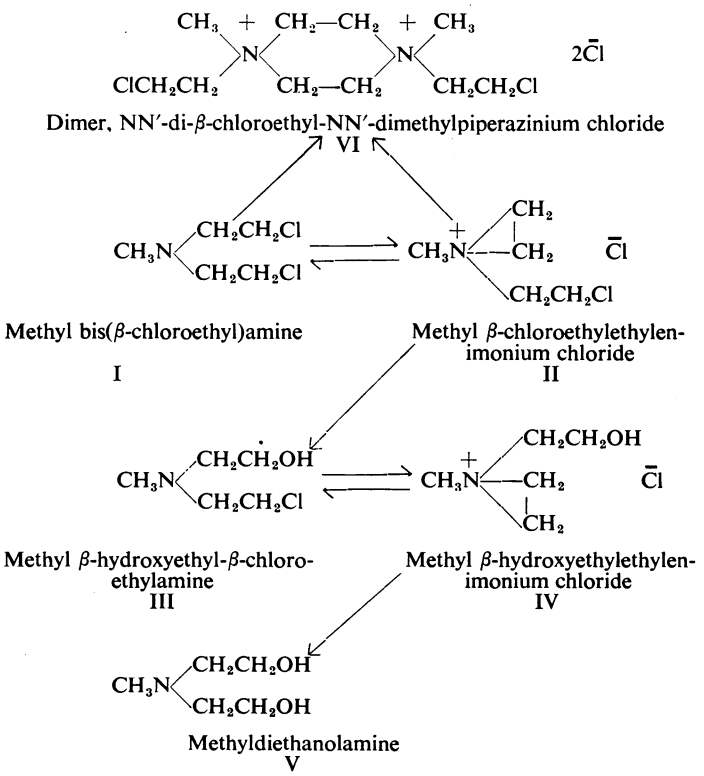
Nitrogenous vesicants or "nitrogen mustards," which are bis(β-chloroethyl)-amine derivatives, differ from mustard gas which is bis(β-chloroethyl)sulphide in some of their biological actions although many pharmacological effects are common to both types of compound. The pharmacology of these substances

REACTIONS OF METHYL BIS(β-CHLOROETHYL)AMINE IN AQUEOUS SOLUTION



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has been summarized by Gilman and Philips (1946). Two differences in behaviour are that aqueous solutions of the bis(β-chloroethyl)amines (1) develop an acute toxicity, associated with a rapid convulsive and lethal action, and (2) remain toxic indefinitely, while aqueous solutions of mustard gas become non-toxic on standing. Methyl-bis(β-chloroethyl)amine is much more soluble in water than is bis(β-chloroethyl)sulphide and aqueous solutions have been found by Hanby, Hartley, Powell, and Rydon to yield the products shown above (Hanby and Rydon, 1946 ; Hanby *et al.*, 1946). The reactions indicated are inhibited by acid, and aqueous solutions of the hydrochlorides of the vesicant bases are stable.

Toxicity of alkyl-bis(β-chloroethyl)amines

Freshly made aqueous solutions of the hydrochlorides of some of the nitrogenous vesicants were given subcutaneously to mice and rats (Table I) and orally to rabbits for determination of the LD50. When the substances were given as fresh aqueous solutions in doses near the LD50, death occurred several days after dosing, and the most usual post-mortem finding was congestion of the gut, which was filled with liquid material. Small doses of the fresh nitrogenous vesicants are similar to mustard gas in their toxic action. The toxicities of methyl-bis-

TABLE I

TOXICITY OF THE HYDROCHLORIDES OF NITROGEN VESICANTS ON SUBCUTANEOUS INJECTION INTO MICE AND RATS

Alkyl-bis(β-chloroethyl)amines Alkyl-$N(CH_2CH_2Cl)_2$ Alkyl group	Mice		Rats	
	LD50 approximate mg./kg.	Number of mice used	LD50 approximate mg./kg.	Number of rats used
Methyl	4	36	2	12
Ethyl	1	40	1	12
Propyl	0·5	19	0·5	16
*iso*propyl	0·5	24	2	8
β-chloroethyl	2	16	2	12

(β-chloroethyl)amine and ββ′β″-trichloroethylamine are of the same order as the toxicity of mustard gas (between 2 and 5 mg. per kg. body weight). Ethyl-bis(β-chloroethyl)amine and propyl-bis(β-chloroethyl)amine are possibly more toxic (with LD50 of 0.5 to 1 mg. per kg. body weight). Lethal doses of fresh aqueous solutions of the nitrogen mustards or of their hydrochlorides cause mice or rabbits to salivate after a short time and to die several hours or days afterwards. Oral administration gave less definite results, but the lethal doses of methyl-bis(β-chloroethyl)amine varied from 3 to 6 mg. per kg. for rabbits to 10 mg. per kg. for rats and mice. Ethyl-bis(β-chloroethyl)amine and ββ′β″-trichloroethylamine were lethal to rabbits in doses of 2 to 6 mg. per kg. *per os.*

Death occurred after 3 to 17 days, during which diarrhoea and loss of weight occurred. At death congestion of the stomach and intestines was usually present.

The lethal action of the reaction products of nitrogen mustards with water

Alkyl-bis(β-chloroethyl)amines react with water in neutral solution to produce toxic compounds with different pharmacological properties. Hanby *et al.* (1946) showed that the first reaction products include ethylenimonium compounds (as formula II). Other reactions lead to the formation of dimers (VI), hydroxyethylchloroethylamines (III), and diethanolamines (V), but except for the hydroxyethylchloroethylamines these substances are not toxic. The pharmacology has been most fully investigated with methyl-bis(β-chloroethyl)amine, but substances with similar properties are produced from the other dichloroethylamines.

TABLE II

SURVIVAL TIMES OF MICE RECEIVING SUBCUTANEOUS INJECTIONS OF 1 G./100 ML. SOLUTIONS OF METHYL BIS(β-CHLOROETHYL)AMINE (10 ML. PER KG.) AT ROOM TEMPERATURE (15°)

Age of solutions	Survival times with aqueous solutions (final *p*H 4·5)	solutions in 3%$NaHCO_3$	solutions of the hydrochloride
Hours			
2	2½ hours	—	2½ hours
6	12 ,,	—	—
18	35 mins.	14 mins.	—
24	40 ,,	16 ,,	—
48	17 ,,	16 ,,	3 hours
72	19 ,,	20 ,,	2½ hours
96	18 ,,	6–12 hrs.	100 min.
Weeks			
1	18 ,,	failed to kill	125 min.
2	19 ,,	—	110 min.
3	20 ,,	—	55 min.
4	18 ,,	—	—
5	21 ,,	—	—
8	22 ,,	—	—
12	19 ,,	—	—

Methyl-bis(β-chloroethyl)amine is soluble to the extent of 1 g. in 100 ml. water and if a solution is kept it becomes more acutely toxic, i.e., the same amount of material injected into mice kills them in less time (Table II). Subcutaneous injection (10 ml. per kg.) into mice of a 1 g./100 ml. solution, which has been kept for two days at room temperature and has attained equilibrium, causes death in about 16 minutes. With higher doses mice die more rapidly (e.g., in 10 minutes with 200 mg. per kg. and in about 6 minutes with 400 mg. per kg.) and with lower doses more slowly. The variations in survival times of mice are indicated in Table VI. In Figs. 1 and 2 and Tables II to V the survival times are mean times for 2 or 4 mice injected with the same preparation. If the survival time is plotted against the dose injected (in 10 ml. of neutral solution per kg.

body weight) on a logarithmic scale a straight line is obtained (Fig. 1). This simple relationship between the logarithm of the dose and the survival time makes it possible to estimate the amount of the convulsive substance in solutions

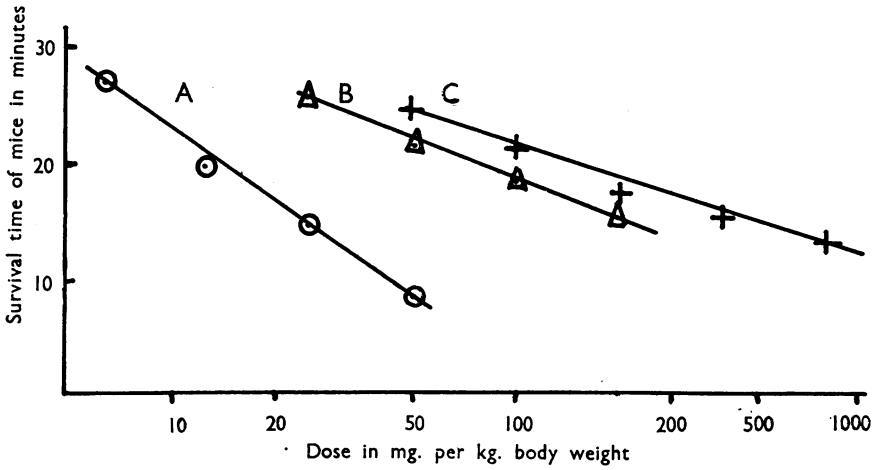

FIG. 1.—Relationship between dose and survival time of mice injected with:
A—Methyl-β-chloroethyl-β-hydroxyethylamine after one hour in neutral solution.
B—Methyl-bis(β-chloroethyl)amine (1%) after four days in aqueous solution.
C—Methyl-bis(β-chloroethyl)amine (8%) after four days in aqueous solution.

if injections are made under standardized conditions. The survival time is longer if the injected solution is acid (below *p*H4) or if the mice are cold. If the drug is injected into cold mice it has a hypnotic action and death occurs after several hours. The rate at which the vesicant base is transformed into the convulsive

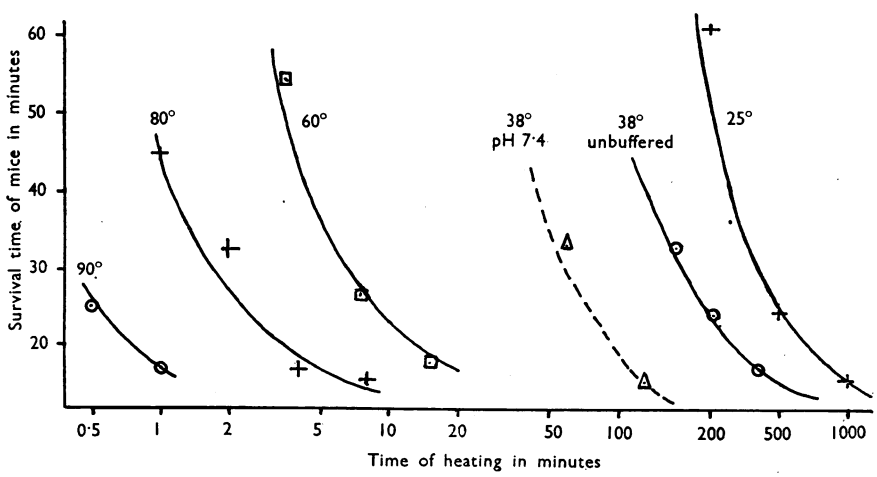

FIG. 2.—The relationship between the survival time of mice injected with 1% methyl-bis(β-chloroethyl)amine in aqueous solution and the time of heating the solution at different temperatures.

derivative was followed at different temperatures by determination of the survival times of mice injected with the solutions. The results (Fig. 2) show that the change is more rapid at higher temperatures. The Q_{10} for the reaction was calculated from the data as 2.5 and the corresponding heat of activation as 21,000 calories. In these experiments it was noticed that when an aqueous solution (1 g./100 ml.) of methyl-bis(β-chloroethyl)amine was heated the liquid became cloudy, presumably owing to the lower solubility of the base at higher temperatures; the cloudiness however disappeared rapidly, the solution becoming clear owing to the formation of easily soluble products. The formation of the acute toxic agent is also more rapid in neutral solution (*p*H 7.4 buffered with $NaHCO_3$ and CO_2) than in the acid solution (*p*H 4.5) produced by the reaction of methyl-bis(β-chloroethyl)amine with water. Fig. 2 shows the reaction at 38° for buffered and unbuffered solutions. The convulsive substance is, however, unstable in neutral or alkaline solution. Other data showing the effect of variations in acidity on the action of the acute toxic agent are given in Table III.

TABLE III

EFFECT OF *p*H AT TIME OF INJECTION ON SURVIVAL TIMES OF MICE INJECTED WITH OLD SOLUTIONS OF METHYL BIS(β-CHLOROETHYL)AMINE

Doses as equivalent of original amine

*p*H	Survival time of mice	
	50 mg. per kg.	200 mg. per kg.
3·6 with citric acid (M/15 buffer)	Failed to kill	12 hours
4·5 with water	43 mins.	20 mins.
6·0 with Na_2HPO_4 (M/15 buffer)	22 „	14 „
7·0 „ „ „ „	20 „	16 „
8·2 „ „ „ „	24 „	15 „

TABLE IV

SURVIVAL TIMES OF MICE INJECTED SUBCUTANEOUSLY WITH VARYING DOSES OF DIFFERENT CONCENTRATIONS OF FOUR-DAY-OLD SOLUTIONS OF METHYL BIS(β-CHLOROETHYL)AMINE IN WATER

Original concentration of solution (mg./100 ml.)	Survival times in minutes after injection of doses (mg./kg.)							Dose killing mice in 20 min. mg./kg.
	500	250	200	125	100	50	25	
10·0	18	22	—	29	—	—	—	350
5·0	11	15	—	26	—	—	—	170
1·0	—	—	12	—	17	23	—	70
0·5	—	—	10	—	16	24	60	70

The relative amount of convulsive material formed from methyl-bis(β-chloroethyl)amine varies with the original concentration, being less with high concentrations (Table IV), possibly because of the formation of the dimer and of acid limiting the hydrolysing reactions.

Subcutaneous injection of the acutely toxic material rapidly produced partial paralysis combined with incoordination of movements and a kinetic tremor. After a few minutes mice have extremely violent convulsions and die. Post-mortem examination of mice dying after such convulsions has not revealed any typical changes.

