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A METHOD OF ESTIMATING CURARE-LIKE ACTIVITY ON THE ISOLATED PHRENIC NERVE DIAPHRAGM PREPARATION OF THE RAT

BY

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(Received September 24, 1946)

Preparations of curare have recently been introduced into medicine for two purposes. They have been used to reduce the force of the muscle contractions evoked in shock therapy of schizophrenia (Bennett, 1940; Cummins, 1942; Wolfe, 1945); they have also been used to obtain full muscular relaxation in anaesthesia, particularly in conjunction with cyclopropane (Griffith and Johnson, 1942; Mallinson, 1945; Gray and Halton, 1946) for operations in the upper part of the abdomen. The preparations used hitherto have been an extract of curare known as intocostrin, prepared by E. R. Squibb and Sons, and *d*-tubocurarine chloride, the pure active principle isolated by King (1935) and prepared by Burroughs Wellcome and Co. The experiments described in this paper have been carried out with the latter.

As the supply of curare is limited there is a need for substitutes. At the same time a quick test method for comparison with curare is required. Previous workers have mostly used the isolated frog nerve-muscle preparation. The frog's gastrocnemius is less suitable than the sartorius muscle, because in the latter preparation the muscle is very thin and differences in diffusion rates of different compounds are negligible. Ing and Wright (1931) found the nerve sartorius preparation of *Rana esculenta* very suitable because the muscle recovered completely after poisoning, and consequently one preparation could be used to test several compounds. But if only small frogs (*Rana temporaria*) are available, the nerve sartorius preparation is too delicate for routine tests. Also, most authors (for references, see Ing, 1936) have estimated either concentrations of drugs which paralysed the muscle completely or concentrations which just failed to cause complete paralysis; after such severe poisoning recovery is very slow, and routine tests would take a very long time. Testing curare extracts in Squibb's laboratories, Holaday (quoted by Bennett, 1941) developed the head-drop method in the rabbit. Curare solutions are slowly infused intravenously until the animal is incapable of holding up its head. The amount per kg. necessary to produce this effect in rabbits is estimated. Results obtained by this method, together with those obtained on the acetylcholine contractions of the frog's gastrocnemius,

were claimed to be reproducible with an error of ± 1 per cent. The authors do not say whether several extracts could be compared in one animal or whether several rabbits were used for each estimation. Also, it seems to be more desirable to use a method with a clearer endpoint.

Recently Bülbring (1946) described a mammalian isolated nerve-muscle preparation, the rat's phrenic nerve-diaphragm, which has several advantages: the rat's diaphragm is a thin muscle which gives constant contractions for many hours and allows drugs which are applied to it to be washed out readily. A method of estimating curare-like substances has been worked out using this preparation.

METHOD

The rat's phrenic nerve-diaphragm preparation was set up as described by Bülbring (1946). The fan-shaped muscle strip was stimulated indirectly to maximal contractions by condenser discharges from a neon-lamp circuit. The contractions of the muscle were recorded by an isotonic lever. The muscle was immersed in a bath containing Tyrode solution with twice the normal amount of glucose. Pure oxygen or a mixture of 95 per cent oxygen and 5 per cent CO_2 was supplied in fine bubbles by means of a gas distribution tube. The bath was kept at a constant temperature between 37–38° C. Its capacity was 100 ml. Between the addition of one dose of tubocurarine and the next there was an interval of 13 minutes during which the Tyrode solution was changed four times and the regular stimulation of the muscle was not interrupted. The routine procedure was as follows: a dose of tubocurarine was added to the bath and allowed to act for 3 min. after which the Tyrode solution was changed; 2, 4, and 6 min. later the solution was changed again, after which the preparation was left for 5 min. before adding the next dose of tubocurarine. By keeping to this schedule it was possible to obtain a satisfactory recovery of muscle contractions and a constant response to a given dose of tubocurarine, provided a dose was chosen which did not produce a complete abolition of muscle contractions but only a partial inhibition, preferably of not more than 50 per cent.

The percentage inhibition was determined by measuring the height of contraction just before tubocurarine was added to the bath, and again 3 min. later. Thus if the first figure was 76 mm. and the second figure was 58 mm., then the contraction was reduced to 76.5 per cent of its original height, and the inhibition was said to be 23.5 per cent.

The effect of the rate of stimulation.—There are very few references in the literature to any relation between the effect of curare and the rate of stimulation. In 1935 Briscoe, using the quadriceps muscle of the decerebrate cat, found that the action of curare in diminishing rigidity was greater when the stimulation was fast than when it was slow. It was easy to determine the effect of different frequencies of stimulation on the action of tubocurarine on the isolated preparation. The response to a given dose of tubocurarine was almost constant, except for the response to the first dose applied to a fresh preparation, or to a preparation left without tubocurarine for an interval; such a response was less than later responses. It was found that an increase in rate of stimulation correspondingly increased the action of tubocurarine (Fig. 1). At a rate of 5 per min., the addition of 0.09 mg. *d*-tubocurarine chloride diminished the muscle contractions in 3 min. by 11.5 per cent, while at a rate of 20 per min. the diminution was

36.5 per cent. No experiment was done at a rate greater than 20 per min. because, presumably owing to fatigue, the muscle then recovered very slowly from the effect of the tubocurarine. A quantitative study of the relation between rate and effect was made in different experiments, and the results of two experiments are given in Fig. 2, which shows the relation between the logarithm of the concen-

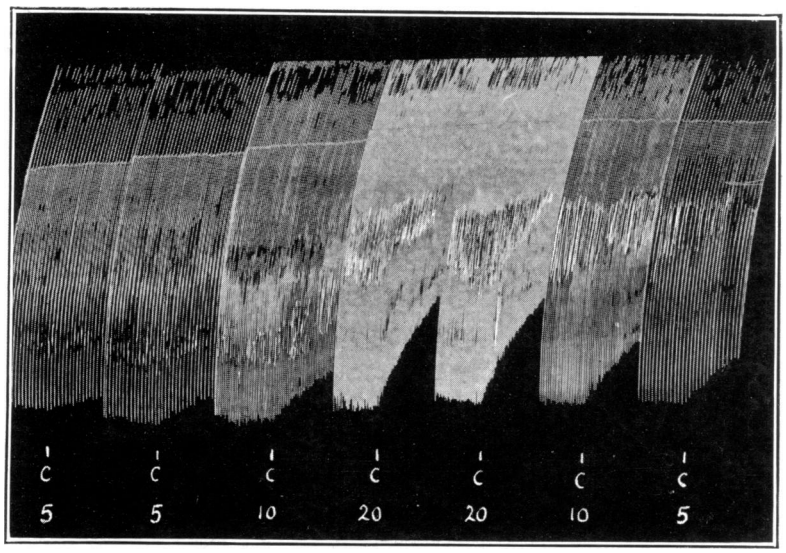

FIG. 1.—Rat. Isolated diaphragm. Record of muscle contractions in response to maximal stimulation of the phrenic nerve ; contractions downwards. Effects of constant doses of 90 μg. *d*-tubocurarine chloride (C) at different rates of stimulation indicated below.

tration of tubocurarine and the percentage inhibition of muscle contractions. In these experiments a diminution in the rate of stimulation from 20 to 5 per min. required, in order to obtain the same effect, that the dose of tubocurarine should be increased 1.2 times. It was also observed that when the stimulation was stopped for 5 min. at the moment of adding tubocurarine, and then restarted for 3 min., the reduction of contractions in this period was less than when the stimulation was continued for 8 min. without interruption. It is evident that the effect produced by tubocurarine is greater when the muscle is working and that it is a function of the rate of stimulation. A variation in the strength of stimuli had no influence on the action of tubocurarine.

The effect of carbon dioxide.—The curare action was less when pure oxygen was used than when the gas mixture consisted of 95 per cent oxygen and 5 per cent carbon dioxide (Fig. 3). The action of tubocurarine in the presence of 5 per cent CO_2 was about 2.5 times that in the presence of pure oxygen. Thus

the inhibition produced by 0.1 mg. *d*-tubocurarine chloride in 100 ml. was about 30.5 per cent with oxygen, but 74 per cent with a mixture of oxygen and CO_2. This increased action of tubocurarine by the gas mixture is most likely due to the action of CO_2, as Jacobs and Stewart (1942) have found that CO_2 catalyses

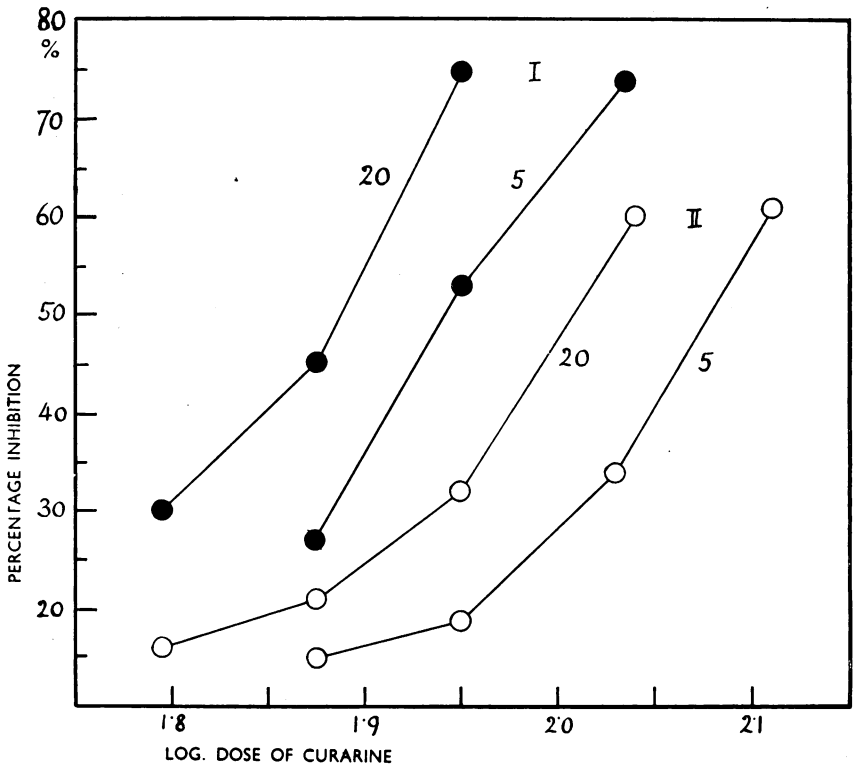

FIG. 2.—Isolated phrenic nerve diaphragm of the rat. Abscissae = logarithm of tubocurarine concentration. Ordinates = percentage inhibition of muscle contractions. The effect of different rates of stimulation (20 and 5 per min.) on the action of tubocurarine is shown in two experiments (I and II).

the diffusion of ions through membranes. The pH of the fluid in the bath, determined by an indicator method, was 8.8 when oxygen was used, and 7.6 when the mixture of oxygen and CO_2 was turned on.

Estimation of unknown samples of tubocurarine.—The quantitative study of a drug such as tubocurarine, which causes cessation of some physiological process which is not well understood, is often fraught with difficulties; this is especially true when the drug is not rapidly destroyed in the tissue and consequently continues to exert its action if it is not removed either by the circulation as *in vivo* or by washing as *in vitro*. However, the isolated rat's nerve diaphragm

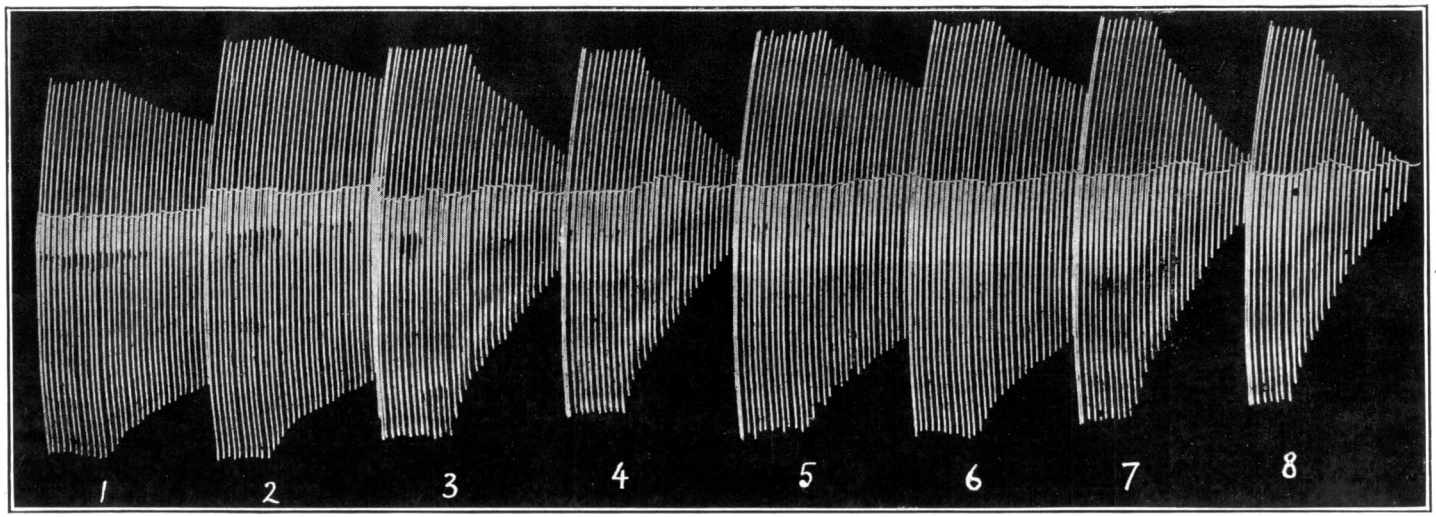

FIG. 3.—Record as Fig. 1. A constant dose of 100 μg. *d*-tubocurarine chloride was added at 1, 2, 5, and 6 during oxygenation with pure O_2; at 3, 4, 7, and 8 during oxygenation with 95 per cent O_2 and 5 per cent CO_2.

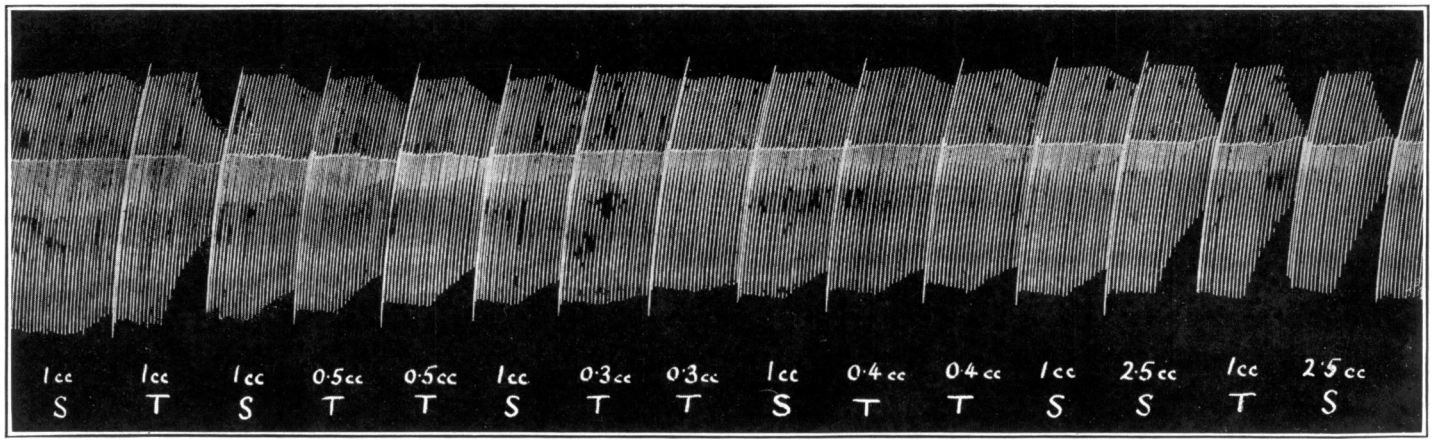

FIG. 4.—Record as Fig. 1. Illustration of the estimation of an unknown tubocurarine solution (T) by comparison with a known solution (S). For detailed description see text.

preparation gives results which are quite reproducible ; in many preparations as many as 36 doses could be tested. The results in Table I show the regularity with which tubocurarine was found to exert its action in five different experiments. In these the rate of stimulation was 5 per min.

TABLE I

Concentration of *d*-tubocurarine chloride μg. per ml.	Percentage inhibition					
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Mean ± S.E.
1·0	14	8	13	15	13	12·6 ± 1·2
1·4	30	19	30	39	32	30·0 ± 3·2
1·8	42	44	54	57	49	49·2 ± 2·5
2·0	62	61	67	70	67	65·4 ± 1 7

In ten experiments an unknown solution of tubocurarine was estimated by comparison with a known solution. The rate of stimulation in the first two experiments was 20 per min., in the other eight it was 5 per min. Each time the effect of equal doses was observed at first and then the effects of the test solution (T) and the standard solution (S) were bracketed as closely as possible. Fig. 4 is an example of the procedure: 1 ml. S was weaker than 1 ml. T ; 0.5 ml. T was stronger, 0.3 ml. T was weaker than 1 ml. S ; 0.4 ml. T

TABLE II

ESTIMATION OF UNKNOWN SOLUTIONS OF *d*-TUBOCURARINE CHLORIDE (T) BY COMPARISON WITH A STANDARD SOLUTION (S) CONTAINING 200 μG. PER ML.

Date	Rate of Stimulation per min.	Effect of equal doses	Bracket effect ml. T>or<ml.S	Geometrical mean of T equivalent to 1 ml. S	Observed value (μg. per ml.)	Actual value (μg. per ml.)	Percentage error
30 Nov. '45	20	T < S	0·77 > 0·45 0·54 < 0·45	1·42	141	150	− 6·0
3 Dec. '45	20	T = S	—	—	200	220	−10·0
2 Sept. '46	5	T < S	1·4 > 1 1·0 < 1	1·18	169·5	166	+ 2·1
3 Sept. '46	5	T > S	0·8 > 1 0·5 < 1	0·63	317	333	− 4·8
4 Sept. '46	5	T < S	1·3 > 1 1·0 < 1	1·14	175·5	180	− 2·5
5 Sept. '46	5	T > S	0·5 > 1 0·3 < 1	0·39	513	500	+ 2·6
5 Sept. '46	5	T < S	1 < 1 1·2 > 1	1·09	183·5	200	− 8·8
6 Sept. '46	5	T > S	0·5 > 1 0·3 < 1	0·39	513	475	+ 8·0
9 Sept. '46	5	T > S	0·5 < 1 0·66> 1	0·57	350	380	− 7·1
10 Sept. '46	5	T > S	0·6 < 1 0·48 > 0·6	0·69	290	270	+ 6·9

was equal to 1 ml. S ; similarly, 1 ml. T was equal to 2.5 ml. S. As S contained 200 μg. curarine per ml. T was estimated to contain 500 μg. ; actually it contained 475 μg. per ml. Not all experiments were so accurate that the arithmetical mean of the bracketing results could be used and it was found that the geometrical mean usually gave the better result. Table II shows the values obtained in all the experiments treated in this way.

Discussion

The use of the rat's phrenic nerve-diaphragm preparation for the estimation of curare-like activity offers several advantages over current methods. It is an isolated mammalian preparation which is sufficiently thin to allow substances to exert their action quickly and also to be washed out readily. It is therefore possible to observe an effect within 3 min. and, if an action has taken place, to observe another effect 10 min. later. As the preparation remains in good condition for many hours and doses can be administered at 13 min. intervals, a large number of estimations can be made in one day.

Summary

A method is described for estimating curare-like activity using the isolated phrenic nerve-diaphragm preparation of the rat. The preparation is easily set up, results are readily reproduced over many hours, and the error of the method is about 6 per cent.

I wish to thank Prof. Burn and Dr. Bülbring for their encouragement and guidance in this work.

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THE RELATIVE ACTIVITY OF PROSTIGMINE HOMOLOGUES AND OTHER SUBSTANCES AS ANTAGONISTS TO TUBOCURARINE

BY

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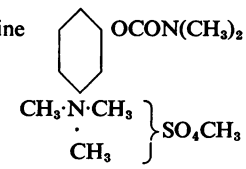
(Received September 24, 1946)

The need for substances which antagonize curare has arisen with the recent introduction of the drug into medicine. There is a serious danger of giving too much and causing respiratory failure. While it is true that this occurrence will usually demand artificial respiration, the length of time for which it must be continued can be greatly reduced by the injection of a suitable antagonist. The substances investigated have been the following:

Prostigmine



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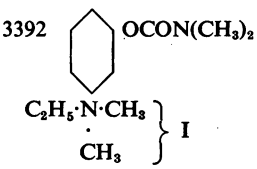

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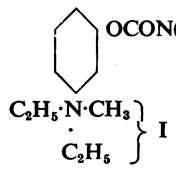
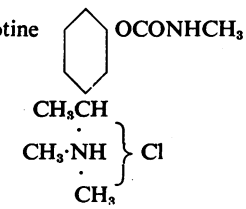
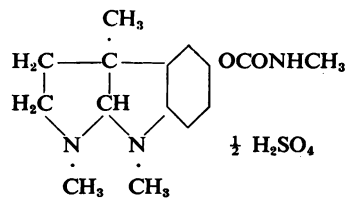


Miotine



Eserine



In addition, guanidine and dimethyl guanidine have been tested, since guanidine is stated by Minot, Dodd, and Riven (1939, 1941) to be a more useful substance for treating myasthenia than prostigmine itself.

The first series of experiments was made on the isolated nerve-muscle preparation of the rat, recently described by one of us (Bülbring, 1946). Experiments

were then performed on the whole animal to see whether the relationships observed in the isolated nerve-muscle preparation held also for muscle in its natural surroundings. Some toxicity tests were made on mice in order to compare the therapeutic indexes of the compounds. Finally, experiments were carried out to determine the relative anticholinesterase action of the prostigmine homologues in the Warburg apparatus.

Experimental Results

1. *The isolated phrenic nerve–diaphragm preparation of the rat*

The method has been described in detail by Bülbring (1946) and also in the preceding paper by Chou. The isolated strip of diaphragm was stimulated through the phrenic nerve by maximal single shocks at a constant rate of 5 per min. The procedure followed was first to determine the percentage inhibition produced by tubocurarine alone within a standard time of 3 min.; the height of the muscle contraction was measured just before tubocurarine was added and also the height of contraction at the end of 3 min., during which tubocurarine was present. The percentage diminution was calculated from these two figures. Between one dose of tubocurarine and the next there was an interval of 15 min. during which the

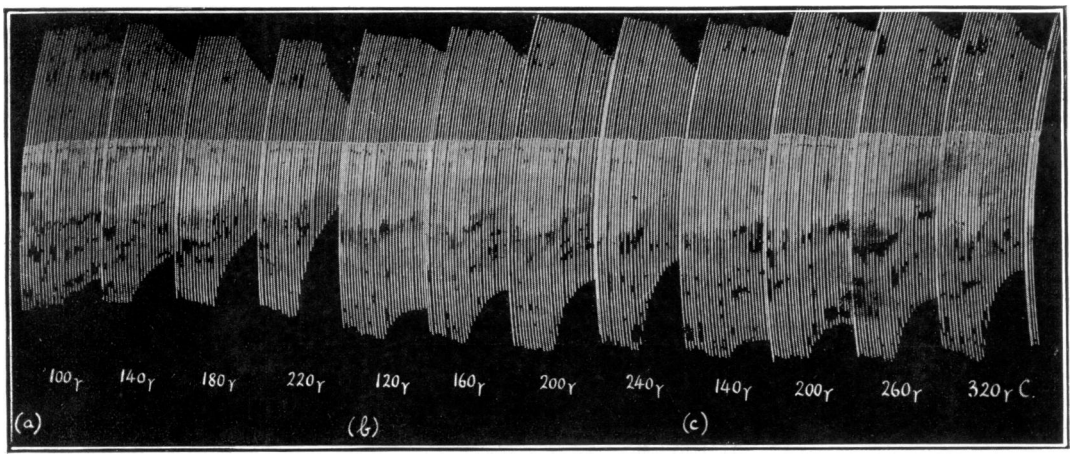


FIG. 1.—Rat. Isolated phrenic nerve–diaphragm preparation. Record of muscle contractions (contractions downwards); rate of stimulation 5 per min. The inhibition caused by four increasing doses of tubocurarine alone (a) is compared with that of four larger doses in the presence of the antagonist 3392, (b) in a concentration of 5×10^{-9}, and (c) in a concentration of 10^{-8}.

fluid in the bath was changed four times as described in the preceding paper for the estimation of tubocurarine. At the beginning of each experiment three or four concentrations of tubocurarine alone were tested. Next the antagonist was added to the bath 1 min. before tubocurarine was added, and the inhibition which the latter produced in 3 min. was determined. An example is given in Fig. 1. The first portion of the record shows the action of four increasing concentrations of tubocurarine alone, while the later portion shows the action of four greater concentrations in the presence of a fixed concentration of the

prostigmine homologue 3392. An attempt was made to match the inhibitions produced by tubocurarine alone with similar inhibitions when both tubocurarine and the antagonist were present together. This attempt was made not only for one concentration of the antagonist but for three or four.

The results were plotted on a graph, as shown in Fig. 2, where the abscissae are the log doses of tubocurarine and the ordinates are the percentage inhibitions. Curve A shows the relation when tubocurarine alone was used; curve B

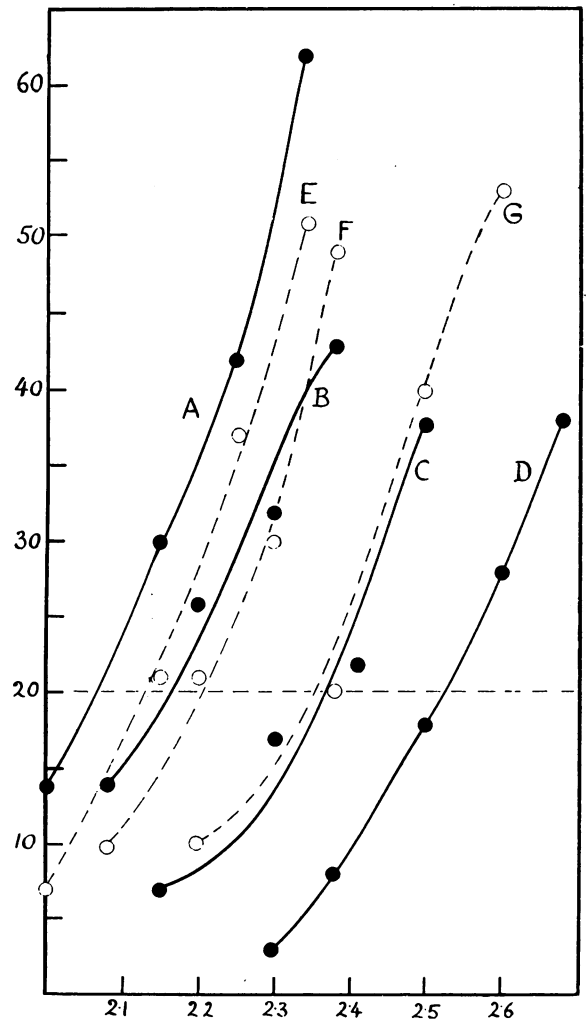


FIG. 2.—Assay of anti-curare activity. Abscissae = log dose of tubocurarine. Ordinates = percentage inhibition. The dose of tubocurarine causing 20 per cent inhibition can be estimated for tubocurarine alone (A). It has to be increased with increasing concentrations of antagonists—i.e., $B = 5 \times 10^{-9}$ 3392, $C = 10^{-8}$ 3392, $D = 3 \times 10^{-6}$ 3392. E, F, and G represent results with corresponding concentrations of prostigmine, which is shown to be weaker than 3392.