While the amount of the acute toxic agent can be assayed by subcutaneous injection into mice, the amount of the original base can be estimated by its toxic effect when given orally to rats. If a mixture of the nitrogen mustard and the acute toxic substances are given subcutaneously to mice, the mice die rapidly before the nitrogen mustard itself can cause death. If the mixture is given orally to rats the absorption is so slow that the acute toxic material has very little action and the rats die from the effect of the nitrogen mustard.

The acute toxic material was not precipitated by addition of 20 volumes of acetone or of excess bromine; both reagents precipitated non-toxic material, probably including polymerized derivatives. The acute toxic material was precipitated by phosphotungstic acid and could be recovered from this precipitate by cautious treatment with barium hydroxide.

The nature of the acutely toxic material

From a solution of the reaction products of methyl-bis(β-chloroethyl)amine with water a number of products have been isolated (Hanby and Rydon, 1946). Of these the dimer (VI) was found to be non-toxic. Mice were injected with 500 mg. per kg. of the fresh solution and of solutions kept for 1, 2, 4, 24, and 48 hours without suffering any obvious ill effects. Other related substances which were found to be non-toxic at the same dosage level when injected as fresh solutions or as 24-hour-old solutions included dimethyl-bis(β-chloroethyl)-ammonium iodide, N-hydroxyethyl-morpholine methiodide, N-methyl-morpholine hydrochloride, β-chloroethylamine and β-chloroethylmethylamine.

Solutions of the half hydrolysis product (III) were acutely toxic, killing mice in short times with convulsions (Fig. 1 and Table V), but as the acute lethal action did not decrease on standing in aqueous solution for 30 minutes, it is

TABLE V

THE SURVIVAL TIMES OF MICE (IN MINUTES) INJECTED WITH AQUEOUS SOLUTIONS OF SUBSTANCES WHICH CAUSE RAPID DEATH

Substance	Survival times (min.) after doses (mg. per kg.)				
	200	100	50	20	10
Methyl-β-hydroxyethyl-β-chloroethylamine (III), fresh ..	—	14	15	31	—
The same after 15 minutes at 25°	—	11	15	25	—
The same after 30 minutes at 25°	—	15	14	24	—
N-β-chloroethyldiethanolamine	10	13	19	23	31
N-β-chloroethyl-N-β-acetoxyethyl-methylamine	—	23	25	50	—

probable that the actual convulsive agent is the methyl hydroxyethyl ethylenimonium chloride (IV), because the open chain compound (III) would probably cyclize rapidly on standing in aqueous solution. The concentration of the open chain form would, therefore, decrease without loss of acute toxicity. The ethylenimonium form would also be formed from the open chain form fairly quickly on injection into the body.

Analogous convulsive poisons

Other alkyl-bis(β-chloroethyl)amines react with water to form substances with an acute toxic action (Table VI). Ethyl-bis(β-chloroethyl)amine appears to be the most potent of these compounds in this respect while propyl-bis(β-chloroethyl)amine is very much less active when allowed to react with water.

TABLE VI

THE SURVIVAL TIMES OF MICE (IN MINUTES) INJECTED WITH AQUEOUS SOLUTIONS OF VESICANTS WHICH HAD BEEN ALLOWED TO STAND UNTIL EQUILIBRIUM HAD BEEN ATTAINED

Doses given in equivalent of original vesicant

S = survived 21 days

Compound	Survival times (min.) of mice after doses below (mg./kg.)							LD50 approx. mg./kg.
	200	100	50	20	10	5	2	
ββ′Dichlorodiethyl sulphide		S S S S	S S S S	S S S S	—	—		>100
Methyl bis(β-chloroethyl) amine	15 17	17 19	20 22	23 26	27 34 41 S	80 S S S		5–10
Ethyl bis(β-chloroethyl) amine	17 14	14 14	15 12	17 18	17 24 21 S	60 S S S		5–10
Propyl bis(β-chloroethyl) amine	84 200	800 800	800 48 hr.	48 hr. 48 hr.	48 hr. 10 days	15 days S		5
*Iso*propyl bis-(β-chloroethyl)amine	25 29	20 17	1,000 2 days	1,000 2 days	S S	S S		10–20
ββ′β″Trichloroethylamine		15 19 20 23	61 100 26 28	23 20 32 40	45 3 days 3 days 3 days	3 days 4 days 4 days 5 days	5 days 6 days 13 days —	2
Fresh ββ′β″Trichloroethylamine		16 17 18 18	19 19 20 21	30 27 52 38	3 days 4 days 6 days 6 days	4 days 4 days 5 days 6 days	3 days 13 days	2

$\beta\beta'\beta''$-Trichloroethylamine differs from the other nitrogen vesicants in being rapidly lethal before it has reacted with water; this compound is only very slightly soluble in water, but reacts with water to give bis(β-hydroxyethyl)-β-chloroethylamine (which can give rise to an imonium form) and triethanolamine (Crane and Rydon, 1946).

Discussion

The nitrogen vesicants are extremely toxic substances and it is possible that the high toxicity is in part due to the fact that they are lipoid soluble materials of low molecular weight which will probably penetrate cells readily; having penetrated they may combine with essential cell constituents or form toxic ionized imonium salts which will be less able to pass out of the cell. There will thus be " one way traffic " of the toxic compound into cells. The toxic effects of these substances include (1) vesication, (2) haemoconcentration, (3) diarrhoea, (4) inhibition of mitosis, and (5) the acute or convulsive effect described in this paper. The vesicant action is certainly due to the parent amines and the convulsive action to the reaction products with water.

Summary

Alkyl-bis(β-chloroethyl)amines are vesicants, more soluble in water than mustard gas, which react with water to form compounds with acute convulsive action. The convulsive compounds appear to be ethylenimonium chlorides. The reaction with water is such that equilibrium is attained, so that an aqueous solution remains toxic indefinitely.

I am indebted to the Director-General of Scientific Research (Defence), Ministry of Supply, for permission to publish this work, and to Dr. H. N. Rydon for supplying many of the substances used.

References

Crane, C. W., and Rydon, H. N. (1946). *J. chem. Soc.*, in the press.
Gilman, A., and Philips, F. S. (1946). *Science*, **103**, 409.
Hanby, W. E., Hartley, G. S., Powell, E. O., and Rydon, H. N. (1946). *J. chem. Soc.*, in the press.
Hanby, W. E., and Rydon, H. N. (1946). *J. chem. Soc.*, in the press.

Brit. J. Pharmacol. (1946), **1**, 255.

A METHOD FOR THE EVALUATION OF ANALGESIC ACTIVITY USING RATS

BY

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Various methods for the experimental evaluation of analgesic activity have been described. They are all based upon the change which occurs in the response of the experimental subject to a painful stimulus after dosage with an active compound. Various animal species as well as man have been used as experimental subject and a variety of stimuli employed. In the examination of new compounds for analgesic activity it is undesirable to use the human subject, and if the number of compounds is large the smaller laboratory animals, rats or mice, become the animals of choice. We have tested a number of methods in which small animals were used, but none has proved entirely satisfactory.

In the ideal method the stimulus employed should provoke a characteristic and readily observed response and the intensity of the stimulus required to elicit it should vary little from animal to animal ; the method should yield reproducible results and these should be in agreement with clinical experience.

A method for analgesic assay using rats is described below which in our experience has been found to satisfy these criteria. It is a simple modification of the method of D'Amour and Smith (1941, 1943), which was itself adapted from the work of Hardy, Wolff, and Goodell (1940) in man.

Apparatus and Procedure

The method is based upon the reaction of the rat to a heat stimulus applied to a small area of the tail.

The apparatus can be constructed from material commonly available in the laboratory. It consists essentially of a sheet of asbestos board (e.g., uralite), 1/8 in. thick and about 4 in. square, supported horizontally and having on its upper surface two strips of the same material, 4 in. long, fixed in such a way as to leave a channel about 1/4 in. wide between them. At some point along this channel a hole 1/4 in. in diameter is drilled through the asbestos sheet and a small coil of resistance wire connected through a key to a 6-volt electrical supply fixed beneath it. The wire is of such a gauge and length that with the circuit closed it is raised to a bright red heat.

The rat under test is held in a cylindrical holder of perforated zinc, clamped horizontally ; its tail lies along the channel and over the hole, which must be not more than 1½ in. from its tip. When the animal has become quiet in this position the circuit is closed. After an interval the animal will withdraw its tail from the channel with a sudden and characteristic flick. This interval is timed with a stopwatch and is referred to as the *reaction time*.

Rats weighing between 120 and 160 g. with clean and healthy tails are used for experiment. The heating coil is adjusted initially to such a position that a majority of the animals react at about 5 seconds. The normal reaction time of a number of rats is determined precisely, the mean of three successive determinations at 2-minute intervals being taken, and those for which it is between 4 and 6 seconds are divided into groups of a convenient size. It has been found impracticable to deal with a group of more than six rats at a time. The compound under test is then given by the chosen route and at the desired dose level, and the reaction times of the rats are thereafter determined at 15-minute intervals. Analgesia is reflected in a prolongation of the reaction time; the increase over normal in the mean reaction time of the treated animals is taken as a measure of analgesic effect, and is hereafter referred to as *effect*.

When analgesia is marked, heating may be continued until the tails are severely burned without eliciting any reaction. To avoid unnecessary damage to the tails, heating is never continued for longer than 15 seconds, and if an animal has not reacted in that time analgesia is assumed to be "complete."

In preliminary experiments we were able by this technique to demonstrate analgesic activity for morphine, codeine, and pethidine, given intraperitoneally. Aspirin and phenazone, given by this route, appeared inactive in doses approaching the median lethal, but intravenously they produced readily measurable effects at much lower doses. In view of this result, intravenous administration seems preferable when new compounds are being examined for analgesic activity for the first time and was adopted for the remainder of our experiments. Injections were made into the tail vein in the third near to the root of the tail. Morphine, codeine, and pethidine were used as their soluble hydrochlorides, phenobarbitone as its soluble sodium salt, aspirin as its sodium salt by neutralizing the acid with N. NaOH to *p*H 6–6.5, phenazone in aqueous solution, and hashish as described below p. 262).

RESULTS

One drug examined fully by the procedure described above was pethidine hydrochloride. The mean analgesic effect at varying times after single intravenous doses of 1 to 15 mg./kg. is shown in Table I, where the number of rats used at each dose is also given, and plotted in Fig. 1. In computing mean values a reaction time of 15 seconds was assigned to animals showing "complete" analgesia.

TABLE I

ANALGESIC EFFECT OF PETHIDINE

Dose mg./kg. (i.v.)	No. of rats	Mean effect in seconds at the following times after injection					
		15 mins.	30 mins.	45 mins.	60 mins.	75 mins.	90 mins.
15	5	10·8	10·6	4·7	2·2	1·2	0·3
10	9	8·1	6·4	2·9	1·3		
7·5	10	6·8	2·5	0·9	0·2		
5	10	3·3	1·3	0·4	0		
2·5	9	3·3	1·7	0·4			
1	9	0·3	−0·1	−0·2			

It will be seen from Fig. 1 that the highest recorded effect was obtained 15 minutes after dosing. This was the case for other drugs examined in the same way.

In order to use this method for assay, the relationship between dose and response must be determined, and to do this it is necessary to decide on a measure of the response to any given dose. For this we could take either (a) the effect at a given time after dosing, or (b) the time taken to produce a given effect. The former is the easier to determine and the more reliable. The recorded effect at 15 or at 30 minutes after dosing or a value obtained by smoothing the curve might be used, but there is little to be gained by the latter procedure since by far the

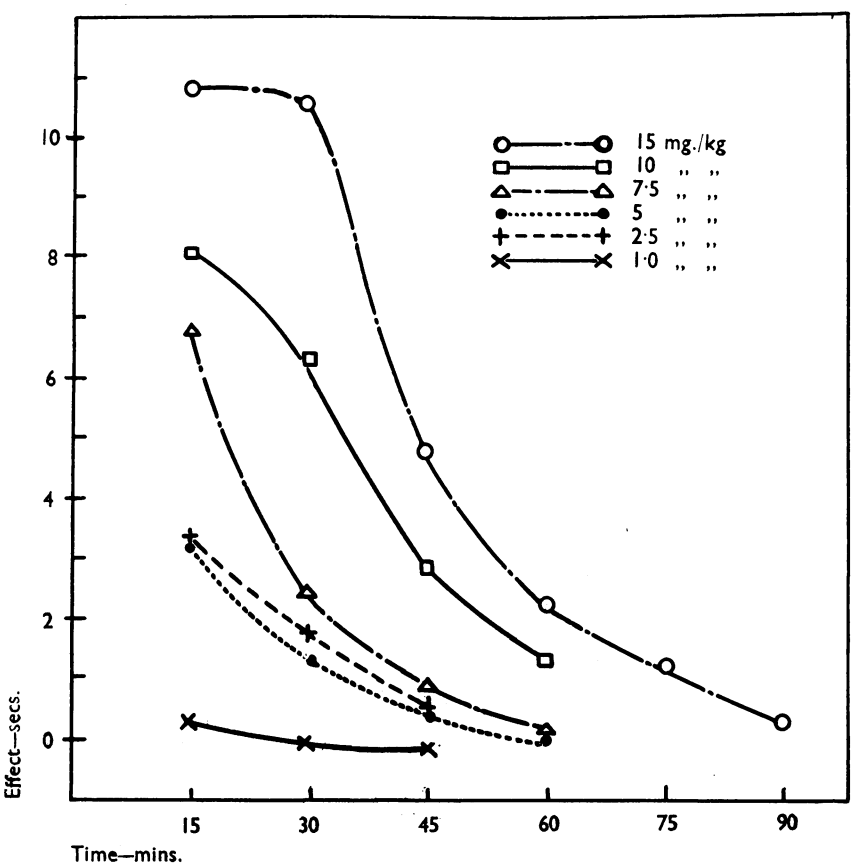

FIG. 1.—Analgesic action of pethidine. Effect plotted against time for doses from 1 to 15 mg./kg.

greatest source of error is the variation between the rats themselves. From an examination of the results for pethidine and other drugs, the recorded effect at 15 minutes was found to be a better measure than that at later times because a smoother and steeper curve was thereby obtained for the relationship between dose and response. It was therefore adopted as the basis of assay.

The reaction time at any given time after dosing for each rat is expressed as an average of three readings. The variation between repeat readings is considerably less than the variation between individual rats and therefore no worthwhile gain in accuracy can be obtained by taking more than three. A further appreciable increase in accuracy could only be obtained by increasing the number of rats used.

The mean effects 15 minutes after injection, the doses, and the number of rats per dose for a number of drugs are given in Table II. In Figs. 2 *et seq.* the mean effect at 15 minutes is plotted against log. dose.

TABLE II

Drug	Test								
Morphine	A	Dose mg./kg. ..	10	5	4	3	2·5	2	1·5	1
		No. rats	3	3	9	10	10	10	10	11
		Mean effect (secs.)	10·5	10·8	9·5	8·2	4·7	3·6	1·9	0·2
Codeine	A*	Dose mg./kg. ..	25	20	15	12·5	10	7·5	5	
		No. rats	4	5	9	10	10	—	4	
		Mean effect (secs.)	10	9·9	10·1	7·8	1·1	—	0·4	
	B	No. rats	—	—	10	10	10	10	10	
		Mean effect (secs.)	—	—	8·7	5	5·1	3·2	1·1	
Pethidine	A	Dose mg./kg. ..	15	10	7·5	5	2·5	1		
		No. rats	5	9	10	10	9	9		
		Mean effect (secs.)	10·8	8·1	6·8	3·3	3·3	0·3		
Hashish distillate	A	Dose mg. kg. ..	3	2	1	0·75	0·5	0·25	0·1	
		No. rats	10	10	10	10	10	10	10	
		Mean effect (secs.)	9·1	8·1	6·5	4	1·7	3·5	0·8	
Phenazone	A	Dose mg. kg. ..	500	350	250	150	100			
		No. rats	9	10	15	15	10			
		Mean effect (secs.)	9·8	8·3	6·2	3·8	1·8			
Aspirin	A	Dose mg. kg. ..	725	600	500	425	350			
		No. rats	10	9	10	10	10			
		Mean effect (secs.)	7·4	6·4	5·4	3·9	2·7			
	B	No. rats	8	9	10	10	10			
		Mean effect (secs.)	8·8	6·3	4·1	4·1	2·9			
Pheno-barbitone	A	Dose mg./kg. ..	100	75	50	37·5	25	17·5	10	
		No. rats	26	28	32	30	35	30	29	
		Mean effect (secs.)	0·4	1·3	1·8	2·1	3·9	1·8	0·5	

* The figures for Test A were obtained in preliminary experiments before the apparatus and technique were standardized.

The relationship is substantially linear over a wide range for all the drugs examined with the exception of phenobarbitone, which will be discussed below. For extreme maximal and minimal effects the curve flattens out as, of course, it must. No transformation of the effect variable is thus required provided reasonable care is exercised with extreme values. In certain cases it is well to omit

results obtained with doses at the extreme ends of the range—for example, of the figures given in Table II, that obtained with 10 mg./kg. morphine should be ignored. In an assay of the activity of a sample of a known drug, a range of doses which produces intermediate effects, e.g., 2 to 8 seconds, should be used.

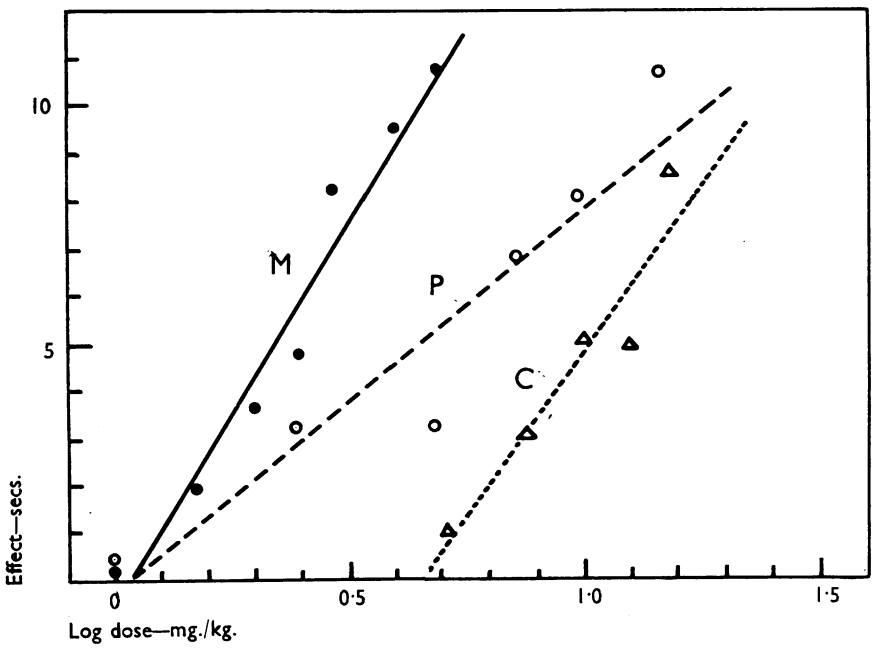

FIG. 2.—Analgesic action of morphine (M), codeine (C), and pethidine (P) administered intravenously. Effect at 15 minutes after injection plotted against log. dose.

Sources of error

The main source of error in determining the log. dose–response line is the variation between individual rats. In our experiments, this variation was related to the magnitude of the mean effect, being least for maximal and minimal effects. Except for these extreme values, however, the standard deviation was practically constant for different doses. Its average value was about 3 seconds for all the drugs examined.

Another source of error is that different results may be expected from experiments carried out on different days. When comparing the activity of two or more samples of any drug, the variation due to this factor may be eliminated by ensuring that in each group tested at one time all samples are equally represented. There is evidence that error has arisen from this source in the results reported here, but owing to the accidental arrangement of the treatments it has not been serious.

An overall measure of the error in these experiments may be obtained by calculating the variance about the best line of fit. Combining the results of all the experiments except those with phenobarbitone, we find that the variation about the linear regressions is no greater than expected from the standard deviation of 3 seconds for individual rats in each group. This indicates that the linear regressions give a satisfactory fit.

From the regressions we may calculate the doses required to produce an increase in reaction time of 5 seconds. These are shown in Table III together with the slopes of the regression lines for the various drugs examined (except phenobarbitone).

TABLE III

Drug	Slope	Standard error of slope	Dose to give mean response of 5 seconds	Standard error	Expected standard error of comparison of two samples with 100 rats each
			mg./kg.	%	%
Morphine	16·1	1·8	2·2	6	6·5
Codeine (Test B) ..	14	2·5	10	7·5	7·5
Pethidine	8·1	1·1	4·6	13	13
Hashish	5·7	0·8	0·72	16	19
Aspirin (Test A) ..	15	3·8	490	7	7
Aspirin (Test B) ..	17·4	4	490	7	
Phenazone ..	11·6	1·7	169	8·5	9

The slopes for morphine, codeine, aspirin, and phenazone do not differ significantly from one another. The slopes for pethidine and hashish are significantly lower than the remainder with the possible exception of phenazone.

The standard errors quoted above refer to the reproducibility within the conditions of the experiment. It is to be expected that larger variations would arise, especially in the dose required to give a mean response of 5 seconds, if a different batch of animals were used or if the experiment were repeated at a different time. Some measure of control is obtained by selecting rats with normal reaction times of 4 to 6 seconds, but we cannot say how effective this is in controlling the response at any given dose level.

The figure in the last column in Table III represents the standard error of a comparison of the activity of two samples of each drug when 100 rats are used for each sample and the following conditions are observed:

(a) That the two samples are equally represented in each group of rats treated and tested together.

(b) That the order in which the doses are given is randomized.

(c) That the dose range is so chosen that the mean effect in all cases falls on the linear portion of the log. dose–response curve. (Simplification of the analysis results if the doses are taken at equal logarithmic intervals.)

If the experiment continues over two days the whole test should be considered as two complete replicates, with 50 rats per sample in each. This idea can be carried further and the experiments regarded as 4 complete replicates with 25 rats per sample in each. Further subdivision is impracticable.

For comparing the analgesic potency of different drugs we adopt as a standard of comparison the dose of each required to produce an increase in mean reaction time of 5 seconds. This increase represents approximately 50 per cent of the maximum increase which can be measured.

The doses of various analgesics required to produce this effect are shown in Table IV.

TABLE IV

Drug	Dose required to produce an increase in mean reaction time of 5 seconds	
	Intravenous administration	Intraperitoneal administration
	mg./kg.	mg./kg.
Morphine	2·2	10
Codeine	10	30
Pethidine	4·6	30
Aspirin	490	Inactive
Phenazone	169	,,
Hashish distillate	0·72	,,
Phenobarbitone	*25	,,

* This dose produced an increase of only 4 seconds, the maximal obtainable with phenobarbitone.

Further observations upon the analgesic action of these drugs are given below.

1. *Morphine and codeine.*—Both these are active when given by intraperitoneal or intravenous injection. Rats dosed with them and showing pronounced analgesia as indicated by a marked increase in reaction time appeared normal in other respects. From Table IV it appears that morphine is about five times as active as codeine when both are given intravenously and three times as active intraperitoneally. This is in fair agreement with clinical experience and with the results obtained by Woolfe and MacDonald (1944), using mice.

2. *Pethidine.*—The response produced by increasing intravenous doses of pethidine is shown in Figs. 1 and 2. The slope of the log. dose–response curve is somewhat less than that for morphine or codeine, indicating that with increasing doses of this drug its analgesic action, relative to that of either of the others, becomes less.

This result confirms that of Woolfe and MacDonald (*loc. cit.*) and suggests that pethidine is relatively less active than morphine in the control of the more intense forms of pain.

3. *Aspirin and phenazone.*—As already mentioned, aspirin produces detectable effects after intravenous administration only. Even so the dose required to produce any effect is enormously greater than with pethidine or the opiates.

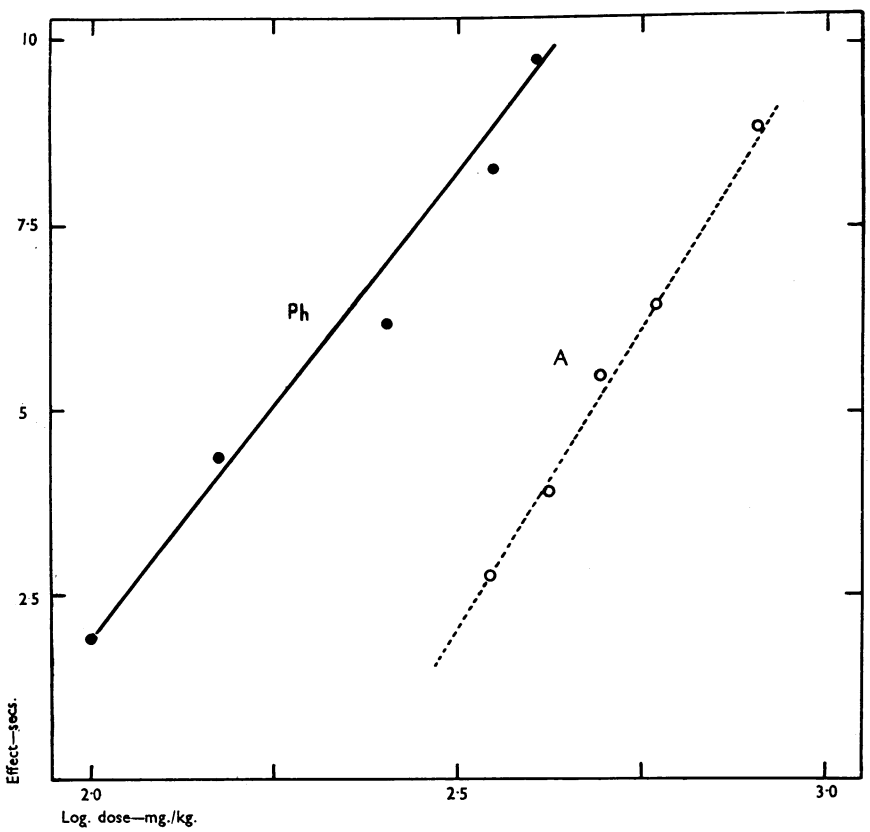

FIG. 3.—Analgesic effect of phenazone (Ph) and aspirin (A), administered intravenously. Effect at 15 minutes after injection plotted against log. dose.

Phenazone has a slight action when given intraperitoneally in large doses just below the median lethal. When given intravenously, it produces clearly demonstrable effects at lower doses (Fig. 3).

4. *Hashish.*—A sample of total distillate of hashish, for which we are indebted to Professor A. R. Todd, was examined by our technique. No analgesic effect was observed when a fine aqueous emulsion of the distillate was given intraperitoneally

or intravenously to rats. With the highest doses given, the animals died in convulsions shortly after being dosed.