TABLE I

COMPARISON OF TUBOCURARINE ANTAGONISTS

Date	Concentration of antagonist	Dose of tubocurarine (μg.) causing 20 per cent inhibition in the presence of		Ratio
		Eserine	Prostigmine	
7 & 10 Dec.	10^{-8}	126	237	0·53
1 & 2 Jan.	10^{-8}	100	114	0·88
9 Jan.	10^{-8}	108	95	1·14
	2×10^{-8}	—	108	
	5×10^{-8}	197	—	
10 Jan.	5×10^{-9}	69	78	0·89
	10^{-8}	91	106	0·86
	3×10^{-8}	124	143	0·87
11 Jan.	5×10^{-9}	96	89	1·08
	10^{-8}	84	130	0·65
	2×10^{-8}	93	132	0·70
			Mean ± S.E.	0·85 ± 0·06
		Miotine	Prostigmine	
19 Feb.	5×10^{-9}	120	117	1·03
	10^{-8}	134	128	1·05
7 March	5×10^{-9}	125	97	1·29
	10^{-8}	199	151	1·32
	3×10^{-8}	215	160	1·35
			Mean ± S.E.	1·21 ± 0·07
		3392	Prostigmine	
26 Feb.	5×10^{-9}	140	134	1·05
	10^{-8}	228	159	1·54
	3×10^{-8}	326	223	1·46
6 March	5×10^{-9}	143	127	1·13
	10^{-8}	226	141	1·61
	3×10^{-8}	393	200	1·97
			Mean ± S.E.	1·46 ± 0·13
		3393	Prostigmine	
7 Feb.	5×10^{-9}	178	145	1·22
	10^{-8}	224	131	1·71
	3×10^{-8}	305	184	1·66
8 Feb.	5×10^{-9}	320	182	1·76
	10^{-8}	—	221	—
	3×10^{-8}	—	321	—
11 Feb.	5×10^{-9}	192	158	1·22
	10^{-8}	312	173	1·86
	3×10^{-8}	416	251	1·66
5 March	5×10^{-9}	257	149	1·74
	10^{-8}	319	162	1·97
	3×10^{-8}	398	184	2·16
12 March	2×10^{-9}	143	—	
	5×10^{-9}	—	127	
	10^{-8}	—	162	
			Mean ± S.E.	1·70 ± 0·09

shows the relation in the presence of 3392 in a concentration of 5×10^{-9} ; curve C shows the relation when the concentration of 3392 was 10^{-8} ; and finally curve D shows the relation when the concentration was 3×10^{-8}.

To make the comparison between one antagonist and another, for example between 3392 and prostigmine, further observations were then made on the same preparation, in which the effect of different concentrations of tubocurarine was observed in the presence of each of a series of concentrations of prostigmine. Results obtained in this way are shown in Fig. 2 as curves E, F, and G. It was found better to arrive at the relative potency of two antagonists by considering only results obtained on the same preparation rather than to compare the effect of one antagonist in one preparation with that of another in a second preparation.

Quantitative expression of results.—From the graph shown in Fig. 2 it was possible to determine the concentration of tubocurarine which caused 20 per cent inhibition in the presence of each of the different concentrations of antagonist.

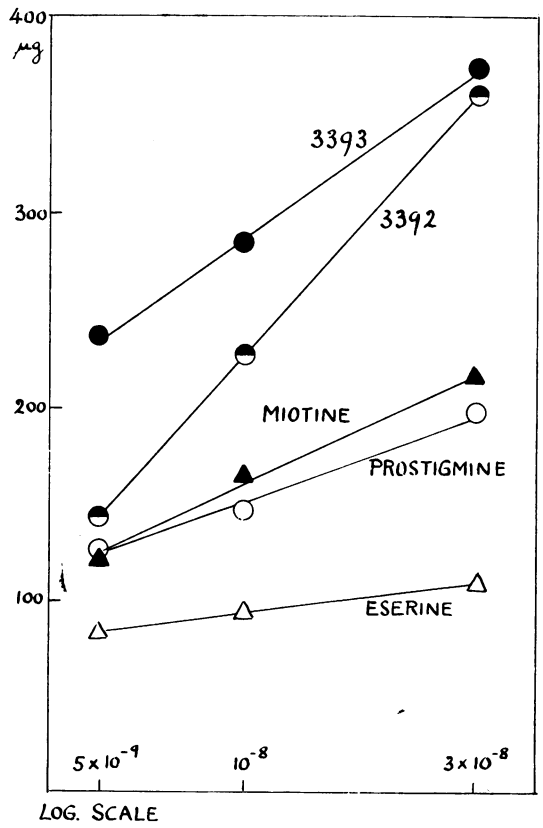


FIG. 3.—The relation between the concentration of antagonist and the dose of tubocurarine required to produce 20 per cent inhibition. Abscissae = log concentration of antagonist. Ordinates = dose of tubocurarine causing 20 per cent inhibition (100 ml. bath).

An expression for the anti-curarine activity of the substances investigated was then obtained, using prostigmine as a standard. The results in Table I represent the experiments in which the different substances were compared.

The ratio of the amount of tubocurarine required to reduce the muscle response by 20 per cent varied little if it was calculated in the presence of the same concentration of each antagonist. If, however, an attempt was made to bracket the effective dose of curarine in the presence of one concentration of an antagonist between two doses in the presence of different concentrations of a second antagonist, the results obtained varied widely. The reason for this difficulty is obvious from Fig. 3, in which all the results obtained were combined; the graph shows that the anti-curare activity of the different substances is raised by different degrees as their concentration is increased. It rises more steeply for 3392 than for prostigmine and less steeply for eserine: thus an increase in the concentration of the antagonist from 5×10^{-9} to 3×10^{-8} requires 57 per cent increase of the dose of tubocurarine in the presence of prostigmine; in the presence of eserine only 32 per cent increase is required, while in the presence of 3392 the dose of tubocurarine has to be increased by 155 per cent.

The general results obtained are set out in Table II, in which prostigmine is given the value 1; the ratios are derived from the data given in Table I.

TABLE II

ANTI-CURARE ACTIVITY RELATIVE TO PROSTIGMINE = 1

Eserine 0·85	3392 1·46
Miotine 1·21	3393 1·70

The interesting result was thus obtained that substitution of the methyl groups attached to the N atom of the basic radical in the prostigmine molecule, first by one ethyl group and then by a second, led to an increase in anti-curare action.

Another comparison was made between eserine, prostigmine, miotine, and 3393, in which tubocurarine and its antagonist were added to the bath in doses which were the same throughout, viz., 0.4 mg. tubocurarine chloride and 1 μg. of the antagonist (100 ml. bath). The degree of inhibition of the muscle contraction was determined after 3 min. exposure to tubocurarine, when tubocurarine and antagonist were added simultaneously, and also when the antagonist was added 3 min., 6 min., and 12 min. before the tubocurarine. The results obtained are shown in Fig. 4, from which it will be seen that there was little difference between eserine and prostigmine; these, however, were weaker than miotine and 3392, which were also similar in activity to one another. Miotine and 3392 were in turn definitely weaker than 3393, which was easily the strongest substance investigated.

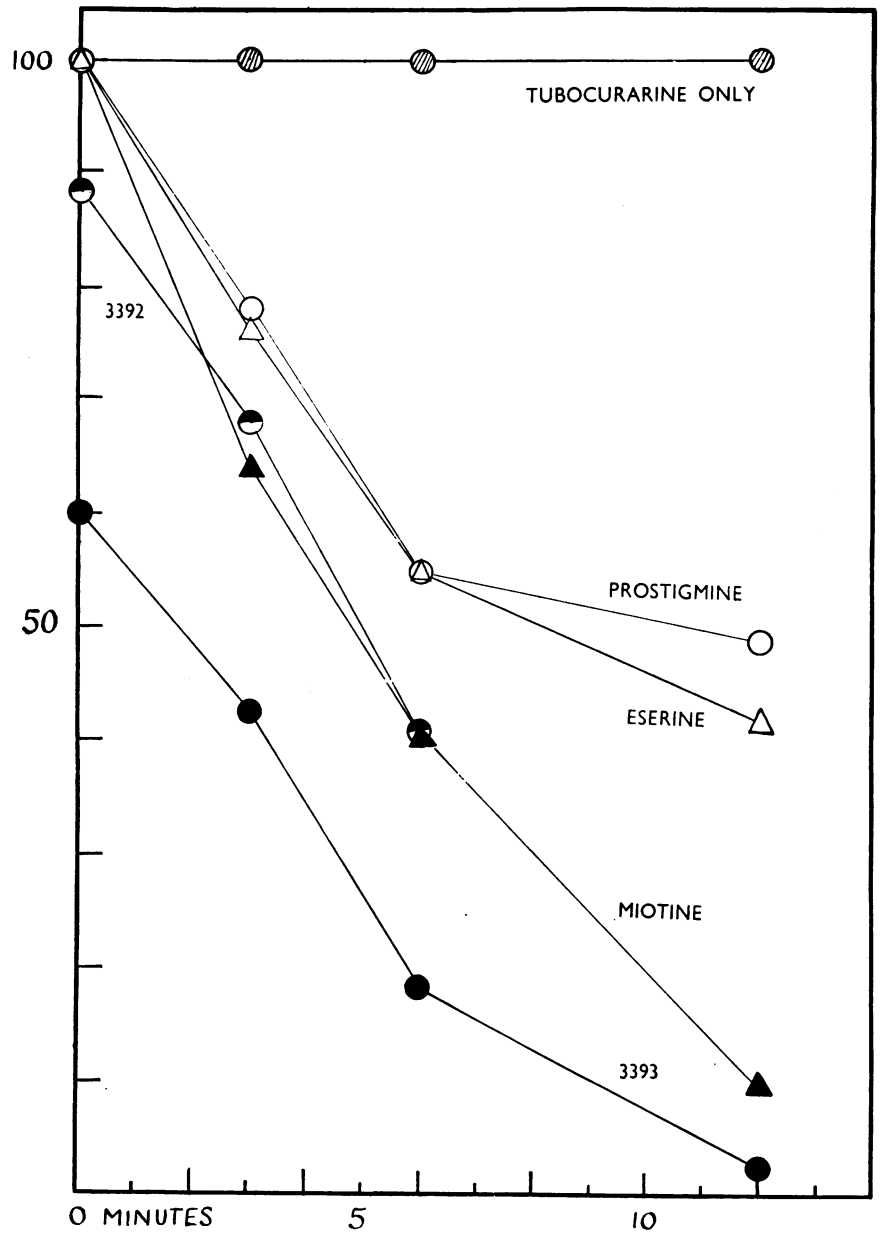


FIG. 4.—The relation between time of exposure and action of tubocurarine antagonists. Dose of tubocurarine was 400 μg. (100 ml. bath); concentration of antagonist was 10^{-8} throughout. Abscissae = time in minutes between the addition of antagonist and the subsequent addition of tubocurarine. Ordinates = inhibition relative to that of tubocurarine alone as 100. When tubocurarine and antagonist were added simultaneously (at 0 min.), eserine, miotine, and prostigmine had no effect. When prostigmine or eserine were allowed to act for 12 min. beforehand the tubocurarine effect was halved; but after 12 min. exposure to 3393 it was almost abolished.

Combination of 3393 and adrenaline.—Bülbring (1946) has shown that when the addition of prostigmine to the isolated diaphragm results in greater contractions, the subsequent addition of adrenaline causes a further augmentation. We have compared the effect of adding 0.25 μg. 3393, together with 50 μg. adrenaline, with the effect of the same dose of 3393 alone. In each comparison

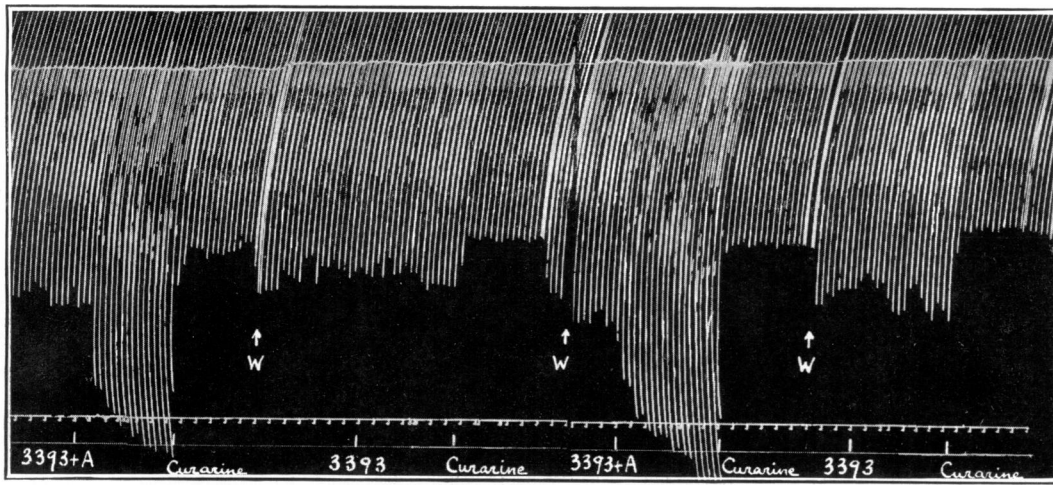


FIG. 5.—Record as Fig. 1. Comparison of anti-curare activity of 3393 alone with that of 3393 combined with adrenaline. Though contractions were greater when 3393 was given together with adrenaline, the final curarine inhibition remained unaltered.

the presence of adrenaline augmented the contractions, as shown in Fig. 5. The adrenaline, however, failed to modify the effect of curarine added 6 min. later, so that the final result was the same whether adrenaline was present or not.