Acetone solutions of the distillate, given intravenously, produced analgesic effects, but the results were vitiated by damage to the tails at the site of injection and the animals developed haematuria. After trials with various solvents we found that by diluting an acetone solution of the distillate with defribrinated rat blood a preparation suitable for assay by our technique was obtained. Great care had to be taken to keep the acetone content to a minimum, otherwise intense haematuria resulted. Not more than 0.1 ml. of acetone solution to every 1 ml. of blood was finally used in our experiments. Used in this way the distillate produced measurable effects in very small doses (0.1 mg./kg.). "Complete" analgesia was

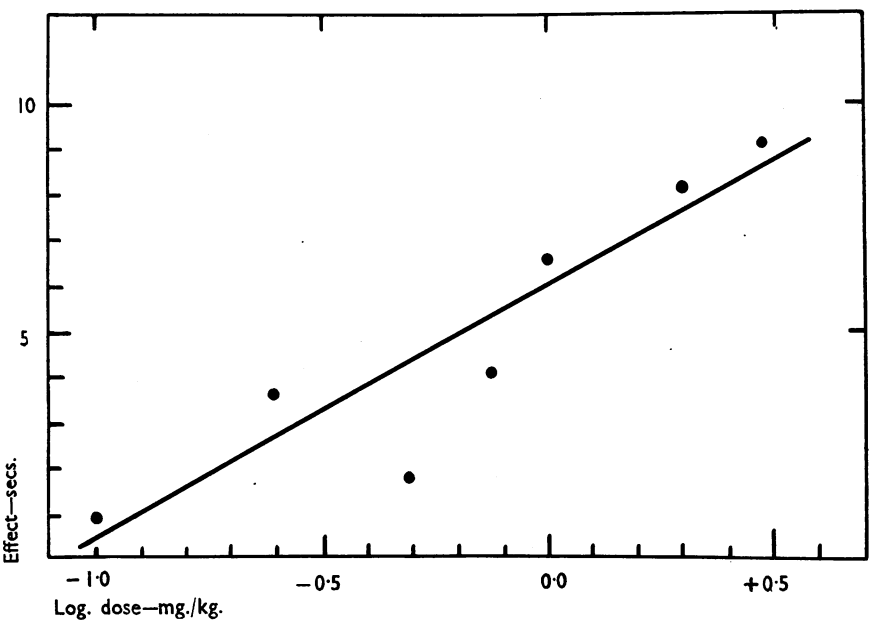

FIG. 4.—Analgesic action of hashish distillate administered intravenously. Effect at 15 minutes after injection plotted against log. dose.

only obtained with a dose fifty times as great as this, i.e., 5 mg./kg., which is very close to the median lethal. The slope of the log. dose–response curve (Fig. 4) was the lowest obtained with any drug examined. This is probably due to the fact that the sample tested was a mixture of the several active constituents of hashish.

5. *Phenobarbitone.*—Barbiturates are reputed to be mild analgesics. We were unable to demonstrate any analgesic effect with phenobarbitone given intraperitoneally in doses up to and including hypnotic doses. With still higher doses, near to the median lethal, an effect is obtained which we attribute to the general depressant action of the drug. On the other hand if phenobarbitone is given intravenously analgesia is produced by doses between 10 to 100 mg./kg. As the

dose is increased from 10 mg./kg. the analgesic effect increases to a maximum which is obtained with a dose of about 25 mg./kg. Further increase in the dose leads to a diminution of the effect until when hypnotic doses are attained analgesia

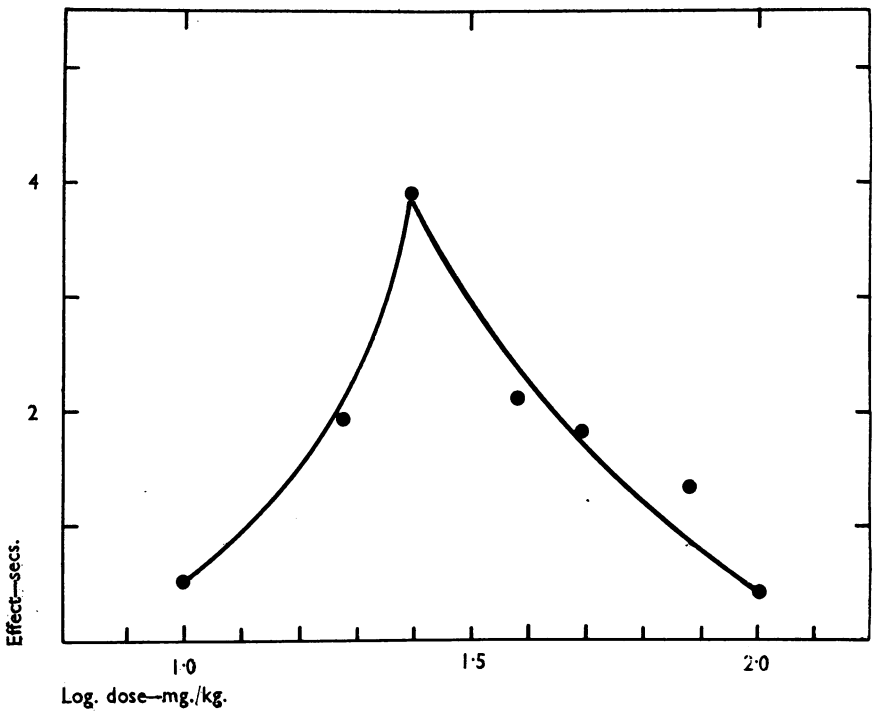

FIG. 5.—Analgesic effect of phenobarbitone administered intravenously. Effect at 15 minutes after injection plotted against log. dose.

is no longer observed. The results reproduced in Table II and Fig. 5 are the means of three different experiments using about 70 rats in each.

It is of interest to note that the analgesic effect of morphine can be demonstrated in rats fully anaesthetized with phenobarbitone. The results demonstrate the complexity of the action of analgesic and hypnotic drugs.

SUMMARY

A new method is described for the detection and evaluation of analgesic activity, making use of the response of rats to a heat stimulus applied to the tail.

By the use of this method the analgesic activity of morphine, codeine, pethidine, aspirin, phenazone, hashish, and phenobarbitone have been compared.

REFERENCES

D'Amour, F. E., and Smith, D. L. (1941). *J. Pharmacol.*, **72**, 74.
Hardy, J. D., Wolff, H. G., and Goodell, H. (1940). *J. clin. Invest.*, **19**, 649, 659.
Smith, D. L., D'Amour, M. C., and D'Amour, F. E. (1943). *J. Pharmacol.*, **77**, 184.
Woolfe, G., and MacDonald, A. D. (1944). *J. Pharmacol.*, **80**, 300.

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THE PHARMACOLOGICAL PROPERTIES OF α : β DIHYDROXY-γ-(2-METHYLPHENOXY)-PROPANE (MYANESIN)

BY

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During a systematic investigation of the pharmacological properties of α-substituted ethers of glycerol the observation was made that certain of these compounds produced paralysis with profound muscular relaxation. Administration of small quantities of these substances to mice, rats, or guinea pigs caused tranquillization, muscular relaxation, and a sleep-like condition from which the animals could be roused. Larger doses produced ataxia, which was followed by paralysis. The animals did not react to painful stimuli and were unable to turn over when placed on their backs; all muscles were well relaxed and quite limp. Paralysis was followed by complete recovery. Excitement, tremors, twitchings, or convulsions did not occur at any time after administration of the drug.

The relative toxicity and effectiveness of the most active compounds after subcutaneous administration to white mice are shown in Table I, together with their melting points and solubilities in water.

Suspensions prepared by mixing one volume of a 40 per cent w/v alcoholic solution of the drug with 7 volumes of an aqueous 5 per cent w/v gum acacia solution were used because of the low water solubility of several of the drugs. The inability of an animal to turn over when placed on its back, together with the absence of any movements of the limbs, was taken as the criterion of paralysis. The animals were observed for 14 days after administration of the drug but any deaths usually occurred within 24 hours of the administration. The observed percentages of effect were treated according to Dragstedt and Lang's (1928) method and the probits of these deduced percentages were plotted against the logarithms of the doses. The median paralysing and lethal doses were then found graphically and the standard errors estimated according to the method of Miller and Tainter (1944). The therapeutic index was expressed by the ratio:

$$\frac{\text{median lethal dose}}{\text{median paralysing dose.}}$$

Most of the 143 compounds which were examined produced paralysis only in doses which were lethal to a proportion of the animals. No correlation of paralysing activity with chemical constitution was observed. α:β-dihydroxy-γ-

(2-methylphenoxy)-propane, the *o*-tolyl ether of glycerol, was the most potent and safest of all the compounds examined and had the widest margin between the paralysing and lethal doses. This substance has been named 'myanesin.' In

TABLE I

MEDIAN LETHAL AND PARALYSING DOSES OF α-SUBSTITUTED ETHERS OF GLYCEROL IN WHITE MICE AFTER SUBCUTANEOUS ADMINISTRATION

$R\text{-}O\text{-}CH_2.CHOH.CH_2OH$ R-substituent	Melting point	Water solubility % w/v at room temp.	No. of mice	LD50 ± S.E. mg. per kg.	PD50 ± S.E. mg. per kg.	Therapeutic index LD50/PD50
n-Butyl	liquid	1·0	60	2,800 ± 150	1,480 ± 54	1·89
n-Amyl	liquid	0·1	80	2,000 ± 100	870 ± 49	2·30
Iso-Amyl	liquid	0·1	50	2,100 ± 130	1,240 ± 80	1·69
n-Hexyl	liquid	0·05	90	2,230 ± 50	1,060 ± 70	2·15
Phenyl	55–57° C.	0·7	90	1,680 ± 65	920 ± 98	1·82
p-Chlorophenyl	77–79° C.	0·08	120	920 ± 86	420 ± 46	2·19
2:4-Dichlorophenyl	74–76° C.	0·001	60	840 ± 44	540 ± 53	1·55
p-Bromophenyl	74–75° C.	0·03	50	1,160 ± 57	840 ± 44	1·38
o-Tolyl (Myanesin)	70–71° C.	1·09	120	1,000 ± 56	325 ± 20	3·07
m-Tolyl	63–66° C.	0·5	80	1,470 ± 89	570 ± 51	2·58
p-Tolyl	74–75° C.	0·2	90	1,270 ± 61	530 ± 39	2·39
p-Ethylphenyl	67–69° C.	0·08	50	1,450 ± 67	820 ± 38	1·77
p-Methoxyphenyl	80–81° C.	0·8	70	1,610 ± 50	940 ± 74	1·72

the following paper a description of its pharmacological properties is given. A preliminary note on its pharmacology (Berger and Bradley, 1946) and a report on its clinical action have been published.

Chemical and physical properties of myanesin.—Myanesin is a colourless, odourless, crystalline solid, melting point 70–71° C. Solubility at 22° C. is 1.09 g. per 100 ml. water, but relatively stable supersaturated solutions can easily be obtained by cooling solutions prepared at higher temperatures. It is very soluble in ethyl alcohol and propylene glycol. Urea and its derivatives, particularly ethyl urea, greatly increase the water solubility of myanesin. Solutions of the drug are stable, can be sterilized by heating, and are compatible and freely miscible with solutions of sodium chloride, glucose, and derivatives of barbituric and thiobarbituric acid.

Toxic and paralysing doses in mice.—The median lethal and median paralysing doses after intravenous administration of aqueous solutions to mice were 322 ± 11 mg. per kg. and 150 ± 6 mg. per kg. respectively. The injections were spread over a period of 1 minute. The results obtained after intraperitoneal administration to mice are given in Table II.

Effect on rabbits.—Rabbits injected intravenously with 10 to 12 mg. per kg. showed the head drop sign which is used in the standardization of certain curare preparations (Bennett, 1941). Intravenous injection of 50 mg. per kg. caused complete paralysis about 10 seconds after termination of the injection. The animal,

although unable to move, gave the impression of being conscious. Skeletal muscles were flaccid. The corneal reflex was present, but the retraction reflex was absent. Breathing was regular, mainly diaphragmatic and somewhat

TABLE II

INCIDENCE OF PARALYSIS AND DEATH, AND DURATION OF INDUCTION AND PARALYSIS, IN MICE AFTER INTRAPERITONEAL ADMINISTRATION OF AQUEOUS SOLUTIONS OF MYANESIN. TWENTY MICE WERE USED AT EACH DOSE LEVEL

Dose mg. kg.	Paralysed per cent	Died per cent	Mean duration of induction min. and sec.	Mean duration of paralysis min. and sec.
150	0	0	—	—
175	65	0	2′ ± 6″	12′ ± 1′42″
200	70	0	2′ ± 6″	13′ ± 4′18″
225	90	0	1′48″ ± 12″	23′ ± 4′12″
300	100	0	1′36″ ± 12″	25′ ± 2′48″
350	100	0	1′12″ ± 6″	56′ ± 6′42″
500	100	5	0′48″ ± 2″	61′ ± 12′24″
550	100	35	—	—
600	100	45	0′48″ ± 6″	120′ ± 4′18″
650	100	60	—	—

LD50, 610 ± 10·1 mg. kg. PD50, 178 ± 8·8 mg. kg. Therapeutic index, 3·42.

increased in rate. The posterior half of the animal remained paralysed for a longer period of time than the anterior half. The rabbits regained muscular power about 7 minutes after termination of the injection and did not show any untoward symptoms, either immediate or delayed. The injection of larger doses caused paralysis of longer duration. The largest tolerated dose on rapid intravenous injection was about 100 mg. per kg. Doses of 200 mg. per kg. injected at a rate of 100 mg. per minute, and 350 mg. per kg. injected over a period of 30 minutes, were tolerated. The animals recovered about 20 minutes after termination of the injections and remained well.

Absorption and fate.—The drug was well absorbed from the blood, muscle, subcutaneous tissues, peritoneal cavity, stomach, and rectum and could be effectively administered by any of these routes. Clearance rates were investigated in rabbits and cats. A 1.8 per cent aqueous solution of myanesin was injected at a constant rate into the marginal ear vein of non-anaesthetized rabbits for several hours. Cats were anaesthetized with ether and prepared for recording blood pressure and respiration ; the myanesin solution was infused into the femoral vein. As soon as the infusion was started, no further ether was given, as the procedure caused paralysis with complete relaxation. It was found that both rabbits and cats tolerated the infusion of at least 4.5 mg. of myanesin per min. per kg. body weight for 3 or 4 hours. This procedure caused paralysis with complete relaxation. Apparently myanesin was quickly metabolized or changed to a physiologically inactive compound in the body. The urine collected from rabbits after large doses of the drug did not cause paralysis in mice.

The drug did not possess cumulative action and tolerance to it did not occur. A minimum effective dose given daily to mice produced the same effect on 14 successive administrations.

Toxic doses caused death by respiratory failure. The heart, as a rule, continued beating after the respiration had ceased. Even after rapid intravenous injection of toxic doses struggling and convulsions were not observed.

Chronic toxicity.—Young growing rats were fed for 9 weeks on a diet containing 2 per cent of myanesin. Each rat consumed on the average 0.18 g. of the drug per day. All the animals survived and continued to grow, although they did not gain weight as rapidly as the controls, which were litter mates. This was due to the fact that the experimental animals consumed less food than the controls, possibly because of the unpalatability of the drug-containing diet.

The animals were sacrificed 63 days after the beginning of the experiment. Six (30 per cent) out of 20 treated rats showed calculi in the bladder and also had small abscesses in the submaxillary glands. The kidneys, liver, spleen, lungs, heart, and suprarenal glands did not show any macroscopical or microscopical lesions.

The effect on blood pressure and respiration.—Experiments were carried out on rabbits and cats under ether and hexobarbitone anaesthesia. An intravenous injection of 30 mg. myanesin did not cause any alterations in blood pressure or respiration. Fairly rapid intravenous administration of 50 mg. caused a slight or moderate fall of blood pressure amounting to 25 to 40 mm. Hg. There was also a decrease in rate and an increase in depth of the respiratory movements. Both respiration and blood pressure returned to normal after 4 to 8 minutes. Large intravenous doses of the order of 125 mg. caused large falls of blood pressure and cessation of respiratory movements for 10 or 15 seconds, but respiration restarted spontaneously and the animals recovered. The blood pressure depression may have been caused partly by accommodation of more blood in the muscles owing to muscular flaccidity. Doses of 150–200 mg. intravenously caused respiratory arrest and death.

Comparison with curare.—In frogs flaccid paralysis and cessation of respiratory movements were obtained after intralymphatic injections of 3 to 10 mg. per frog (*R. temporaria*). The frogs remained paralysed for several hours, but recovered completely by the next day. In paralysed animals faradization of the sciatic nerve failed to produce muscular contractions, whereas direct stimulation of the muscle itself always produced contractions. The threshold for direct stimulation during the pre-paralytic and paralytic stage remained unchanged. The results indicate that the drug may possess a curare-like action when administered in large doses. The effect of myanesin on mammalian nerve-muscle preparations was similar to that of curare, but large and nearly lethal doses were required for its production.

The paralysis of the muscle to indirect but not to direct stimulation, which was observed only when large doses of myanesin were given, may be due either to a block at the myoneural junction (curare-like action) or to direct action on the nerve (local anaesthetic action). Isolated nerve-muscle preparations could not be usefully employed for the demonstration of curare-like action because myanesin on local application impaired the conductivity of the nerve.

Physostigmine antagonism.—The effect of physostigmine sulphate and of prostigmin on the action of myanesin was investigated in frogs and mice. Physostigmine (0.02 mg. per frog) somewhat accelerated recovery from paralysis caused by the injection of 3 mg. myanesin (mean recovery time of 9 control frogs 326 ± 29 min., mean recovery time of 9 physostigmine frogs 237 ± 27 min., $t = 2.23$, $P < 0.05 > 0.02$). Physostigmine also caused resumption of respiratory movements and the appearance of generalized muscular fibrillation.

In mice subcutaneous injections of prostigmin (0.15 mg. per kg.) somewhat accelerated recovery from paralysis caused by myanesin. Larger but tolerated doses of prostigmin when injected together with non-lethal doses of myanesin caused death in the majority of animals. Neither prostigmin nor physostigmine abolished the effect of lethal doses of myanesin. This suggested that the curare-like activity of myanesin accounted for only a part of the effects produced by this drug.

The anticonvulsive action.—The depressant action of myanesin on the central nervous system was demonstrated and located by its power to antagonize convulsions produced by central nervous system stimulants. Convulsions were produced by leptazol or strychnine and antagonized by simultaneous administration of myanesin or soluble hexobarbitone. All solutions contained $2\frac{1}{2}$ per cent w/v gum acacia and were injected subcutaneously in a volume of 0.4 ml. per 20 g. mouse.

TABLE III

THE ANTAGONISM OF MYANESIN AND SOLUBLE HEXOBARBITONE TO LEPTAZOL AND STRYCHNINE CONVULSIONS ON SUBCUTANEOUS ADMINISTRATION TO MICE

Leptazol mg./kg.	Strychnine mg./kg.	Myanesin mg./kg.	Hexobarbitone mg./kg.	No. of mice	Per cent convulsed	Per cent died
120	—	—	—	60	96·6	53·3
120	—	500	—	20	15·0	0·0
120	—	400	—	30	46·6	3·3
120	—	200	—	40	92·5	42·5
120	—	—	50	20	0·0	0·0
—	1·33	—	—	109	68·9	49·6
—	1·33	100	—	140	19·3	2·8
—	—	50	—	50	40·0	12·0
—	1·33	—	100	100	97·0	29·0
—	1·33	—	50	20	95·0	35·0

Large doses of myanesin diminished the incidence and severity of leptazol convulsions (Table III). Mice receiving 500 mg. per kg. myanesin survived and usually did not convulse ; 400 mg. per kg. markedly reduced mortality but did not prevent the occurrence of convulsions ; smaller doses were almost ineffective.

Hexobarbitone, on the other hand, prevented the occurrence of both convulsions and deaths in a dose of 50 mg. per kg.

In the control of strychnine-induced convulsions myanesin proved very much more effective than soluble hexobarbitone. Myanesin in a dose of 100 mg. per kg. (about one tenth of the LD50) prevented the occurrence of convulsions and deaths in the majority of animals, and 50 mg. per kg. still markedly reduced the severity of convulsions. Hexobarbitone (100 mg. per kg., or about one fourth of the LD50) did not decrease the incidence of convulsions and was not very effective in saving the lives of the animals. Myanesin effectively suppressed convulsions in doses which by themselves did not cause paralysis, whereas hexobarbitone was ineffective even in narcotic doses.

The powerful effect of myanesin in strychnine convulsions suggests that the compound exerts a depressant action on the reflex excitability of the spinal cord. The antagonistic action of myanesin could not be due merely to a curare-like action, because intocostrin, a purified curare preparation, when administered in a similar way did not antagonize but somewhat aggravated the effects of strychnine; this may be due to an additional stimulation of the cord, a well-known side-effect of curare (Sollmann, 1944).

The potentiating effect on barbiturate anaesthesia.—Aqueous solutions of hexobarbitone or myanesin, or mixtures of these substances, were injected intraperitoneally in a volume of 0.4 ml. per 20 g. mouse. The loss of the righting reflex was taken as the criterion of narcosis. The results of these experiments are set out in Table IV.

TABLE IV

POTENTIATION OF BARBITURATE NARCOSIS BY MYANESIN. TWENTY MICE WERE USED AT EACH DOSE LEVEL. INTRAPERITONEAL ADMINISTRATION

Soluble hexobarbitone mg./kg.	Myanesin mg./kg.	Per cent narcotized	Mean duration of narcosis min. and sec.	Mean duration of induction min. and sec.	Remarks
50	—	0	—	—	Excitation, twitching
—	100	0	—	—	Subdued
50	50	75	17′54″±1′50″	3′21″± 8″	Relaxation, no excitation
50	100	100	32′4″±3′6″	2′11″± 7″	Relaxation
70	—	85	14′30″±2′58″	3′35″±15″	Twitching
70	50	100	30′37′±4′24″	2′ 5″± 7″	Relaxation, no excitation
70	100	100	41′10″±3″37″	2′ 5″±19″	Complete relaxation

The mice which received soluble hexobarbitone only were excited, jumpy, had muscular fibrillations, and sometimes rolled over along their longitudinal axis. The animals to which myanesin was also administered exhibited a strikingly different picture. They lay quietly on their backs with all muscles profoundly relaxed, did not show twitching and did not react to painful stimuli or loud noises. An ineffective dose of myanesin injected together with an ineffective dose of

soluble hexobarbitone caused narcosis without excitement in the prenarcotic stage and muscular relaxation during narcosis. Small doses of myanesin also increased the depth and duration of hexobarbitone anaesthesia.

The synergistic effect of myanesin on barbiturate anaesthesia could also be demonstrated in the rabbit. Thirteen rabbits were injected intravenously with 15 mg. per kg. soluble hexobarbitone; the mean duration of narcosis was 1 minute 48 seconds (S.E. $\pm$23 seconds). One week later the same animals were injected with 30 mg. per kg. myanesin intravenously; muscular relaxation was observed but the animals still showed righting reflexes. After a 10 days' rest, myanesin and soluble hexobarbitone, in the above-mentioned doses, were injected together. Deep narcosis lasting almost twice as long as that obtained with hexobarbitone alone was observed (3 minutes 28 seconds, S.E. $\pm$27 seconds). This prolongation of the mean duration of narcosis after myanesin was statistically significant ($t=3.32$, $P<0.01$, >0.001).

Other pharmacological properties.—The local anaesthetic activity of myanesin was tested on the rabbit's cornea, using 10 rabbits for each concentration of the drug. Procaine was instilled into the other eye. The concentrations causing abolition of the wink reflex in one half of the animals with myanesin and procaine were 0.1 per cent and 0.2 per cent respectively, but procaine caused local anaesthesia of longer duration than myanesin. Intracutaneous injections of myanesin and procaine into guinea pigs caused local anaesthesia of about equal duration. It is concluded that myanesin possesses a local anaesthetic activity about equal to that of procaine.

Myanesin did not have any antipyretic or analgesic activity in doses insufficient to cause paralysis. In dilutions of 1 : 10,000 it did not affect the tonus or contractions of the isolated rabbit duodenum. Contractions of the isolated guinea pig ileum brought about by 1.0 μg. histamine could be partially relaxed by the drug in a dilution of 1 : 5,000. Doses of 200 mg. per kg. subcutaneously did not influence the blood sugar of rabbits.

A 0.2 per cent solution did not possess any irritating properties when applied to the eye, but a 0.4 per cent solution caused transient hyperaemia. Intramuscular, subcutaneous, intracutaneous and percutaneous administration of a 1.8 per cent aqueous solution did not cause irritation.

Discussion

The increased reflex excitability of the spinal cord due to strychnine poisoning can be counteracted by the administration of paralysing doses of curare or of anaesthetic doses of barbiturates. Myanesin, on the other hand, antagonizes the action of strychnine in doses which do not cause paralysis or narcosis. From this it may be inferred that myanesin produces muscular relaxation and potentiates narcosis by exerting a depressant action on the reflex excitability of the spinal cord. The curare-like effect of myanesin is unlikely to be of significance in the production of muscular relaxation by therapeutic doses. The weak

antagonistic effect to leptazol shows that the midbrain is influenced only when large doses are given. Myanesin does not appear to act on the brain because it does not affect consciousness and never causes prenarcotic excitation. It cannot, therefore, be classed among the general anaesthetics.

Animals which have been completely paralysed by myanesin continue to breathe spontaneously and recover from the effects of the drug without the aid of artificial respiration. Lethal doses of myanesin eventually paralyse the diaphragm also. It may be concluded that the diaphragm is less affected by myanesin than other muscles and that it is the last muscle to be paralysed. In this respect myanesin also differs from curare. With curare complete muscular paralysis without arrest of respiration can hardly be produced and curarization of the respiratory muscles does not seem to be markedly less complete than that of the other skeletal muscles (Cohnberg, 1946).

In preliminary clinical trials myanesin has proved useful for the production of muscular relaxation during light anaesthesia. It may also be of value in cases of spastic paralysis and for the prevention of traumatic accidents during convulsive shock therapy.

SUMMARY

1. A number of α-substituted ethers of glycerol produced transient relaxation and paralysis of skeletal muscles in small laboratory animals.

2. Of the 143 compounds which have been examined, $\alpha:\beta$-dihydroxy-γ-(2-methylphenoxy)-propane, named myanesin, was the most potent and also possessed the widest margin of safety between paralysing and lethal doses.

3. Myanesin in non-paralysing doses effectively antagonized strychnine convulsions, but was not very effective against leptazol convulsions. Myanesin also counteracted prenarcotic excitement and increased the duration of barbiturate anaesthesia.

4. Myanesin in small doses produced depression of the reflex excitability of the spinal cord. Larger doses had an ascending depressant action on the central nervous system. Myanesin also possessed local anaesthetic properties.

5. Myanesin may prove useful for the production of muscular relaxation during light anaesthesia.

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REFERENCES

Bennett, A. E. (1941). *Am. J. Psychiat.*, **97**, 1040.
Berger, F. M., and Bradley, W. (1946). *Lancet*, in the press.
Cohnberg, R. E. (1946). *J. Lab. clin. Med.*, **31**, 866.
Dragstedt, C. A., and Lang, V. F. (1928). *J. Pharmacol.*, **32**, 215.
Miller, L. C., and Tainter, M. L. (1944). *Proc. Soc. exp. Biol., N.Y.*, **57**, 261.
Sollmann, T. (1944). *A Manual of Pharmacology*, 6th edition, page 417. Philadelphia and London: Saunders.

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THE SYNTHESIS, TOXICITY AND ANAESTHETIC POTENCY OF TWO NEW LOCAL ANAESTHETICS

BY

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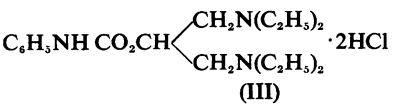
Einhorn in 1899 demonstrated the production of local anaesthesia by esters of *p*-aminobenzoic acid and in 1905 introduced *p*-aminobenzoyldiethylaminoethanol (procaine) as a local anaesthetic. Since then an enormous number of compounds chemically related to procaine have been prepared and many of them have proved useful for the production of local anaesthesia in one or another of several fields. On the other hand, comparatively few studies on the local anaesthetic properties of esters of phenylcarbamic acid (II) have been made, although chemically they are closely related to esters of *p*-aminobenzoic acid (I) as shown below:

$NH_2C_6H_4COO—$ (I) $\qquad$ $C_6H_5NHCOO—$ (II)

The object of the present paper is to report our studies upon the synthesis, toxicity, and local anaesthetic activity of two new esters of phenylcarbamic acid, viz., sym. bis-(diethylamino)*iso*propyl phenylurethane dihydrochloride (III) and sym. dipiperidino-*iso*propyl phenylurethane dihydrochloride (IV).