TABLE III

EFFECT OF GUANIDINE ON MUSCULAR INHIBITION BEFORE AND AFTER TUBOCURARINE (PHRENIC NERVE–DIAPHRAGM PREPARATION OF THE RAT)

Date	Dose of guanidine	Time added before or after tubocurarine	Dose of tubocurarine μg.	Percentage inhibition
Feb. 15	Nil	—	80	47
	1 mg.	5′ before	80	37
	1 mg.	5′ after	80	41
	Nil	—	80	71
Jan. 22	Nil	—	120	37
	1 mg.	with tubocurarine	120	36
	1 mg.	5′ before	120	18
	1 mg.	10′ before	120	12
	1 mg.	20′ before	120	12

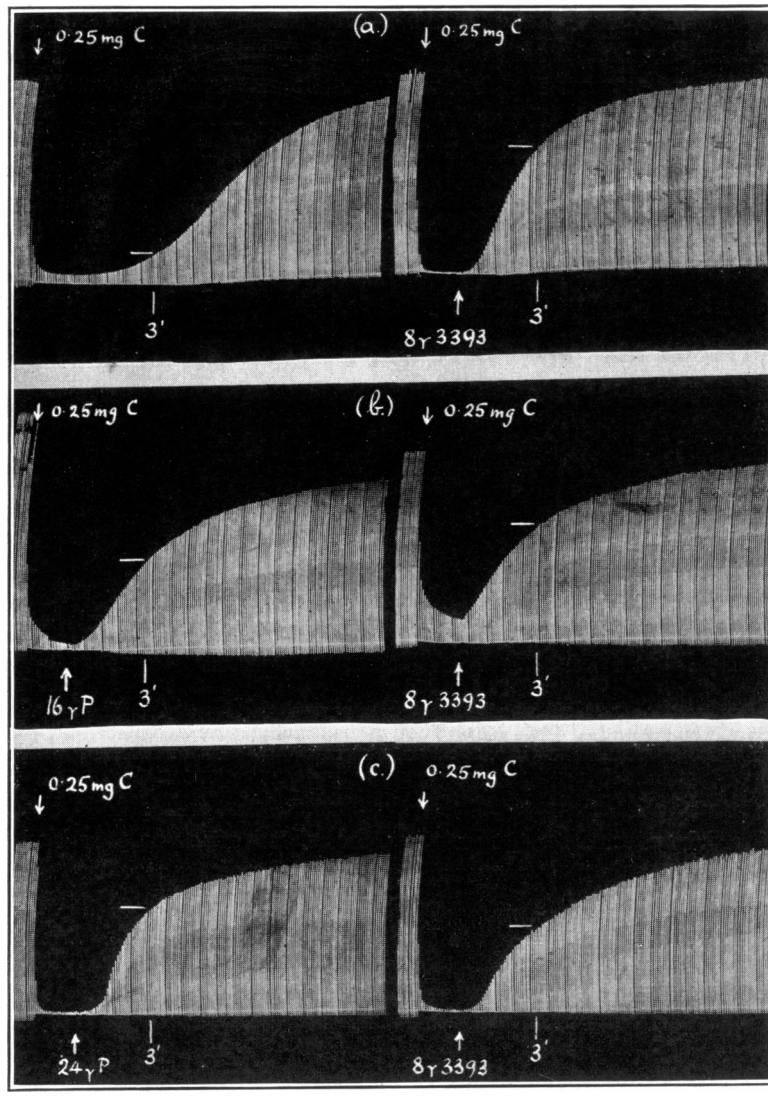


FIG. 6.—Cat. Sciatic gastrocnemius (contractions upwards). Rate of stimulation 12 per min. (a) Recovery from the effect of 0.25 mg. tubocurarine compared with that after 8 μg. 3393. (b) The same after 16 μg. prostigmine and 8 μg. 3393. (c) After 24 μg. prostigmine and 8 μg. 3393.

Guanidine and dimethylguanidine.—Guanidine is known to exert an action on skeletal muscle, producing fibrillary twitching in low concentration and muscular paralysis in high concentration (Meighan, 1917). It has been used for patients suffering from myasthenia gravis, and some observers record consider-

able improvement (Dodd, Riven, and Minot, 1939, 1941). The present experiments show that the decurarizing action of guanidine on the rat's nerve-muscle preparation is very weak. Concentrations below 1 μg. per ml. have no action. When the concentration is increased to 10 μg. per ml. a feeble decurarizing activity is shown within a period of 9 min. Even at this concentration, the action is uncertain (see Table III). However, a greater effect was obtained by prolonging the time before adding curarine. Thus when 1 mg. guanidine was added to the bath 10 min. before adding 120 μg. curarine, the inhibition produced was diminished from 36 to 12 per cent. When larger doses of curarine (e.g., 0.4 mg.) were used, guanidine had no effect. Dimethylguanidine, which also produces fibrillary twitching, was similarly tested. Its effect was feebler and less definite than that of guanidine.

2. *Observations on the cat*

Experiments were carried out comparing the anti-curare activity of the different substances in the whole animal. For this purpose decerebrate cats were used. The left hindleg was prepared so as to record the contraction of the gastrocnemius muscle when the sciatic nerve was stimulated. The details of the preparation were as given by Bülbring and Burn (1941); injections were made into the bifurcation of the aorta through a cannula tied into the right external iliac artery, and the tension developed in the gastrocnemius was recorded; the resting tension was about 1 kg. The sciatic nerve was stimulated by condenser discharges from a neon-lamp circuit at a constant rate, which was 8 per min. in some experiments and 20 per min. in others. The dose of tubocurarine was chosen to produce a paralysis lasting about 20 min. before recovery was complete; this varied in different experiments from 0.25–0.75 mg. In testing the action of prostigmine or of its homologues, the dose was injected at a precise interval, usually 1 min. after the injection of the tubocurarine. An example of the effect of tubocurarine alone and of tubocurarine followed by 3393 is given in Fig. 6 (a). No close comparison between the different substances was found possible by this method, but in a series of experiments an approximate relation was established by determining the percentage recovery at a given time (from 3–12 min. in different experiments) after the injection of the tubocurarine antagonist. Thus in Fig. 6 (b) it was shown that 3 min. after 16 μg. prostigmine the muscle contractions had recovered 38 per cent of their height compared with 56 per cent recovery 3 min. after 8 μg. 3393. Therefore 16 μg. prostigmine was weaker than 8 μg. 3393. Similarly in Fig. 6 (c) 24 μg. prostigmine was stronger than 8 μg. 3393.

The final result obtained from a comparison of the two substances on five cats is illustrated in Table IV. In the first experiment it was demonstrated that prostigmine was clearly weaker than 3393 when the two were injected in equal doses. In the second experiment it was shown that prostigmine had, however, more than one-third of the action of 3393. Finally, in each of three experiments prostigmine was found to have approximately 50 per cent of the activity of 3393. These results agree with those obtained on the rat's diaphragm. However, when prostigmine was compared with 3392 in the whole animal, no difference was observed between them. It should be pointed out that in each experiment several comparisons of the selected doses of the two tubocurarine antagonists were made.

TABLE IV

RECOVERY OF MUSCLE CONTRACTIONS IN THE CURARIZED SCIATIC-GASTROCNEMIUS PREPARATION OF THE CAT AFTER PROSTIGMINE AND 3393. (PR. = PROSTIGMINE)

Date	Time of injection	(1) Tubocurarine mg.	(2) Antagonist and dose (μg.)	Interval between injections (1) & (2) min.	Interval between injection (1) and measurement min.	Percentage recovery	Result
12.2.46	2.45	0·30	— —	—	4	5	
	4.05	,,	3393 4	1	4	64	
	4.45	,,	Pr. 4	1	4	11	3393 > Pr.
	5.24	,,	Pr. 20	1	4	58	
	5.54	,,	3393 20	1	4	74	
8.3.46	3.10	0·75	3393 4	8	12	68	
	3.41	,,	Pr. 12	8	12	76	
	4.10	,,	3393 4	8	12	36	3393 < 3 × Pr.
	4.42	,,	3393 4	8	12	28	
	5.13	,,	Pr. 12	8	12	90	
29.3.46	12.40	0·25	Pr. 16	1	11	10	
	1.07	,,	3393 8	1	11	10	3393 = 2 × Pr.
1.4.46	11.58	0·25	— —	—	3	14	
	12.28	,,	3393 8	1	3	61	
	12.58	,,	Pr. 16	1	3	38	
	1.28	,,	3393 8	1	3	56	3393 = 2 × Pr.
	2.10	,,	Pr. 24	1	3	60	
	2.40	,,	3393 8	1	3	45	
	3.20	,,	Pr. 16	1	3	68	
					Interval between injection (1) and reappearance of contractions		
27.3.46	12.29	0·75	3393 4	1	3 min. 40 sec.		
	12.59	,,	— —	—	4 ,, 10 ,,		
	1.29	,,	Pr. 8	1	3 ,, 40 ,,		
	2.10	,,	3393 8	1	3 ,, 15 ,,		3393 = 2 × Pr.
	2.46	,,	Pr. 16	1	3 ,, 25 ,,		
	3.24	,,	3393 8	1	3 ,, 15 ,,		
	3.48	,,	3393 16	1	2 ,, 25 ,,		
	4.06	,,	Pr. 32	1	2 ,, 55 ,,		

3. *Observations on toxicity*

It remained to compare the toxicity of these substances in order to discover whether the ratio of the toxic dose to the active dose was greater for 3393 than for prostigmine.

White mice were used. Different doses of prostigmine and 3393, calculated per 20 g. mouse, were injected into the tail vein. The LD50 of 3393 was 1.2 μg. per 20 g. mouse, that of prostigmine was 3.2 μg. per 20 g. mouse. When atropine

(40 μg. per 20 g. mouse) was given intraperitoneally 1 hour beforehand, the toxicity of both substances was reduced to less than half. For 3393 the LD50 was 2.7 μg. per 20 g. mouse, for prostigmine it was 7.9 μg. per 20 g. mouse. Thus the ratio without atropine was 2.7 and in the presence of atropine 2.9. As the anti-curare activity of 3393 compares with that of prostigmine as 2:1, and the toxicity as 2.7:1 and in the presence of atropine as 2.9:1, the therapeutic index of 3393 is slightly less favourable than that of prostigmine.

4. *The inhibition of cholinesterases by prostigmine and related substances*

Prostigmine and similar substances are inhibitors of cholinesterases, but it is still under discussion whether or not their anti-curare effect is fully explained by their affinity to cholinesterase. One of us (Chou) has therefore compared the inhibitory action of prostigmine, miotine, and the two homologues of prostigmine on the enzymic hydrolysis of acetylcholine. Schweitzer, Stedman, and Wright (1939) have compared the inhibitory action of prostigmine and 3393 on the enzymic hydrolysis of butyrylcholine by horse serum; they found a stronger inhibition with 3393. Horse serum, according to Mendel, Mundell, and Rudney (1943), contains chiefly a non-specific cholinesterase, and we have therefore examined preparations from three different sources, viz. (1) human plasma, (2) human red cell haemolysate, and (3) suspensions from the dog's caudate nucleus. Human plasma, like horse serum, contains a non-specific esterase, but red cells and brain suspensions contain a specific cholinesterase.

The rate of hydrolysis was followed manometrically using Warburg's method. The gas mixture was 95 per cent N_2 and 5 per cent CO_2 and the total reaction volume was made up to 3 ml. The physiological solution employed was Krebs's bicarbonate-Ringer. The experiments were carried out at a temperature of 38° C. In all experiments inhibitor and enzyme were filled into the main compartment of the conical manometric flasks; the reaction was started by tipping the acetylcholine from the side bulb. The amount of carbon dioxide liberated from the 3rd to the 33rd min. after tipping was used for calculating the percentage inhibition. In all experiments the concentration of acetylcholine bromide used as substrate was 6×10^{-3}M.

(a) Experiments on human plasma.—The hydrolysis of acetylcholine was strongly inhibited by all three members of the prostigmine series (Table V) with

TABLE V

THE PERCENTAGE INHIBITION OF CHOLINESTERASE IN HUMAN PLASMA BY THE PROSTIGMINE SERIES

Inhibitor	Percentage inhibitions at				
	10^{-6}M		10^{-7}M		
Prostigmine	89	90	54	53	40
3392	—	94	—	—	59
3393	—	96	81	—	80

inhibitor concentrations of 10^{-6}M and 10^{-7}M. The percentage inhibitions were in the order: Prostigmine <3392 <3393.

(b) Experiments on human red cell haemolysate.—The red cell esterases are known to be closely related to the enzyme of nervous tissue. In our experiments the inhibitions were less than with the plasma esterase ; this may be explained by the high affinity of the red-cell enzyme to acetylcholine.