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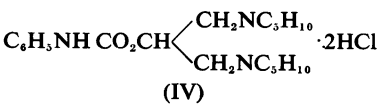

(IV)

EXPERIMENTAL

*Sym. bis-(diethylamino)*iso*propylalcohol.*—A mixture of sym. dichloro*iso*propylalcohol (10 g.) and diethylamine (22.6 g.) was refluxed for sixteen hours. It was then acidified with dilute hydrochloric acid (30 ml.) and washed with ether so as to remove any unchanged sym. dichloro*iso*propylalcohol. The addition of solid sodium hydroxide (15 g.) to the aqueous solution precipitated an oil which was taken up in ether. The ethereal extract was dried over potassium carbonate and then distilled to remove the solvent and excess diethylamine. The residue upon further distillation gave 11.0 g. (or 70% of the theoretical yield) of pure sym. bis-(diethylamino)*iso*propyl alcohol, b.p. 93°/6.2 mm. or 230–232°/769 mm. Berend (1884) obtained the same compound by a different process and gave its boiling point as 234.5°.

*Sym. bis-(diethylamino)*iso*propyl phenylurethane dihydrochloride* (III).—Sym. bis-(diethylamino)*iso*propylalcohol (4 g.) previously cooled to 0° was mixed thoroughly with phenyl-*iso*cyanate (2.63 g.) and allowed to stand at 0° for one hour and then at room

temperature overnight. Since the phenylurethane thus formed failed to solidify upon cooling to −12°, it was treated with 40 ml. 10% hydrochloric acid. The resulting solution, after being freed from oily impurities by means of animal charcoal and dried *in vacuo* over sulphuric acid and solid sodium hydroxide, gave 7.1 g. of almost pure sym. bis-(diethylamino)*iso*propyl phenylurethane dihydrochloride, m.p. 205–210°. It crystallized from absolute alcohol in colourless crystals, extremely soluble in water, m.p. 210–211° (bath preheated to 200°).

Found: C, 54.93; H, 8.42; N, 10.48. $C_{18}H_{33}O_2N_3Cl_2$ requires: C, 54.80; H, 8.44; N, 10.66%.

Sym. dipiperidino-iso*propylalcohol.*—Sym. dichloro-*iso*propylalcohol (6 g.) and piperidine (16 g.) were mixed and allowed to stand at 0° for one hour and then at room temperature for twenty hours. The mixture was then heated at 60–70° for six hours and finally on a boiling water bath for five minutes. The mixture was diluted with water (20 ml.) and extracted several times with ether. The ethereal extract was dried over potassium carbonate, evaporated on the water bath and filtered to remove solid impurities. Pure sym. dipiperidino-*iso*propylalcohol distilled at 144–145°/6.5 mm.; yield, 6.9 g., or 66% of the theoretical. Vassiliadès (1937) gave 172–173°/12 mm. as the boiling point of the compound.

Found: C, 69.29; H, 11.39. Calcd. for $C_{13}H_{26}ON_2$: C, 68.96; H, 11.58%.

The 3.5-dinitrobenzoate was prepared, which crystallized from petroleum ether (b.p. 30–50°) in yellowish plates, m.p. 98–100°.

Found: C, 57.21; H, 6.73. Calcd. for $C_{20}H_{28}O_6N_4$: C, 57.11; H, 6.71%.

Sym. dipiperidino-iso*propyl phenylurethane.*—Sym. dipiperidino-*iso*propylalcohol (4 g.) and phenyl*iso*cyanate (2.72 g.) were mixed at 0°; the reaction took place immediately with evolution of heat. After being kept in ice for a half-hour the mixture was allowed to stand at room temperature for sixteen hours. The resulting product, sym. dipiperidino-*iso*propyl phenylurethane, was extracted with petroleum ether (b.p. 40–60°) in a Soxhlet apparatus. It crystallized in colourless prisms, melting at 106–107°. Yield, 5.3 g., or 87% of the theory.

Found: C, 69.60; H, 8.87; N, 11.91. $C_{20}H_{31}O_2N_3$ requires: C, 69.51; H, 9.05; N, 12.17%.

Sym. dipiperidino-iso*propyl phenylurethane dihydrochloride* (IV).—The hydrochloride was prepared by dissolving the phenylurethane (1.5 g.) in ice-cold 5% hydrochloric acid (30 ml.) and evaporating the solution *in vacuo* over sulphuric acid and solid sodium hydroxide. The hydrochloride melted at 242–243° (bath previously heated to 230°) and crystallized from warm dilute hydrochloric acid in colourless needles, readily soluble in water. The yield was quantitative.

Found: C, 57.15; H, 7.95; N, 9.75. $C_{20}H_{33}O_2N_3Cl_2$ requires: C, 57.39; H, 7.95; N, 10.05%.

Toxicity

The determination of the LD50 of sym. dipiperidino-*iso*propyl phenylurethane dihydrochloride (hereafter referred to as PP) and sym. bis-(diethylamino)-*iso*propyl phenylurethane dihydrochloride (hereafter referred to as DP) was carried out on mice according to Kärber's method. For the purpose of comparison, the LD50s of cocaine and procaine were also determined. All drugs were given intraperitoneally. Table I shows that PP and DP are about as toxic as cocaine but several times more toxic than procaine.

TABLE I

THE LD50 OF PP, DP, COCAINE, AND PROCAINE

Drug	Dose, mg./kg.	Proportion killed	LD50, mg./kg.
PP	50	0/10	
	70	2/10	
	90	3/10	108
	110	7/10	
	130	10/10	
DP	90	0/10	
	110	1/10	
	130	3/10	151
	150	7/10	
	170	10/10	
Cocaine	60	0/12	
	70	1/12	
	80	4/12	91
	90	6/12	
	100	12/12	
Procaine	300	0/10	
	400	2/10	
	500	5/10	564
	600	7/10	
	700	10/10	

In order to compare the toxic action of the above drugs upon respiration and circulation, experiments were made on etherized cats, and the doses of the drugs which caused an appreciable depression of blood pressure and respiration were determined. The drugs were injected intravenously. The average results obtained on six cats are shown in Table II. These results show that PP and DP have an action on blood pressure and respiration more or less similar to that of cocaine but stronger than that of procaine.

TABLE II

DOSAGE OF PP, DP, COCAINE, AND PROCAINE REQUIRED TO PRODUCE AN APPRECIABLE DEPRESSION OF BLOOD PRESSURE AND RESPIRATION

Drug	Dose, mg./kg.	
	Circulation	Respiration
PP	1·0	1·8
DP	1·1	2·2
Cocaine	1·0	1·7
Procaine	6·0	7·0

ANAESTHETIC POTENCY

The anaesthetic potencies of PP and DP were compared with those of cocaine and procaine by the rabbit's cornea method and the intradermal weal method.

These methods were chosen because they resemble more closely the clinical methods of applying local anaesthetics and also because they appear to give more reliable results than other methods (Sinha, 1936).

Rabbit's cornea method.—In this method the cornea was flooded with the drug solution and washed thoroughly with saline solution at the end of five minutes. The winking reflex was tested from time to time until it was completely restored to normal. Since there was marked individual variation in the sensitivity of the rabbit's eye toward anaesthetics and in some animals the sensitivity varied from time to time, comparisons with cocaine were made by applying the drug to be tested alternately to the same eye of a rabbit whose sensitivity showed little or no variation with time. In order to provide time for the recovery of the eyes, an interval of six to seven days was allowed between tests. Rider (1930) and Sinha (1936) have both asserted that repeated applications of cocaine render a cornea less sensitive to the drug. On the other hand, the writers found more often a slight increase rather than a decrease in the sensitivity of the rabbit's eye when cocaine was applied to it every six to seven days. Table III shows the anaesthetic potency of PP and DP and cocaine as determined by the rabbit's cornea method. The results are the average of two or three more or less consistent determinations, discordant results being discarded. It will be seen from Table III that while PP is about twice as potent as cocaine, DP is only one half as potent. Since the toxicity of these agents is about equal to that of cocaine, these results would warrant a clinical trial of these drugs as local anaesthetics for the production of surface anaesthesia.

TABLE III

THE ANAESTHETIC POTENCY OF PP AND DP AS DETERMINED BY THE RABBIT'S CORNEA METHOD

Drug	Duration of anaesthesia in minutes in rabbits numbered 1 to 9										Efficiency ratio
	1	2	3	4	5	6	7	8	9	Mean	
PP 0·25%	10	11	23	14	19	17	—	—	—	16	2
DP 1%	14	—	—	—	17	16	17	19	15	16	1/2
Cocaine 0·5% ..	10	10	23	13	15	17	17	21	18	15	1

Intradermal weal method.—The method used was to inject 0.1 ml. of a sterilized solution intracutaneously on the flexor surface of the forearm. The duration of anaesthesia was determined by the time during which there was a loss of sensation of the injected area to pin pricks. Table IV shows the results obtained on three subjects; it will be seen that PP and DP were 12.5 and 5 times as potent as procaine respectively. Since the toxicities of PP and DP are

about six and four times that of procaine respectively, these results indicate that PP and DP may be of clinical value as local anaesthetics for the production of conduction anaesthesia.

TABLE IV

THE ANAESTHETIC POTENCY OF PP AND DP AS DETERMINED BY THE INTRADERMAL METHOD

Drug	Duration of anaesthesia in minutes in different subjects				Efficiency ratio
	Ho	Fan	Hsia	Mean	
PP 0·02%	11	12	14	12·7	12·5
DP 0·05%	13	11	10	11·3	5·0
Procaine 0·25% ..	10	11	9	10·0	1·0

ACKNOWLEDGMENTS

We are obliged to Mr. M. J. Chang, of the New Asia Pharmaceutical Research Institute, for microanalyses, and to the China Foundation for the Promotion of Education and Culture for a research grant awarded to one of us (Y. T. H.).

SUMMARY

Two new phenyl urethanes, namely sym. bis-(diethylamino)*iso*propyl phenylurethane and sym. dipiperidino-*iso*propyl phenylurethane, were prepared. The dihydrochlorides of both exhibited marked local anaesthetic activity when tested by the rabbit's cornea and the human intradermal weal methods. In view of their activities and toxicities relative to cocaine and procaine they are considered worthy of clinical trial.

REFERENCES

Berend, L. (1884). *Ber. dtsch. chem. Ges.*, **17**, 511.
Rider, T. H. (1930). *J. Amer. chem. Soc.*, **52**, 2115.
Sinha, H. K. (1936). *J. Pharmacol.*, **57**, 199.
Vassiliadès, C. (1937). *Bull. Soc. chim.*, Paris, Sér. 5, **4**, 1131.

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THE ANTIHISTAMINE SUBSTANCE 2786 R.P.

BY

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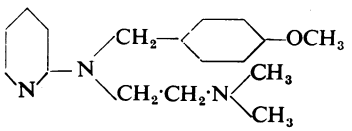
Bovet and Walthert (1944) have described the properties of a substance which is known as 2786 R.P., and called by them neoantergan ; it is *N*-dimethylaminoethyl *N*-*p*-methoxybenzyl-α-aminopyridine.



This substance powerfully inhibits the action of histamine in animals and has been used clinically with success in the treatment of chronic urticaria and other conditions like hay fever. Benadryl (dimethylaminoethylbenzhydryl ether hydrochloride) was introduced by Loew, Kaiser, and Moore (1945) a year later, and Dr. H. O. Schild informs us that when tested on the guinea-pig ileum in comparison with benadryl, neoantergan is 7 to 18 times more powerful against histamine, depending on the length of contact with the ileum, and 60 to 70 times less powerful against acetylcholine.

We have examined neoantergan by several of the methods used by Bovet and Walthert, and have confirmed their main results. We have also examined neoantergan by several other methods which will now be described.

Experimental Observations

Action on isolated auricles.—Very little stress was given by Dale and Laidlaw (1910), in their original description of the properties of histamine, to the stimulant action of histamine on cardiac tissue. The stimulant action was first demonstrated by Gunn (1926). It is not, however, nearly so striking in the isolated heart perfused by Langendorff's method as when the auricles of the rabbit heart are dissected and suspended in a bath of well-oxygenated Ringer's solution at 28° C. Oxygenation is provided by a gas distribution tube at the bottom of the vessel. In these circumstances the addition of histamine in such amount that

the concentration of base is 1 in 1 million causes a large augmentation of the beat; the effect is seen twice in succession in Fig. 1. Neoantergan was then

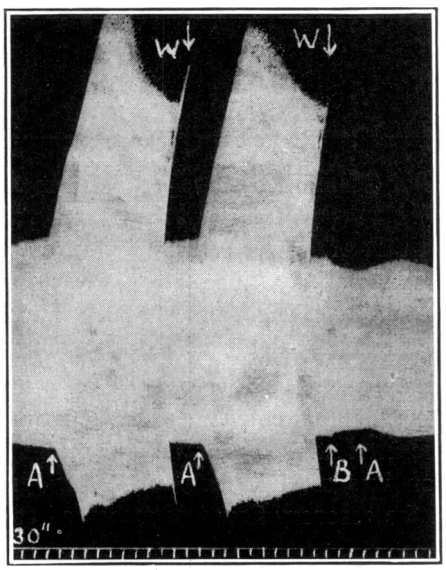


FIG. 1.—Isolated auricles of rabbit heart. At A, histamine was added to the bath (0.1 mg. to 100 ml.). Note the great increase of amplitude. At B, 0.5 mg. neoantergan was added, after which the addition of histamine was without effect.

added to the bath, and in its presence the addition of histamine was without effect.

Action on the driven auricles.—Dawes (1946) has recently described a preparation to measure the action of quinidine on cardiac tissue. The isolated auricles of the rabbit are arranged in contact with electrodes so that they can be made to beat at an imposed rate. A maximum rate can be determined beyond which they cannot follow the stimuli applied, and this maximum rate is reduced when the auricles are exposed for a given length of time to a given concentration of quinidine, or of a substance having a similar action. We have tested neoantergan by this method, and have found that it is approximately twice as active as quinidine. Table I gives the results obtained in one of the experiments. A comparison was undertaken of the toxicity of neoantergan for mice with that of quinidine, both substances being given by intraperitoneal injection. It was found that neoantergan was approximately twice as toxic as quinidine, the LD50 of neoantergan being approximately 120 mg. per kg., whereas that of quinidine was approximately 225 mg. per kg. (About 30 mice were used for each substance.) Neoantergan is thus of equal value to quinidine for its action on the heart; it is twice as active, but twice as toxic. It should be noted that

TABLE I

COMPARISON OF NEOANTERGAN WITH QUINIDINE ON THE ISOLATED AURICLES OF THE RABBIT

Substance	Dose in mg. in 100 ml. bath	Percentage reduction in maximal rate
Quinidine	0·25	7·9
	0·5	17·2
Neoantergan ..	0·125	8·3
	0·25	15·3

the figure for the LD50 of quinidine by intraperitoneal injection differs from that given by Dawes (1946). Dawes's figure was 135 mg. per kg. The difference between our figure and that of Dawes serves to illustrate the point that figures for toxicity have no general validity, since they can vary by as much as 100 per cent in the same laboratory at different times. Such figures are only of use for comparing two substances at the same time (compare Burn and Greville, 1931).

Action on coronary flow.—Histamine is well known to produce a dilatation of the coronary vessels of the cat, and since histamine is here relaxing smooth muscle we were interested to examine the action of neoantergan. The Langendorff preparation was set up, the heart being perfused with Ringer's solution at 37° C. and the coronary flow was measured as the outflow from the

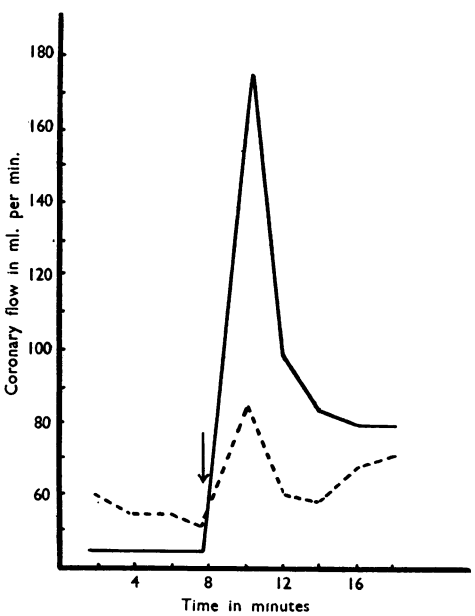


FIG. 2.—Abscissae: time in min. Ordinates: coronary flow in ml. per min. recorded in the heart–lung preparation of the dog. At the arrow 0.1 mg. histamine was injected into the sup. vena cava with the effect shown in the continuous line. Later 0.1 mg. neoantergan was injected followed 1 min. later by 0.1 mg. histamine. The effect of the histamine was that shown in the broken line.

heart. The results in different experiments were similar, and the following is an example. The injection of 25 μg. histamine caused an increase in coronary flow from 4.4 ml. per min. to 12.0 ml. per min. When this effect had finally passed off in about 6 min., neoantergan was injected in a dose of 25 μg. and followed by 25 μg. histamine. The coronary flow increased to 6 ml. per min. only, and returned in 2 min. to the previous rate.

A similar experiment was carried out in the heart–lung preparation of the dog, in which the coronary flow was recorded by inserting a Morawitz cannula in the coronary sinus. The result is shown in Fig. 2. These experiments show that neoantergan reduces the effect of histamine in relaxing the smooth muscle of the coronary arteries.

Action on blood vessels.—The vessels of the rabbit's ear perfused with Ringer's solution by the method of Gaddum and Kwiatkowski (1938) are constricted by small doses of histamine. We have found that this constrictor action is extremely sensitive to the presence of neoantergan and disappears when very small amounts are injected. Fig. 3 shows that the constrictor action of 0.4 μg.

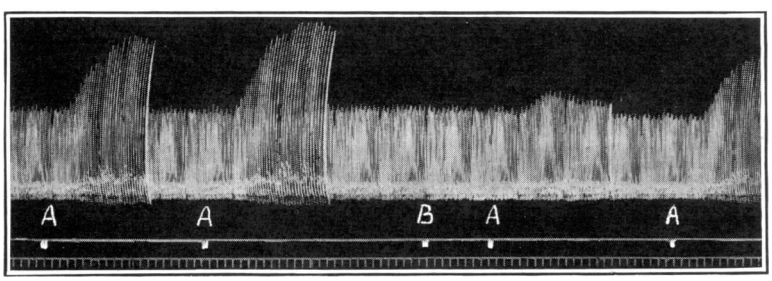


FIG. 3.—Perfusion of rabbit's ear with Ringer's solution; outflow recorded by Gaddum's drop-timer. At A, 0.4 μg. histamine injected. At B, 0.01 μg. neoantergan injected.

histamine was almost abolished by the previous injection of 0.01 μg. neoantergan, and we have observed that a significant reduction was produced by 0.001 μg. neoantergan.

In experiments in which blood flowed through the vessels, the effect of neoantergan on histamine was much less. In the hind-leg of the dog perfused by a pump, the vasodilator action of 3–5 μg. histamine was in two experiments greatly diminished and in a third little affected by the previous injection of 10 μg. neoantergan. In the cat anaesthetized with chloralose, when the spleen volume and the blood pressure were recorded, the intravenous injection of neoantergan abolished the action of histamine on the spleen, but not the depressor action on the blood pressure, even when a dose as large as 10 mg. was injected. (See Fig. 4.) On the other hand, in cats anaesthetized with ether, the injection of 2.5 mg. neoantergan greatly diminished the action of histamine on the blood pressure, so that even 80 μg. produced less fall than 10 μg. previously.

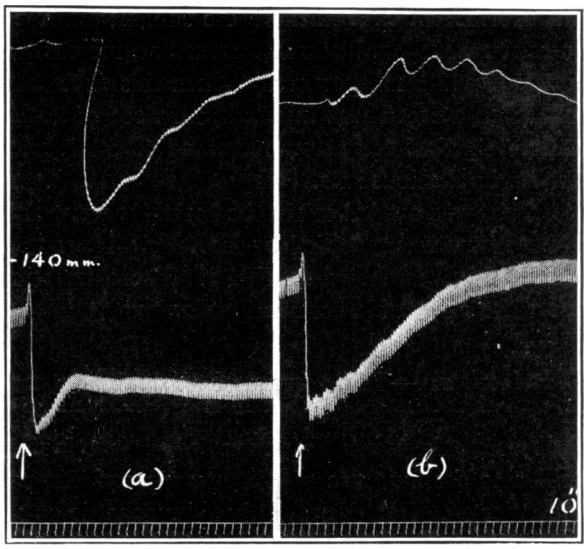


FIG. 4.—Cat ; chloralose. Upper record is spleen volume ; lower record is blood pressure. At the arrow 10 μg. histamine was injected intravenously. Between (a) and (b) 2.5 mg. neoantergan was injected. Note the abolition of the action on the spleen, while the blood pressure effect remains.

In the rabbit anaesthetized with urethane, the injection of 5 mg. neoantergan somewhat reduced the duration of the fall of blood pressure produced by 0.25 mg. histamine, but wholly abolished the stimulant action of this dose of histamine on the respiration.

Action on the uterus.—The common description of histamine as a stimulant of smooth muscle not only neglects the inhibitory action of histamine on the smooth muscle of the coronary vessels of the cat and dog, but also the inhibitory action on the uterine muscle of the rat. We have already seen that the inhibitory action on the coronary vessels is reduced by neoantergan ; we have failed, however, to observe that neoantergan, in concentrations which have no effect on the uterus, reduces the inhibition of the rat uterus by histamine. This result confirms Bovet and Walthert (1944), but we do not confirm their statement that neoantergan has little antihistamine action on the guinea-pig uterus. Fig. 5 illustrates this effect produced by neoantergan in a concentration of 1 in 500 million.

Action as a spasmolytic.—Neoantergan is an antagonist of acetylcholine on the isolated rabbit intestine, though its effect is slight. The stimulant action of 5 μg. acetylcholine was reduced to less than half by the presence of 0.5 mg. neoantergan in a bath of 100 ml. This atropine-like effect is feeble compared with its antihistamine action on guinea-pig ileum, in which tissue the stimulant action of 1 μg. histamine is reduced to less than half by 0.1 μg. neoantergan.

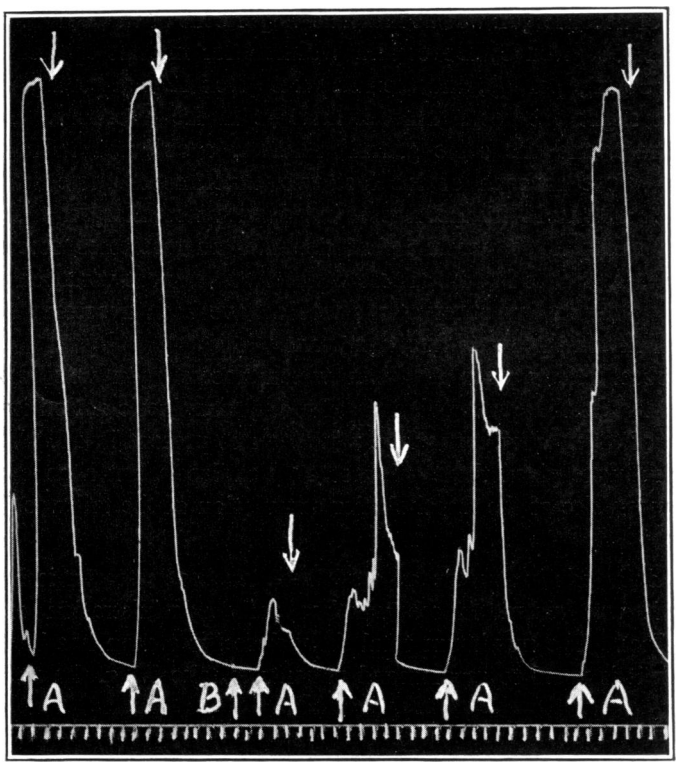


FIG. 5.—Isolated uterus of guinea-pig in bath of 100 ml. At A, 3 μg. histamine added to the bath. At B, 0.2 μg. neoantergan added. The bath was changed as soon as each contraction passed its maximum.

Local anaesthetic action.—Since neoantergan has some atropine-like action, though very little, it was also tested for local anaesthetic action, using the guinea-pig intracutaneous test as described by Bülbring and Wajda (1945). After preliminary observations had shown that a local anaesthetic action was present, a comparison was made with procaine, in which solutions of neoantergan of strengths 0.06, 0.12, and 0.5 g./100 ml. were compared with solutions of procaine of strengths 0.25, 0.5, and 1.0 g./100 ml. The mean result was that neoantergan is 3.1 times as active as procaine by this test.

Analgesic action.—Neoantergan was tested for analgesic action by the method described by Thorp (1946). One group of rats was injected with pethidine, one group was injected with neoantergan, and one group was injected with saline. After determining the threshold for stimulation of the tail, the injections were given intravenously into the tail vein. Rats injected with 5.0 mg. per kg. pethidine then required a much stronger stimulus, the mean increase of threshold being 90.2 per cent.

The dose of neoantergan was as much as 40 per cent of the lethal dose, assuming that the LD50 given by Bovet and Walthert for mice is applicable to rats. This figure is 30 mg. per kg., and the rats were given 12 mg. per kg. The injection caused a slight but not significant increase in the threshold. Each group contained 5 rats, and the same general result was obtained in four separate experiments. Since in each of the last two experiments there was a rather greater rise of threshold in the rats injected with neoantergan than in those injected with saline, it is possible that neoantergan possesses some analgesic action, though, if so, it is feeble. There was, however, great variation among the rats in a group, and many observations would have been necessary to establish that the small rise in threshold was significant.

Further observations were made in which neoantergan was injected subcutaneously. Again there was evidence of a slight analgesic action in some rats when a dose of 30 mg./kg. was given; it was not present in all. When doses of 100 mg./kg. were given there was not only analgesia but a general narcotic effect. The mean rise of the threshold for stimulation of the tail was 80 per cent after one hour.

Effect of daily administration on growth.—Since neoantergan is intended for clinical use it was tested to see whether daily administration exerted any deleterious effect. The maximum total daily dose for a man has been taken as 1.0 g., though it is usual to recommend patients to take 0.1 g. three times a day

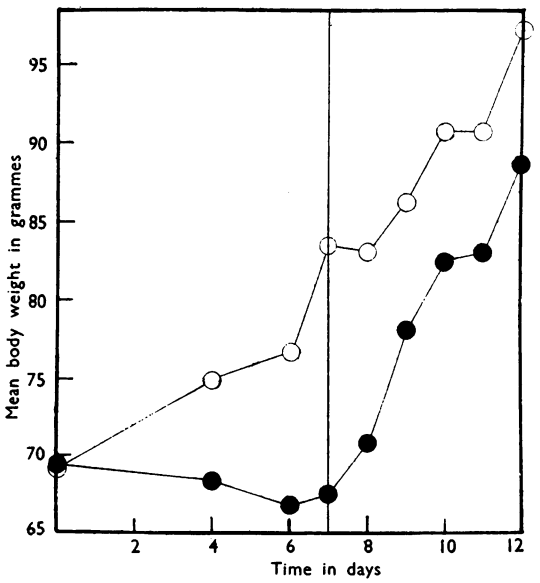


FIG. 6.—Abscissae: time in days; ordinates, mean body weight in grammes. White circles show the increase in body weight of 5 control rats; black circles show the body weight of 5 rats given 25 mg./kg. neoantergan twice daily by subcutaneous injection, for 7 days. After the seventh day the injections were stopped.

at first. This dose is then doubled, and may be recommended four times a day; or again, the dose may be trebled. A dose of 1.0 g. for a man of 70 kg. is equivalent to 14 mg./kg.