Prostigmine and its homologues were examined in a molar concentration of 10^{-6} and the percentage inhibitions were: prostigmine, 70 ; 3392, 82 ; and 3393,

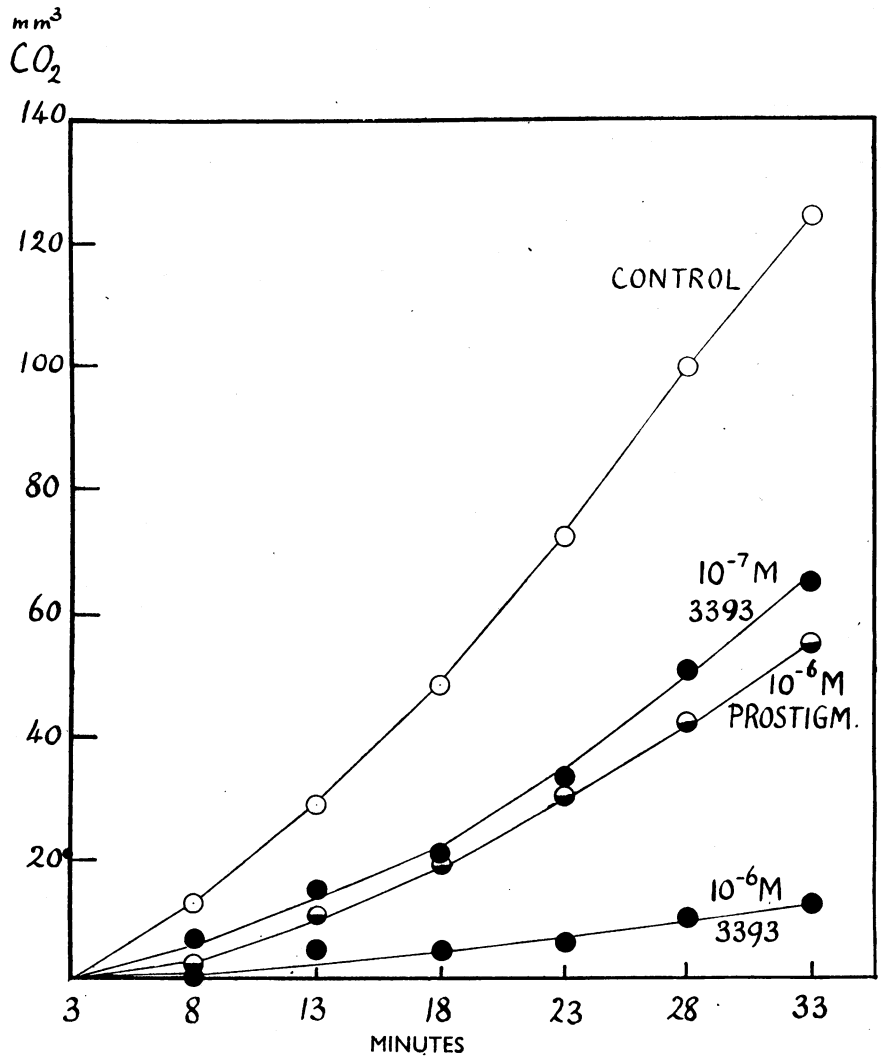


FIG. 7.—Inhibition of cholinesterase in human red corpuscles haemolysate. Abscissae = time in minutes. Ordinates = mm.^{3} CO_2.

94 per cent. Thus, as for the plasma enzyme, the order of inhibitory activity was: prostigmine <3392 <3393. In order to obtain a more quantitative estimate of the relative inhibitor properties of prostigmine and 3393, the experiment shown in Fig. 7 was carried out. It shows that the inhibition of the red-cell enzyme caused by 10^{-7}M.3393 was only slightly less than that caused by a tenfold molar concentration of prostigmine.

We have also compared the inhibition of the red-cell enzyme by miotine and by prostigmine in two experiments, using the inhibitors at a molar concentration of 10^{-6}. Prostigmine was slightly more effective than miotine.

(c) Experiments with suspension of dog's brain.—The results obtained with a suspension of the caudate nucleus of the dog confirm earlier observations on the high cholinesterase activity of this tissue. In our experiments 10 mg. of brain tissue (fresh weight) were used in each flask. Our results with the three members of the prostigmine series are closely similar to those obtained with human red cells. In 10^{-6}M concentration, the percentage inhibitions were: prostigmine, 60; 3392, 78; and 3393, 91 per cent.

Discussion

It is not known whether the anti-curare activity of substances which inhibit cholinesterase is solely due to this property or whether another mechanism is involved. In the experiments described in this paper it was found that substitution in the prostigmine molecule of one or two of the methyl groups attached to the N atom of the basic radical by ethyl groups led to an increase in anti-curare activity; similarly, this change led to an increase in anti-cholinesterase activity. Thus the order of potency was found to be prostigmine <3392 <3393. But while the substitution of two methyl groups in the prostigmine molecule by two ethyl groups doubled the anti-curare activity, it caused a greater increase in the inhibition of cholinesterase. When miotine, which does not resemble prostigmine so closely, was tested, it was found to have the same anti-curare activity as prostigmine, but to be definitely weaker in inhibiting cholinesterase. In the prostigmine homologues the change in anti-curare action and anti-cholinesterase action is in the same direction, though not parallel. It remains doubtful whether a general parallelism exists between the two properties for substances other than those of closely related chemical structure.

Summary

1. The activity of prostigmine homologues, eserine, and miotine as antagonists to tubocurarine was estimated on an isolated muscle preparation and in the whole animal.

2. The isolated phrenic nerve–diaphragm preparation of the rat was found to be a quick method for comparing large numbers of compounds. The results

obtained with this method were in good agreement with those obtained *in vivo* on the cat's sciatic gastrocnemius.

3. The activity of prostigmine homologues as inhibitors of cholinesterase increases in the same direction as their anti-curare activity. It is doubtful whether a similar parallelism holds for molecules of different structure.

We wish to express our thanks to Dr. H. Blaschko for his great help and advice with the manometric experiments.

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EFFECT OF STREPTOMYCIN ON EXPERIMENTAL TUBERCULOSIS IN GUINEA-PIGS

BY

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There have been several favourable reports from U.S.A. on the efficacy of streptomycin as a chemotherapeutic agent, with particular reference to tuberculosis (e.g., Feldman and Hinshaw, 1944; Feldman, Hinshaw, and Mann, 1945; Youmans and McCarter, 1945).

The present paper reports investigations carried out during the past year with a strain of *Mycobacterium tuberculosis* (human), the results of which confirm the observation that streptomycin exerts a marked suppressive action on guinea-pig tuberculosis.

EXPERIMENTAL

Strain.—A virulent strain of *M. tuberculosis* (human), H.418, supplied originally by Dr. Ungar of Messrs. Glaxo, Ltd., was employed for all tests. When a three-week growth from a Löwenstein slope was used, 0.0001 mg. of this strain consistently gave rise to a progressive and fatal disease within 6 months.

Animals.—Guinea-pigs from the laboratory-bred stock and weighing approximately 500 g. were used. All animals received an ample diet, including green vegetables daily, and cod liver oil twice weekly.

Streptomycin.—For the supplies of streptomycin hydrochloride I am indebted to Mr. M. Lumb, M.Sc., of this Division, who prepared the culture filtrates, and to Drs. Short, Peak, Coppock, and Falconer, who supervised the extraction and preparation of the solid. The material used was, in general, of a potency ranging from 89 to 450 u./mg. For injection, the solid material was dissolved in sterile saline or Ringer's solution.

In vitro *Tests*

When the floating pellicle test with Douglas's modification of Long's medium was used, strain H.418 was inhibited completely at 10 u./ml. and there was some inhibition at 1 u./ml. When Youmans's (1944) submerged culture test and the above medium, with 10 per cent ox serum added, were used, the effects varied with the size of the inoculum, as has been noted with other substances. Table I gives typical results of this test, and it will be seen that 6.5 u./ml. inhibited the growth of a small inoculum, of which the control grew well, whilst quite large amounts of streptomycin had little or no effect on much larger inocula.

Tuberculostatic action of the blood of treated guinea-pigs

It was readily shown by the Brownlee test (1945) that the heart blood of treated guinea-pigs inhibited the growth of tubercle bacilli. For this test the blood was mixed with the modified Long's agar medium, and sowed with strain H.418. The blood taken up to three hours after the subcutaneous injection of 1,500 units of streptomycin showed this inhibition of growth of the organisms.

TABLE I

SUBMERGED GROWTH OF *M. TUBERCULOSIS* IN PRESENCE OF STREPTOMYCIN (2 WEEKS)

Concentration of streptomycin	Inoculum size per 10 ml.					
	0·5 mg.		0·05 mg.		0·005 mg.	
Nil (control)	+++	++	++	++	++	++
6·5 u./ml.	+++	+++	++	++	—	—
65 u./ml.	+++	+++	+	+	—	—
650 u./ml.	+++	+++	++	+	—	—

In vivo *Tests*

Test I.—In a preliminary test a daily dosage of 2,000 u./pig/day was given to six guinea-pigs for 8 weeks, 500 units being given subcutaneously four times daily, at 3-hourly intervals, commencing 4 days after the intraperitoneal injection of 0.0005 mg. strain H.418. None of the six controls or the six treated animals died during the 8 weeks of the experiment, but on necropsy the results were considered promising, especially in view of the relatively small dosage. All the guinea-pigs showed macroscopic evidence of the disease: the control animals had very extensive tuberculosis of the spleen, liver, and lungs, and a large lesion at the site of injection; in contrast, none of the treated guinea-pigs had a local lesion and only one had more than scattered tubercles in the spleen and lungs. Spleen cultures of all animals, both treated and controls, were positive.

Test II.—In view of the promising results of Test I and the availability of more streptomycin, a second test was made, using a much larger dose of the drug—i.e., 10,000 u./day/pig. All animals received an intramuscular infecting dose of 0.0001 mg. of strain H.418. Thirty-six guinea-pigs were divided into three groups:

Group I.—12 controls.

Group II.—12 treated for 14 weeks from date of infection.

Group III.—12 treated for 11 weeks, starting 24 days after infection (when positive with 0.05 ml. of 1/100 tuberculin).

Groups II and III received four subcutaneous doses of 2,500 units streptomycin every day at 8.30 a.m., 11.30 a.m., 2.30 p.m., and 5.30 p.m. The animals were weighed fortnightly until the first part of the experiment was terminated after 14 weeks. At this stage nine animals of each group were killed, the remaining three being left without further treatment in order to ascertain the consequences of discontinuing administration of the drug.

Before being killed, each animal was again tuberculin-tested. After necropsy cultures were made from the spleens and portions were injected into healthy guinea-pigs. Sections of spleen, lung, and liver were preserved for histological examination.

An approximate numerical assessment of the macroscopic evidence of disease was carried out by the method of Sher and Kloeck (1946), each organ having the following maximum value:

Lymph nodes	8	Liver	28
Spleen	24	Lungs	40

RESULTS

As can be seen from Table II, the results were encouraging. Six of the nine guinea-pigs treated from the date of infection and two of the eight which did not receive treatment until 24 days after infection showed no macroscopic evidence

of tuberculosis ; the other treated animals showed only slight glandular involvement. All the control animals showed widespread macroscopic tuberculosis which would obviously have been fatal in a few weeks ; spleen, glands, liver, and lungs were involved and the group assessment was 33 (average of the 9 pigs), against 0.6 for Group II and 2.0 for Group III. Before the animals were killed, some of the tuberculin tests on the treated guinea-pigs were doubtful, whereas all those made on control animals were strongly positive.

TABLE II

THE INFLUENCE OF STREPTOMYCIN IN GUINEA-PIG TUBERCULOSIS

Group	Treatment	Length of infection	Deaths	T.B. assessment	
				Individual animals	Group average
I. Controls					
9	Nil	14 weeks	0/9	12, 22, 38, 55, 33, 39, 37, 45, 16	33
3	Nil	22 weeks	1/3	58, 78, 85	74
II. Treated from date of infection					
9	10,000 u./day for 14 weeks	14 weeks	0/9	0, 0, 0, 0, 2, 2, 2, 0, 0	0·6
3	10,000 u./day for 14 weeks, then nil for 8 weeks	22 weeks	0/3	24, 8, 26	19
III. Treated when tuberculin positive (24 days after infection)					
9	10,000 u./day for 11 weeks	14 weeks	*1/9	2, 2, 0, 0, 2, 4, 4, 4	2·2
3	10,000 u./day for 11 weeks, then nil for 8 weeks	22 weeks	0/3	49, 49, 39	44

* This death (premature) was not due to T.B.

Spleen cultures and injection of spleens into normal guinea-pigs.—All spleens were cultured on Löwenstein medium. Six out of nine of the control samples gave a positive response, whilst of the three negative ones two were macroscopically infected. In both Group II and Group III two spleens out of nine gave a positive result.

Half of each spleen of eleven of the treated guinea-pigs (six from Group II, five from Group III) was ground with sand and Ringer's solution, and the supernatant fluid from each preparation was injected into a normal animal. The two controls showing the least degree of infection were also tested in this way. In all cases, except one of Group III, the guinea-pigs developed widespread tuberculosis in two months.

Development of tuberculosis on cessation of streptomycin treatment.—Three guinea-pigs from each group were left on test after cessation of streptomycin treatment in order to ascertain whether the disease would flare up. After two months, one control had died, and all other pigs were tuberculin positive and showed at necropsy macroscopic tuberculosis of spleen, lungs, and glands. As would be expected, the disease was far more extensive in the controls than in the treated pigs, but even the latter showed marked glandular and spleen involvement. Assessments are given in Table II.

Toxicity.—At the beginning of the experiment streptomycin made the animals' skins very sensitive and there was considerable irritation. This effect was not observed after several weeks, and may have been due to the impurities present in the early batches used. At necropsy there were no signs of any toxic effect and all pigs appeared healthy throughout the experiment and were gaining in weight.

Discussion

The results, although of necessity limited to small numbers of animals owing to the small amounts of streptomycin available, confirm Feldman's work (1944, 1945) regarding the marked suppressive effect of the drug on experimental tuberculosis of guinea-pigs. Under our experimental conditions, 10,000 u./day were effective, although the results of spleen culture and the flare-up tests showed that virulent and viable bacilli were still present after 14 weeks' treatment. During treatment the disease did not progress beyond minor involvement of glands and spleen, and in several pigs it was macroscopically absent. The presumption is that if the experiment had been continued longer these pigs would not have developed the disease so long as treatment was maintained, whereas the controls were obviously succumbing to generalized tuberculosis. Feldman's failure to eliminate tubercle bacilli from the body has also been confirmed by these experiments. It is obviously important to discover whether or not more extensive treatment will cure the disease entirely. This and other related studies are now in hand in these laboratories.

Summary

1. The marked suppressive effect of streptomycin on experimental tuberculosis of guinea-pigs has been confirmed.

2. Under our conditions the disease was not entirely eliminated from the treated animals.

The author wishes to thank Sir Jack Drummond, F.R.S., and Mr. C. E. Coulthard for their interest in this work, and Miss B. Bailey, B.Sc., for her assistance in the *in vivo* work.

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THE RELATIONSHIP BETWEEN SURVIVAL TIME AND DOSAGE WITH CERTAIN TOXIC AGENTS

BY

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In toxicity experiments with chemical warfare agents it was frequently noticed that animals which had received the largest doses died more quickly than those receiving smaller doses. The investigations described here were carried out in order to find whether a quantitative relationship existed between dosage and survival time and, if so, how best this relationship could be expressed and used. It has not been possible for the present authors to pursue this investigation very far, but in view of the interesting results so far obtained it is hoped that others may extend this study to other substances.

The substances used in our investigations were mustard gas, $S(CH_2.CH_2Cl)_2$, and phosgene gas, $COCl_2$. When breathed by animals these substances exert their effect in quite different ways: animals dying after exposure to phosgene consistently showed, at autopsy, a picture of pulmonary oedema; those dying after exposure to mustard gas vapour, however, presented a more varied pathology, pseudo-membranous tracheitis, pulmonary oedema, broncho-pneumonia, and enteritis being the predominant autopsy findings.

The survival times of the animals were quite different: with mustard gas animals may die from the direct effect of the vapour 20 days after exposure, whereas with phosgene nearly all animals die in the first 48 hours.

Mice exposed to mustard gas

Mice were exposed to mustard gas vapour in a constant-flow apparatus. The advantage of this type of apparatus over the static chamber is that the concentration of gas does not tend to fall off during the exposure as it does with the latter owing to adsorption on to the surface of the chamber and the animals' fur. In the constant-flow apparatus a continuous flow of air-gas mixture of the required concentration is drawn through the chamber. In the experiments described here the volume of the chamber was 20 litres and the flow of air-gas mixture was 200 litres per minute. The liquid mustard was evaporated into the air stream at the required rate by means of an electric heating coil. The atmosphere in the chamber was continuously sampled throughout the exposure and accurate determinations (within about 1 or 2%) of the concentrations in the chamber could be made.

240 male albino mice (of weight 25–30 g.) were mixed up together and 8 groups of 30 mice selected at random. The groups were exposed all on the same day to 8 different

concentrations of mustard vapour. The exposure time in each case was 10 minutes. The exposures were carried out in wire cages which had separate compartments for each mouse in order to avoid the possibility of the mice huddling together and breathing through each other's fur.

The mortalities at the end of successive 24-hour periods are shown in Table I.

TABLE I

DISTRIBUTION OF SURVIVAL TIMES IN GROUPS OF 30 MICE EXPOSED TO VARIOUS CONCENTRATIONS OF MUSTARD VAPOUR FOR 10 MIN. IN A CONSTANT-FLOW APPARATUS

Concentration in mg./cu.m.		Time in days																								Total mortality
		2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	
A	67									2		2			2			1				1					8/30
B	93						5			1		2			3	2				1						1	15/30
C	116					1	1			1	1	1	4		3			2		2	1			1			18/30
D	160				1		1	3	5		2	2		1		1			1								17/30
E	260			4	2	2	2	2	6	7		1	3									1					30/30
F	300				5	8	2	4	2	3		2						1	3								30/30
G	375			5	9	7	4	3	2																		30/30
H	505		3	11	10	6																					30/30

Analysis of results

(i) As a preliminary analysis the median time for each group was plotted against the dosage ; in general, it was clear that median survival time decreased with increase of dosage, but not as a linear function ; in fact, a smooth curve drawn through the points resembled a rectangular hyperbola. A second very noticeable feature of the data was the increase in spread of the observations in the groups with larger average survival times.

(ii) The original data were converted to logarithms and, in the groups where all the animals died, the means and standard deviations were computed in the usual way. In the groups in which some animals survived, estimates of the means and standard deviations were obtained by plotting the logarithms of the individual observations in each group on a probit scale and estimating the mean from the intersections of the best " eye-fitted " line with the ordinate at 5 probits, and the standard deviation from the reciprocal of the slope of the line (Bliss, 1936, and Gaddum, 1945). It seemed doubtful whether very accurate estimates could be obtained by this method, since there appeared to be a general tendency for the lines to be convex upwards (i.e., for the distributions to be positively skew), and the assumption of normal distribution, on which this method is based, therefore seemed to be invalid. A second very noticeable feature about the data was that in spite of the log transformation the means and standard deviations for the groups were still highly correlated (the product moment correlation coefficient for the unweighted data was about 0.93, which is highly significant : $P = <0.001$).

When these estimates of the mean log survival times were plotted against the logarithms of the dosage there appeared to be a linear relationship. A straight line drawn through the points had a slope of about -1, suggesting a reciprocal relationship between time and dosage.

(iii) The original data were therefore converted to reciprocals, and means and standard deviations of the reciprocal survival times for the groups were found as before. It was noticeable that when the results for each group were plotted on a probability scale there seemed to be no tendency for the points to be curvilinear, which suggested that after this transformation the data were more nearly normally distributed. A second feature of the use of this transformation was the apparent stability of the variance. (The product moment correlation coefficient for the unweighted data was about -0.26, which is quite non-significant: $P \simeq 0.5$.)

The values for the estimates of means and standard deviations for the groups using the logarithmic and the reciprocal transformations are given in Table II.

TABLE II

VALUES FOR THE ESTIMATED MEANS AND STANDARD DEVIATIONS OF TRANSFORMED SURVIVAL TIMES (DAYS)

Group	Mustard dosage (Ct) mg. min./cu.m.	Log transformation (log days)		Reciprocal transformation ($100 \times$ days^{-1})	
		Mean	Standard deviations	Mean	Standard deviations
A	670	1·43	0·31	1·3	6·8
B	930	1·30	0·35	3·9	7·8
C	1160	1·27	0·25	5·2	4·3
D	1600	1·10	0·21	7·1	6·0
E	2600	0·88	0·19	14·6	7·0
F	3000	0·87	0·20	14·6	5·4
G	3750	0·71	0·12	20·1	5·1
H	5050	0·60	0·10	26·0	6·4
		Correlation coeff. between mean and S.D. = 0·93 $P \simeq 0{\cdot}001$		Correlation coeff. between mean and S.D. = − 0·26 $P \simeq 0{\cdot}5$	

When the estimates of the mean reciprocal survival times were plotted against the dosage the points appeared to fall near a straight line.

It is clear that the estimates of mean reciprocal survival time were not of equal reliability, since some were estimated from complete data in the ordinary way, whilst others had to be obtained by graphical methods. Some system of weighting should therefore be used to allow for this. Bliss (1936) and Stevens (1936) have worked out a method for an analogous case in which by successive approximation the maximum likelihood estimates of the means and standard deviations of the truncated normal curve can be calculated to any required degree of accuracy. They also give tables of a factor E which enables one to

calculate the variance of the mean of the truncated curve. This factor E may be used to furnish a weighting factor N/E.

When the data are complete (i.e., when all the animals react), E is equal to unity and the weighting factor becomes equal to N, the number of animals in the group. When the exposure curve is truncated owing to some animals failing to react, N/E will give the equivalent number of animals which would have given as accurate a result if they had all reacted. With the reciprocal transformation, all the points seemed to fit the dosage–probit line and there was no difficulty in deciding at what point truncation* had occurred ; it was in every case determined simply by the proportions of animals dying.

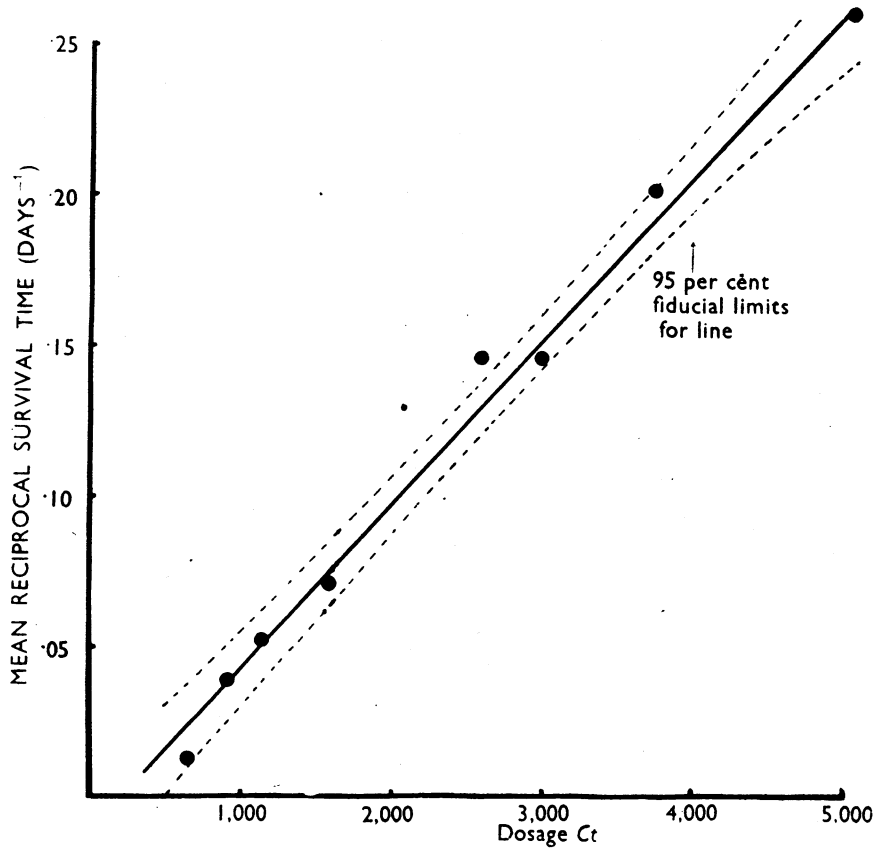

FIG. 1.—*Mustard.* The mean reciprocal survival time for groups of mice exposed to various dosages of mustard gas vapour, showing the line of best fit with its 95 per cent fiducial limits.

*Bliss (1936) dealt with a similar problem (reaction time of organisms when immersed in a toxic solution or gas). His data usually showed a tendency for the points corresponding to the last few organisms to react to fall away from the line. He regarded these organisms as atypical and found the point of truncation by noting where the line appeared to change in slope.

To test the hypothesis that the reciprocal of the survival time was linearly related to the dosage a regression line was fitted between the means of the reciprocal survival times and the dosages. This line, together with the approximate 95 per cent fiducial limits, is shown in Fig. 1.

An alternative hypothesis is that the survival time is proportional to the dosage (i.e., that the line passes through the origin); a second calculation was performed, therefore, and the best-fitting line obtained with the restriction that it should pass through the origin. The "goodness of fit" of the two lines may now be tested, since we may compare the sum of weighted squares of residuals in each case with the pooled "within groups" variance; the ratio of these two quantities will be distributed approximately as χ^2 (since the numbers of observations involved in the between groups variance is large). We obtain:

TABLE III

Hypothesis (1) DOSE AND RECIPROCAL OF SURVIVAL TIME LINEARLY RELATED			*Hypothesis* (2) DOSE AND RECIPROCAL OF SURVIVAL TIME PROPORTIONAL		
χ^2	Degrees of freedom	P (approx.)	χ^2	Degrees of freedom	P (approx.)
3·43	6	0·75	5·03	7	0·5

It is clear that either of the above hypotheses is acceptable.

A distinction is here drawn between the two possibilities, since it seems likely that for some toxic substances a definite "threshold" effect may exist when, although the relationship might be linear, the line would not pass through the origin. It seems useful to keep the more general case in mind, even though in our work we have found no significant departure from proportionality.

TABLE IV

DISTRIBUTION OF SURVIVAL TIMES IN GROUPS OF RATS EXPOSED TO VARIOUS CONCENTRATIONS OF PHOSGENE GAS FOR 10 MIN. IN A CONSTANT-FLOW APPARATUS

Concentration in mg./cu.m.	Time in hours																									Total mortality
	3	3¼	3½	3¾	4	4¼	4½	4¾	5	5½	6	6½	7	8	9	10	11	12	14	16	20	24	30	40	60	
T 151																1			1	1	1	1	1		1	7/12
U 156													1			1	2	1	1		1	1	4	2		14/20
V 300									1		1				1		1		1		1					6/12
W 411										1		1	2			1	2		1	1	1					10/12
X 589							1		2	1	1		1	2		1			1	1		1				12/12
Y 702				1		1		1	1	2	2		1	2			1									12/12
Z 923	1		1	2	3	1		1	4	1	1	1	2	2												20/20

Rats exposed to phosgene gas

The apparatus used for the phosgene exposure is described fully elsewhere (Box and Cullumbine, 1947). A number of groups of albino rats (weight 115–125 g.) were exposed all on the same day to various concentrations of phosgene; as with mice, each rat was exposed in a separate compartment. The exposure time was 10 minutes in each case. The rats were inspected at the times shown and the mortalities recorded. The results are given in Table IV.