The effect of giving a total daily dose of 50 mg./kg. by subcutaneous injection was determined in a group of 5 young rats of 60–70 g. The dose was divided into two parts, half being given in the morning, and half in the evening. Fig. 6 shows the arrest in growth when this dose was given for 7 days, and also shows that when the injections were stopped the rats at once began to grow at the same rate as the group of 5 control rats. The cause of the arrest of growth may have been a decline in the amount of food eaten, though we do not know this; it was probably not due to a toxic effect on the liver or kidney, since growth restarted at once when injections stopped. A second experiment was then carried out in which the total daily dose was 16 mg. per kg. In this experiment there was no effect on the growth of the 5 injected rats as compared with the 5 control rats. Thus a dose equivalent to the maximum human therapeutic dose, when given in two injections daily for 11 days, did not affect the growth of young rats.

Discussion

Perhaps the most striking method of demonstrating the effect of antihistamine substances is to use them to protect guinea-pigs against the action of histamine sprayed into the air from an atomizer. We have used a box with a glass top similar to that described by Bovet and Walthert (1944), in which the guinea-pig is placed. The box is about 12 in. square and 6 in. deep. The nozzle of an atomizer is placed in a small hole in the floor of the box, and a solution of 1 or 2 per cent histamine (base) is sprayed into the box. If a series of guinea-pigs is placed in the box, one at a time, it is seen that they become asphyxiated and collapse in about 90 sec. If they are promptly removed from the box, they recover. The injection under the skin beforehand of 1 mg. neoantergan gives complete and long-lasting protection against the histamine, and we have confirmed Bovet and Walthert's statement that as little as 0.1 mg./kg. gives considerable protection.

The antihistamine action is also easy to demonstrate if a 1 in 1,000 solution of neoantergan is mixed with 1 in 1,000 histamine, and a drop is put on the forearm. If a prick is made through the drop, very little effect of histamine is seen.

Dawes (1946) has pointed out that substances having a quinidine-like action possess other properties in addition. Local anaesthetics, such as procaine and amethocaine, have a quinidine-like action, and so have spasmolytics such as "syntropan" and "trasentin." Substances with an analgesic action like papaverine and pethidine also have a quinidine-like action. We are now able to extend the list to include antihistamine substances, for neoantergan has a quinidine-like action too. It is becoming clearer that there is a large group of substances which possess in common all these different properties, though in

very varying degrees. Neoantergan is not only an antihistamine substance which possesses a quinidine-like action, but it is also a local anaesthetic, and it has a feeble action as an antagonist to acetylcholine on the intestine of the rabbit. It appears to have a slight action as an analgesic which is detectable by the test on the tail of the rat. The chemical relation of neoantergan to other substances with similar properties is not immediately obvious, but many other substances contain a chain resembling dimethylaminoethyl, and also a group corresponding to *p*-methoxybenzyl. In " benadryl " the linkage of the two is effected as an ether. In neoantergan the linkage is through α-amino pyridine ; this is certainly likely to be less toxic than a linkage through aniline, as in the earlier compound antergan (Halpern, 1942).

SUMMARY

1. The properties of the antihistamine substance 2786 R.P., called by Bovet and Walthert neoantergan, have been examined, and the statements of these authors have, in the main, been confirmed. We observed, however, more antihistamine action on the guinea-pig uterus and less on the blood pressure of an animal anaesthetized with chloralose.

2. In addition the following effects have been observed. Neoantergan is a local anaesthetic three times as potent as procaine. It has a quinidine-like action on the auricle, twice as strong as that of quinidine. It abolishes the stimulant action of histamine on cardiac tissue, the dilator action on coronary vessels, and the constrictor action on the vessels of the rabbit ear. It has some analgesic action. When the maximum daily therapeutic dose calculated per kg. is given to young rats daily for 11 days, their growth rate is unaffected, though three times as much causes an arrest of growth.

Our thanks are due to Prof. J. H. Burn for directing this work. The work was done during the tenure by one of us (J. D. P. Graham) of an I.C.I. fellowship awarded by the University of Glasgow. We are indebted to Dr. D. Bovet of the Institut Pasteur for a supply of the material. We also received some from Messrs. May & Baker, Ltd.

REFERENCES

Bovet, D., and Walthert, F. (1944). *Annal. pharmaceut. franc.*, Suppl. No. 4.
Bülbring, E., and Wajda, I. (1945). *J. Pharmacol.*, **85**, 78.
Burn, J. H., and Greville, G. D. (1931). *J. Pharmacol.*, **43**, 645.
Dale, H. H., and Laidlaw, P. P. (1910). *J. Physiol.*, **41**, 229.
Dawes, G. S. (1946). *Brit. J. Pharmacol.*, **1**, 90.
Gaddum, J. H., and Kwiatkowski, H. (1938). *J. Physiol.*, **94**, 87.
Gunn, J. A. (1926). *J. Pharmacol.*, **29**, 325.
Halpern, B. N. (1942). *Arch. internat. pharmacodyn.*, **68**, 339.
Loew, E. R., Kaiser, M. E., and Moore, V. (1945). *J. Pharmacol.*, **83**, 120.
Thorp, R. H. (1946). *Brit. J. Pharmacol.*, **1**, 113.

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THE TOXICITY OF ARSINE ADMINISTERED BY INTRAPERITONEAL INJECTION

BY

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In a previous paper (Levvy, 1946) accurate figures were given for the median lethal dose of arsine for mice. These figures were obtained by determining the arsenic in the entire carcases after subjecting the animals to exposures to arsine which were known to produce 50 per cent mortality. Technical considerations prevented the application of this method for determining the lethal dose to larger animals. In experiments with different species of animal, it was found possible to administer arsine by intraperitoneal injection of the gas in admixture with hydrogen. Since the toxicity of arsine given in this way bore no direct relationship to its toxicity when inhaled, the experiments were discontinued at an early stage. It was considered that, although the number of animals used was small, the results obtained might possess some theoretical interest.

Experimental Results

Mixtures of arsine and hydrogen, prepared and assayed as previously described (Levvy, 1946), were injected from a hypodermic syringe or gas burette. The arsine concentration in the mixture was usually about 10 mg./100 ml. Surviving animals were kept under observation for at least a fortnight after the injection or the last of a series of injections.

Figures for the mortality produced by single intraperitoneal injections of arsine into mice, rabbits, cats, and sheep are shown in Table I. The LD50 of arsine administered in this way appears to have been approximately the same (2.5 mg. AsH_3/kg.) for all species of animal studied. Results obtained when arsine was injected repeatedly at intervals of 24 hours into mice and rabbits are summarized in Table II. For mice, the maximum tolerated daily dose appears to have been about 1.2 mg. AsH_3/kg., approximately half the figure for the LD50 by single injection, while for rabbits the corresponding figure was only 0.5 mg. AsH_3/kg. It is strange that two injections of 1.0 mg./kg. caused a greater percentage mortality in rabbits than a single injection of 2.0 mg./kg.

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TABLE I

LETHAL DOSE OF ARSINE ON INTRAPERITONEAL INJECTION

Animal	Dose, mg. AsH_3/kg.	Mortality	Approx. LD50, mg. AsH_3/kg.
Mouse	2·5	2/6	3·0
	3·75	4/6	
	4·0	6/7	
	5·0	12/12	
Rabbit	1·0	1/2	2·5
	1·5	0/2	
	2·0	0/2	
	3·0	2/2	
	4·0	2/2	
	6·0	2/2	
Cat	2·0	1/2	2·0–2·5
	2·5	1/2	
	3·0	2/2	
	4·0	2/2	
	5·0	2/2	
Sheep	2·0	0/2	3·0
	4·0	2/2	

TABLE II

CUMULATION OF ARSINE INJECTED AT 24-HOUR INTERVALS

Daily dose, mg. AsH_3/kg.	Animals dying on daily injection		Animals surviving daily injection	
	No. of animals	No. of injections before death	No. of animals	Total no. of injections received
Mice:				
0·3	—	—	4	8
0·6	—	—	8	10
1·2	1	5	7	10
	1	8	3	14
1·5	4	3	8	3
2·0	1	2	2	2
	5	3	—	—
2·5	2	1	1	2
	5	2	—	—
Rabbits:				
0·5	1	11	2	11
1·0	5	2	—	—
	1	3	—	—
1·5	3	2	—	—

The clearance of arsine from mice injected with 4.0 mg./kg. is shown in Fig. 1. At intervals after injection, the animals were killed and the arsenic in the carcases was determined (Levvy, 1943). Three mice were killed at each

time interval and the average figure for residual arsine is plotted in the graph. Half of the arsine injected was cleared in the first 24 hours, but most of the remainder was still present after another 18 hours. The clearance process was,

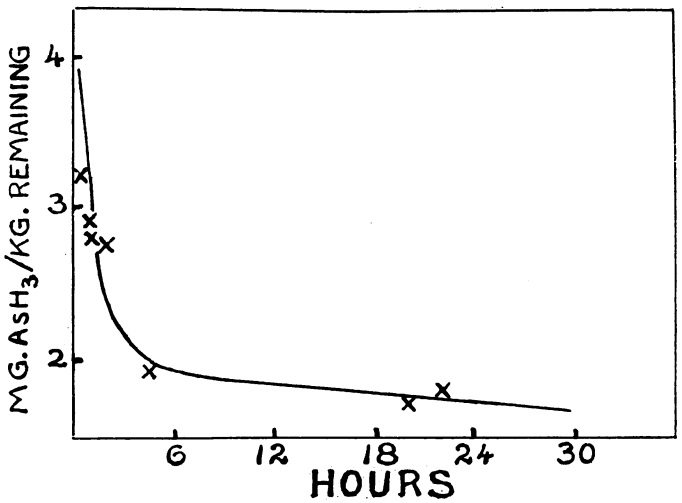

FIG. 1.—Clearance of arsine from mice after intraperitoneal injection. 4.0 mg. AsH_3/kg. injected.

however, rapid enough to explain the tolerance by mice of daily injection with half the LD50.

DISCUSSION

From the results described above it appears unlikely that much arsine injected into the peritoneal cavity escaped fixation by the erythrocytes or the body tissues. Arsine administered in this way, however, is far from exhibiting its maximum toxicity. The toxicity for mice exposed to the gas (Levvy, 1946) was found to increase as the concentration inhaled increased, and with a concentration of 2.5 mg. AsH_3/litre in the atmosphere, the LD50 was only 0.67 mg. AsH_3/kg. It did not approximate to the figure obtained when the gas was given intraperitoneally (2.5 mg./kg.) until the atmospheric concentration had been reduced to 0.25 mg./litre.

In contact with blood, arsine is fixed by the erythrocytes, and undergoes reaction with the haemoglobin. When haemolysis ultimately occurs, the arsenic-containing product or products of the reaction are liberated. Fixation of the arsine occurs so rapidly that it has been held until recently that all arsine entering the body must go through this process. Arsenical poisoning of body tissues was considered, on this view, to be due entirely to the product or products of the reaction in the red blood corpuscles. An arsenite is the most toxic compound likely to be produced in the reaction. The lethal dose of an arsenite administered

parenterally appears to be constant for all species of animal at about 5 mg. As/kg. (Fischl and Schlossberger, 1934). To explain the much lower figure observed for the LD50 of arsine when it was inhaled in high concentration, it was suggested in the previous paper (Levvy, 1946) that part of the gas inhaled escapes fixation by the erythrocytes long enough to be carried in physical solution in the plasma to vital organs and to produce a specific effect in them.

The fact that the LD50 of arsine when it was given intraperitoneally was 2.5 mg./kg. may indicate that the conditions of absorption from the peritoneal cavity are such that the fraction of the gas not fixed by the erythrocytes is the same as during inhalation of arsine from an atmosphere containing 0.25 mg./litre. If absorption of arsine from the peritoneal cavity is at all delayed, it is unlikely that much of the gas can escape fixation in the blood. In this case, the effects on body tissues must be due solely to the arsenic derivative liberated on haemolysis. This possibility is not incompatible with the belief that the compound formed in the blood is an arsenite, since haemolysis may contribute sufficiently to the toxicity for the whole animal to explain the smaller figure for the LD50 on injection of arsine than of arsenite. The alternative explanation, that the figure observed for the LD50 of arsine when it is administered intraperitoneally indicates that as a result of the reaction in the blood the gas is converted into some unknown compound (almost certainly trivalent—see Graham, Crawford, and Marrian, 1946) intermediate between arsine itself and arsenites in the severity of the effects it produces in the rest of the body, cannot be excluded.

SUMMARY

1. It was found possible to administer arsine quantitatively to animals by intraperitoneal injection of arsine-hydrogen gas mixtures.

2. The median lethal dose of arsine administered in this way was approximately 2.5 mg./kg. for all species of animal studied.

3. From cumulation and excretion experiments it was concluded that half the dose injected into mice was cleared from the body in 24 hours. With rabbits, the clearance appeared to proceed more slowly.

4. Possible explanations are discussed of the fact that the median lethal dose of arsine in these experiments was much greater than the lowest figures observed when the gas was given by inhalation.

Permission from the Director-General of Scientific Research (Defence), Ministry of Supply, to publish these results is gratefully acknowledged.

REFERENCES

Fischl, V., and Schlossberger, H. (1934). *Handbuch der Chemotherapie*, p. 359. Leipzig: Georg Thieme.
Graham, A. F., Crawford, T. B. B., and Marrian, G. F. (1946). *Biochem. J.*, **40**, 256.
Levvy, G. A. (1943). *Biochem. J.*, **37**, 598.
Levvy, G. A. (1946). *Quart. J. exp. Physiol.*, in the press.

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