Analysis of results

The results were analysed exactly as before, the features of the distribution of survival time being remarkably similar. Again we found a general decrease in survival time with increase in dosage, which assumed a linear form when plotted on a log–log scale with slope approximately equal to -1. Again the spread of observations was correlated with the mean value and this was still the case on transforming to logarithms ($r=0.87$, $P\simeq 0.01$), but not the case with reciprocal transformation ($r=-0.28$, $P\simeq 0.5$). As before, plots of probit mortality against log dose suggested positively skew distribution with the log transformation, but normal distribution with the reciprocal transformation.

The values for the estimates of means and standard deviations for the individual groups are given in Table V.

TABLE V

VALUES FOR THE ESTIMATED MEANS AND STANDARD DEVIATIONS OF TRANSFORMED SURVIVAL TIMES (HOURS)

Group	Phosgene dosage (*Ct*) mg. min./cu.m.	Log transformation (log hours)		Reciprocal transformation ($100 \times$ hours^{-1})	
		Mean	Standard deviations	Mean	Standard deviations
T	1510	1·45	0·34	2·8	5·0
U	1560	1·43	0·33	3·3	5·2
V	3000	1·34	0·47	4·0	10·9
W	4110	1·07	0·27	8·9	6·0
X	5890	0·94	0·24	13·0	5·7
Y	7020	0·80	0·14	16·7	5·8
Z	9230	0·72	0·13	20·1	5·7
		Correlation coeff. between mean and S.D. = 0·87 P≃0·01		Correlation coeff. between mean and S.D. = −0·28 P≃0·5	

Regression lines were calculated exactly as before and the line obtained on the hypothesis of simple linearity is shown in Fig. 2.

Again the alternative hypothesis of proportionality between mean reciprocal time and dosage was tested, and the values obtained for χ^2 in the test of goodness of fit are given in Table VI.

TABLE VI

Hypothesis (1) DOSE AND RECIPROCAL OF SURVIVAL TIME LINEARLY RELATED			*Hypothesis* (2) DOSE AND RECIPROCAL OF SURVIVAL TIME PROPORTIONAL		
χ^2	Degrees of freedom	P (approx.)	χ^2	Degrees of freedom	P (approx.)
1·81	5	0·9	2·15	6	0·9

As before, neither of these tests demonstrates significant departure from the respective hypotheses.

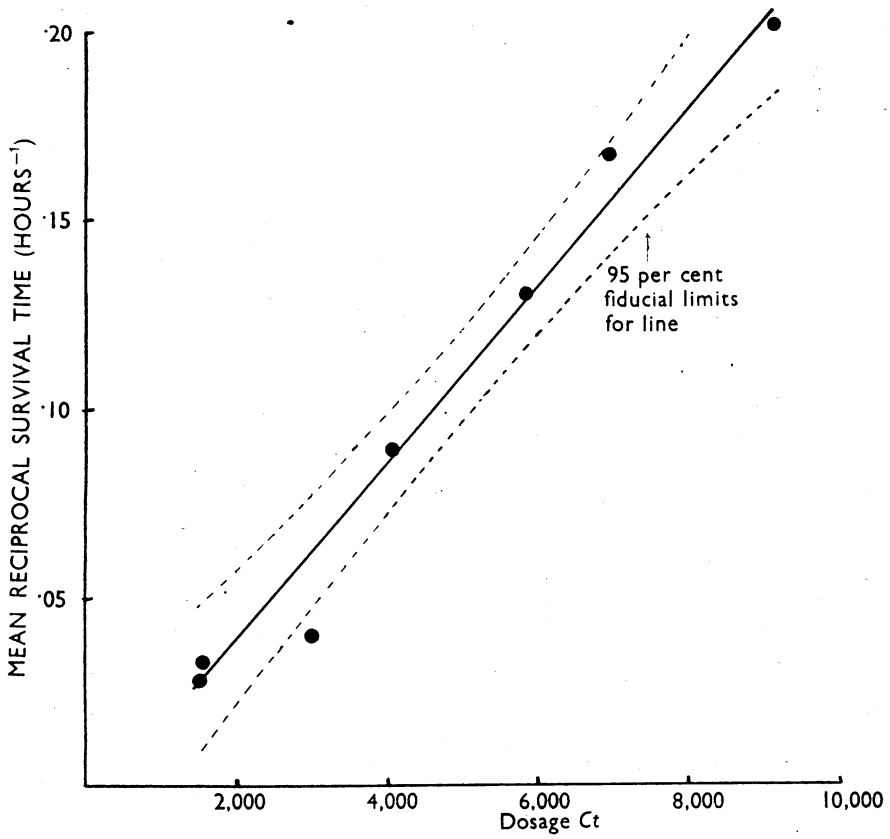

FIG. 2.—*Phosgene.* The mean reciprocal survival time for groups of rats exposed to various dosages of phosgene gas, showing the line of best fit with its 95 per cent fiducial limits.

DISCUSSION

Interpretation and use of the relationship

The use of survival time in pharmacological experiments has been largely confined to the standardizing of cortical extracts (Bulbring, 1937, *et al.*). We

believe that where substantial correlation can be shown between dosage and a function of survival time, its use may be considerably extended. With the two substances and species of animals investigated our experiments suggest: (i) Rate of dying (i.e., the reciprocal of the survival time) is directly proportional to the dosage ; (ii) rate of dying tends to be homoscedastic (i.e., the variance is roughly constant for different survival times) ; (iii) rate of dying is probably more nearly normally distributed than survival time or log survival time. These facts were used in the design and analysis of experiments, including many to assess the value of suggested therapies for chemical warfare agents. In this type of experiment it is a great advantage to be able to test a large number of factors at once in accordance with the principles of experimental design described by Professor R. A. Fisher and his followers. For example, the injection of a certain substance may be suggested as a possible treatment for a certain type of poisoning, but normally there will be many uncertainties, such as the correct dose, the best route of administration, whether it should be given in single or divided doses, and so on. We could, of course, try to guess what would be the most promising combination of these factors, expose a control and treated group to (say) an LD90 of the poison, and observe whether any significant reduction of mortality in the treated group occurred. If it did, then attempts to enhance it by varying other factors could be carried out later. The disadvantages of this type of experimentation are (i) if we guess wrongly we may miss the treatment altogether, (ii) it is very uneconomical in the use of animals, and (iii) it cannot detect interaction between factors.

By means of a continuous variate, such as transformed survival time, many of the factors can be introduced into the first experiment *without increasing its size or losing efficiency in testing significance* (Fisher, 1942). Difficulties occur, however, when attempts are made to use factorial schemes in which percentage mortality is the variate. The most important of these is the lack of range of this type of experiment. Usually we try to get our stock of animals as uniform as possible, but the more uniform we make them the smaller will be the range over which we shall get a response between 0 and 100 per cent mortality. When a number of factors are introduced to be tested simultaneously, the range of the effects will usually be increased ; this will often result in a number of groups with 0 or 100 per cent mortality which have little weight and cannot be compared one with the other. We can widen the range of the experiment by carrying it out at more than one dosage level ; but this type of experiment will almost certainly have a low overall efficiency owing to the number of groups with little weight. Further, if we try to introduce a number of factors and dosage levels, we shall soon have to increase the overall size of the experiment, otherwise the individual group will become too small. In this way when a quantal response is used the advantage of the factorial design tends to be lost. Provided that the groups are large enough, this type of experiment can be analysed by the use of the transformation $x = \sin^{-1}\sqrt{p}$ (where p is the proportion dying).

This transformation overcomes the difficulty of inequality of variance in the groups; its disadvantage, however, is that the transformed variate will not be a linear function of dosage. However, if we use a transformation which *is* a linear function of dosage (i.e., the probit transformation), the weights will depend upon the expected values and be different for different groups. Finney (1943) has given the solution in this case, but his analysis is by multiple regression and becomes laborious when there are many factors, especially if some of the interaction effects have to be included in the analysis.

In view of difficulties of this kind arising out of the use of a quantal response (percentage mortality), wherever it could be shown that some function of survival time was closely correlated with dosage, factorial experiments have been carried out with this function as the variate. The procedure was to give very large doses (usually 3–5 times the LD50), which it was known would probably result in 100 per cent mortality even if the treatments were fairly effective; the therapies were then judged by the increase in survival time. For example, with mustard gas and phosgene, the use of the reciprocal transformation allows the ordinary methods of analysis of variance to be correctly applied. As an additional safeguard the most promising of the treatments may be compared by using percentage mortality and (if the experiment is carried out at more than one dosage level) by calculating the index $I_{LD50} = \frac{\text{LD50 for treated group}}{\text{LD50 for control group}}$; this index will give a true measure of the effectiveness of the treatment and is a similar measure to M (the log of the ratio of the potencies) used by Gaddum (1933). It is interesting to note that I is a function of the percentage mortality and b (the slope of the probit–dose line), so that unless b is known a test at one dosage level using percentage mortality as the variate does not provide us with any information about the *absolute effectiveness* of the treatment; e.g., it is possible for two equally good therapies to give quite different reductions in mortality if the values of b in the two tests are different. However, in the case of survival time, the ratios of the means of the transformed survival times in control and treated groups will give a measure of effectiveness which is independent of the standard deviations of the groups. For instance, we found that, where the reciprocal transformation is appropriate, $I_T = \frac{\text{Median survival time of treated group}}{\text{Median survival time of control group}}$ gives a measure of effectiveness, which is often a good approximation to I_{LD50}.

Even if a relationship can be established between transformed survival time and dosage for a particular case, it will *not* follow that the method outlined here will necessarily be appropriate for the testing of therapies. Examples may occur where a therapy will increase the survival time without reducing mortality. Usually, however, a knowledge of the probable mechanism of a therapy will enable one to judge whether this method of experimentation will be appropriate. In the opinion of the authors the technique can often be used profitably, side

by side with more orthodox methods, and they have conducted many experiments on these lines, some of which appear elsewhere. (Cullumbine and Box, 1946; Box and Cullumbine, 1947.)

Analysis of experiments in which some groups are incomplete

Occasionally when using factorial experiments of the type outlined above, a group or groups will partly, or wholly, survive. When all or most of the animals survive in a group or groups in marked contrast to other groups, usually no analysis will be needed to establish the superiority of the corresponding treatments. Experiments in which there are a few survivors in one or two groups only are not so easily dealt with.

(i) Maximum likelihood estimates of the means in the incomplete groups may be obtained by the method of successive approximation given by Bliss (1936) and Stevens (1936). The solution will then be given by regression analysis using the appropriate weighting coefficients; however, the labour involved in the calculations is scarcely justified.

(ii) Provided at least 50 per cent have died in all groups, the medians of the transformed values may be used and the ordinary analysis of variance procedure adopted. A certain amount of information is lost when this method is used, but with small groups the loss is not large. Pearson and Adyanthaya (1928) give the following values:

Sample size using median	2	3	4	5	7	10
Equivalent sample size using mean	2	3	4	4	5	8

(iii) The means may be estimated in the incomplete groups by graphic analysis and the experiments analysed as though the ordinary analysis of variance technique were appropriate (i.e., as though the means of the groups were all known with equal accuracy). This method is probably sufficiently accurate provided that the number of animals failing to respond is not too great.

(iv) Where the reciprocal transformation is appropriate, the "rate of dying" of an animal which survives is $\frac{1}{\infty}=0$, so that with this transformation we could score these animals as zero and carry out the analysis in the ordinary way. In our opinion this approximation will be more inaccurate than (iii). If the "within groups" variance is used in the analysis the truncated groups should be omitted from its calculation, since the values will be too small. In general it is better to use high order interactions between group means as the "error" estimate. We find that (iii) gives a satisfactory approximation and utilizes all the information without complicating the analysis.

SUMMARY

1. Experiments are described in which mice were exposed to mustard vapour and rats to phosgene gas. The properties of the survival time, its logarithm and reciprocal were investigated. Using the reciprocal transformation, the transformed

variate (rate of dying) was proportional to the dosage, had stable variance, and appeared to be more nearly normally distributed than time or log time.

2. The use in appropriate circumstances of relationships of this nature is discussed and the advantages of factorial experiments using transformed survival time are indicated.

3. Methods are suggested for dealing with the data when in some groups the animals do not all die.

We should like to express our indebtedness to Prof. R. A. Fisher for discussing this problem with one of us and for suggesting the use of the reciprocal transformation.

Our thanks are due to the Director-General of Scientific Research (Defence) for permission to publish these results, and to our colleagues at Porton for their advice and criticism.

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THE EFFECT OF EXPOSURE TO SUB-LETHAL DOSES OF PHOSGENE ON THE SUBSEQUENT L(Ct)50 FOR RATS AND MICE

BY

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It has been suggested that when dogs and goats survive doses of phosgene gas ($COCl_2$) their subsequent susceptibility is apparently lessened. The effect was explained as being probably due to selection; since the more susceptible animals were killed, the remainder would be more resistant and their average susceptibility lower. The experiments described in this report were carried out in order to obtain further information about the matter.

EXPERIMENTAL

Preliminary experiments were carried out to ascertain the highest concentration to which rats and mice could be exposed for 10 min. without causing death. It was found that exposure of rats and mice to dosages (Ct)* of 800 and 600 mg.min./cu.m. ($t = 10$ min.) respectively did not normally produce any deaths, although the animals showed all the symptoms of severe phosgene poisoning. These dosages were therefore used throughout the work as the preliminary or "pregassing" doses.

The exposure to phosgene was carried out in a small chamber (20 litres capacity) in a constant flow of 200 litres/min. of an air–phosgene mixture of the required concentration. The apparatus is shown in Fig. 1. The atmosphere in the chamber was sampled at 10 litres/min. throughout the whole of the exposure.

*The dosage to which animals were exposed is measured here by multiplying the mean concentration (C) measured in mg./cu.m. by the time of exposure t. (Hence Ct in mg.min./cu.m.) The time of exposure (t) was 10 min. in all experiments unless otherwise stated.

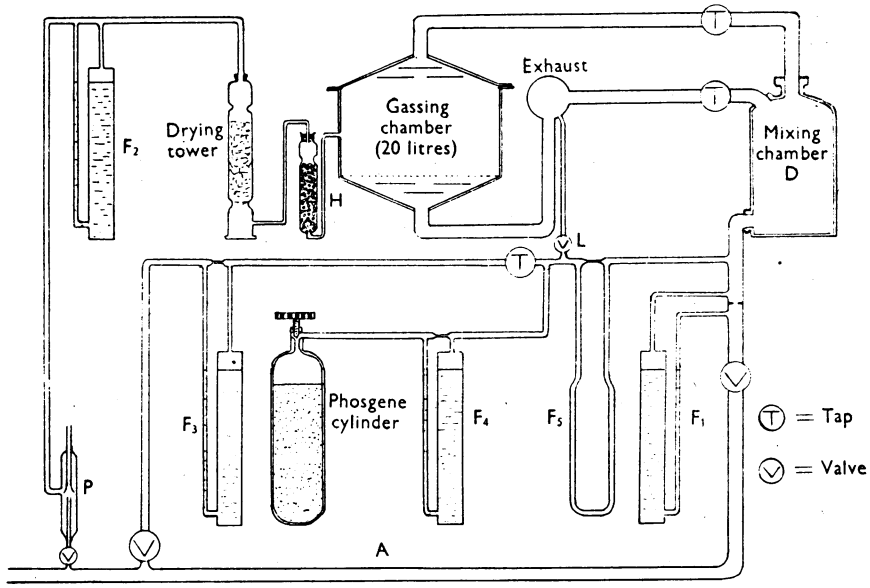

FIG. 1.—The constant-flow phosgene apparatus. 200 litres/min. of air was blown along the main air line A and measured by flowmeter F_1. Phosgene (measured by F_4) was mixed with diluting air (measured by F_3). The mixture regulated by valve L was measured by F_5 and mixed in chamber D with the main air stream. The diluted air–phosgene mixture could then be made to pass through the chamber or run to waste as required. A suction pump (P) drew a continuous sample at 10 litres/min. through the sampling bubbler H.

The effect of phosgene

96 rats (weight 115–125 g.) were taken and divided at random into 4 groups of 24 each. 12 rats in each group were exposed to a dosage (*Ct*) of 800, and the remaining 12 in each group were kept under identical conditions as controls. Five days later each group of 24 was exposed for 10 min. to concentrations of phosgene in the lethal range. The mortalities at the end of 48 hours were:

Dosage (*Ct*) to which rats were exposed (mg.min./cu.m.)	2300	2500	3150	4400	Total
Mortality in controls	8/12	7/11	9/12	11/12	35/47
Mortality in pregassed animals ..	3/12	2/12	3/12	8/12	16/48

Regression lines were fitted between the mortality expressed in probits and the logarithm of the dosage (*Ct*) by the method described by Gaddum (1933), as elaborated by Bliss (1935, 1938) and Fisher and Yates (1943).

Statistical analysis

	Degrees of freedom	χ^2	P
Differences in position of lines	1	17·6	<0·0001
Differences in slope of lines	1	0·2	0·6
Heterogeneity	4	2·2	0·7

L(*Ct*)50 Pregassed 3840 mg.min./cu.m.
„ Control 1880 „
} $I_{L(Ct)50} = 2{\cdot}0$

Where $I_{L(Ct)50}$ is an index of effectiveness obtained by dividing the L(*Ct*)50 for pregassed by the L(*Ct*)50 for control animals.

There is little doubt that when subsequently exposed to the same concentration there is a lower mortality in the pregassed than in the control animals.

Phosgene and hexamine

Larger doses of phosgene for pregassing might produce greater effects, but would result in deaths in the pregassing. It was therefore decided (i) to pregas with a dosage (*Ct*) of 6,000, but to prevent death by administering oral hexamine* immediately beforehand, and to compare this with normal pregassing ; (ii) to use intervals of both 3 and 7 days between pregassing and lethal gassing so as to compare the effect of altering the periods between gassing ; (iii) to carry out the experiment with mice in order to test whether they behaved similarly to rats ; and (iv) to carry out the whole experiment at two different concentrations of phosgene.

96 mice were divided at random into 8 groups of 8, and 2 of 16. The treatment applied to the groups and the resulting mortalities are shown below. The dose of hexamine used was about 2 g. per kg. (0.2 c.c. of a 20 per cent (w/v) solution orally immediately prior to gassing).

	Pregassed 3 days before 2nd exposure with *Ct*		Pregassed 7 days before 2nd exposure with *Ct*		No pregassing
2nd gassing *Ct* in mg.min./cu.m.	600	6000 and hexamine	600	6000 and hexamine	—
2,450	6/8	5/8	6/8	8/8	16/16
1,500	4/8	3/8	2/8	5/8	14/16

Statistical analysis

By a simple extension of the technique described by Bliss (1935), we can fit regression lines for the 4 treatments and control, and compare these for differ-

*Hexamine has a great chemical affinity for phosgene, and oral administration (2 g. per kg.) *immediately* prior to gassing will prevent phosgene poisoning.

ences in position and slope. The variation between slopes is not significant ($\chi^2 = 1.70$ for 4 degrees of freedom, $P \simeq 0.8$). Comparing the lines for position, we find:

Comparison of methods of pregassing	Degrees of freedom	χ^2	P
Time (3 days *v* 7 days)	1	1·55	0·2
Dosage (600 *v* 6000 + hexamine)..	1	1·55	0·2
Comparison of pregassing (in general) with controls ..	1	11·41	<0·001

L(*Ct*)50 pregassed (average) .. .. .. 1630 mg.min./cu.m.
L(*Ct*)50 control .. .. 1020 mg.min./cu.m. } $I_{L(Ct)50} = 1{\cdot}6$

In general pregassing of mice has a highly significant effect. There is no evidence from this experiment that the effect is different after 3 or 7 days. Increase in dosage (*Ct*) of the second gassing from 1,500 to 2,450 mg.min./cu.m. produces a uniform increase of probit in all the groups, and the effect is equally marked at both dosages.

Exposure in the preliminary gassing to a dosage of 6,000 after oral hexamine produces an effect no greater than that of exposure to 600 without hexamine.

Duration of the effect

In order to obtain further information on the duration of the effect, 70 mice were taken and divided at random into 7 groups of 10. One group was kept as a control and the other 6 were exposed to a phosgene dosage of 600 mg.min./cu.m. at 1, 2, 3, 5, 7, and 10 days before the second gassing. The whole 70 mice were then exposed to a dosage (*Ct*) of 5,850 mg.min./cu.m. (i.e., about 3 times the L(*Ct*)50) at the same time. The time of death of the mice was noted. We have shown elsewhere (Box and Cullumbine, 1947) that the reciprocal of survival time is closely correlated with dosage. The survival times were therefore transformed to reciprocals for analysis. The median survival times are given below together with the analysis of variance of the transformed variate. The data are graphed in Fig. 2, using the reciprocal scale.

Second gassing (*Ct.* = 5,850, t = 10) on day Z.

Preliminary gassing on day:	Z-1	Z-2	Z-3	Z-5	Z-7	Z-10	Control
Estimated median survival time (hours):	4·3	5·0	9·1	10·4	7·5	5·3	5·5

ANALYSIS OF VARIANCE OF RECIPROCAL SURVIVAL TIMES ($100 \times HR.^{-1}$)

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio (F)
Between groups ..	6	1529	255	8·8
Within groups ..	63	1802	29	
Total	69	3331		

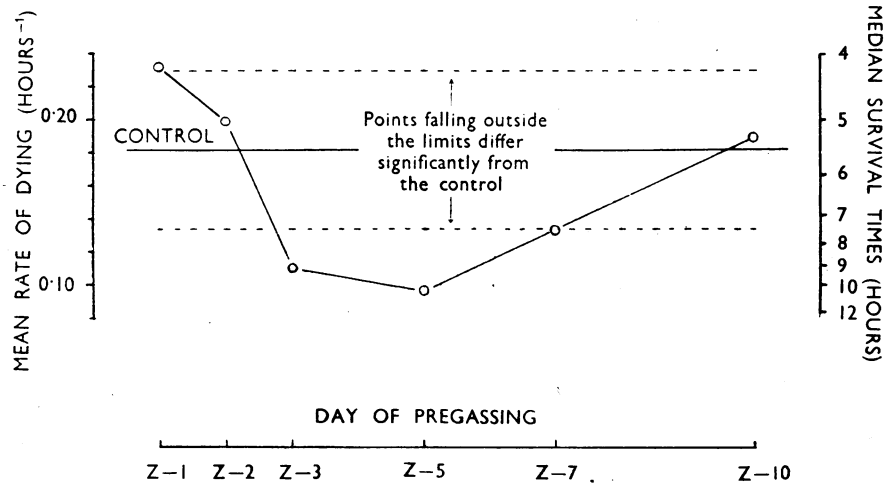

FIG. 2.—The median survival time (plotted as reciprocal) of mice pregassed at different times and exposed to phosgene (dosage $Ct = 5{,}850$, $t = 10$ min.).

It is clear that the variation between groups is highly significant ($P < 0.001$) and that the animals in the group pregassed one day before die significantly faster, whilst those pregassed 3, 5, and 7 days before die significantly slower than the control animals. The index of effectiveness I_T (obtained by dividing survival time in treated group by the survival time in controls) reaches a maximum of 1.9 five days after pregassing.

Pregassing more than once

This experiment was designed to test whether the effect could be increased by exposure more than once to pregassing. The pregassing doses were given 12, 8, and 4 days before the dose in the lethal zone. 96 mice were used and divided at random into 8 groups, A to H, each containing 12 mice.

On day Z−12	groups	C, D, G and H	were exposed to a dosage (Ct) of	600
,, ,, Z−8	,,	D, B, F and H	,, ,, ,,	550
,, ,, Z−4	,,	E, F, G and H	,, ,, ,,	600
,, ,, Z	,,	A, B, C, D, E, F, G, H	,, ,, final dosage of	2,850

MORTALITIES (OUT OF 12) AFTER 48 HOURS

A : 11/12	B : 10/12	E : 7/12	F : 6/12
C : 11/12	D : 12/12	G : 5/12	H : 3/12

The data may be analysed by transforming the proportions to angles using the transformation $x = \sin^{-1}\sqrt{p}$ (where p is the proportion dying). This will have the effect of stabilizing the variance. The technique was exactly as described by Fisher and Yates (1943). The expected values can in this case be obtained by inspection of the data, but in more awkward cases the graphical methods suggested by Richards (1941) have proved helpful.

Analysis of variance of the transformed variate

Effect	Degrees of freedom	Sum of squares	χ^2	P
Z−12 1st Pregassing ..	1	0		
Z−8 2nd „	1	12		
Z−4 3rd „	1	1588	23·4	<0·00001.
Interactions				
1 × 2	1	101		
1 × 3	1	0		
2 × 3	1	101		
1 × 2 × 3	1	12		
Total	7	1814	26·5	<0·001

Theoretical variance $\frac{820 \cdot 7}{12} = 68$

Pregassing on Z–12 and Z–8 days had no effect upon mortality, although pregassing at Z–4 produced a highly significant decrease in mortality in all groups.

Lung damage and the effect

To test whether damage to the lungs was necessary to produce the effect, the following experiment was carried out: 48 rats were divided at random into 4 groups of 12, A, B, C, and D.

Group A was given a pregassing dosage on day Z–5,

B was given a pregassing dosage on day Z–5 immediately after oral hexamine,

C was kept as a control,

and D was given oral hexamine *only* on day Z–5.

A, B, C, and D were all exposed on day Z to a dosage of 3,150 mg.min./cu.m. After the first exposure group B showed no signs of distress. (Rats treated in this way showed no signs of pulmonary oedema at autopsy 12 and 24 hours after gassing: thus it appeared that oral hexamine was completely effective in preventing the action of this concentration of phosgene.) The 48-hour mortality after the second exposure was:

	No hexamine	Hexamine
Pregassed	A : 3/12	B : 9/12
Not pregassed	C : 9/12	D : 9/12

Statistical analysis

	Degrees of freedom	χ^2	P
Pregassing with no hexamine	1	4·17	<0·05

Thus pregassing with no hexamine significantly reduces mortality; pregassing *with* hexamine so that no pulmonary damage is produced is ineffective. It would appear necessary therefore to cause lung damage in order to produce the effect. At first sight this experiment appears to contradict our previous experiment in which mice were gassed with a *Ct* of 6,000 after hexamine. However, in that experiment the administration of oral hexamine was not able to neutralize the whole effect of a very high dosage (6,000) of phosgene.

Duration of lung damage

In order to investigate lung damage produced after different time intervals by the pregassing dosage and to try to correlate it with the effect produced, groups of mice were exposed to the pregassing dose and autopsied after 1, 2, 3, 4, 5, 7, 10, and 14 days. The trachea was tied off before the thorax was punctured and paraffin wax sections prepared; these were examined by Professor G. R. Cameron, whose observations are given in Table I.

It would appear that the oedema is present for the first four days and that no damage is visible after the 10th day. When this result is compared with our experiment on the duration of the effect, the return to normality appears to correspond with a cessation of the effect, which is also absent in the first few days when oedema is most severe.

TABLE I

HISTOLOGICAL EXAMINATION OF LUNGS OF MICE AT VARIOUS TIMES AFTER PREGASSING

All mice were exposed to a dose (*Ct*) of 600 mg.min./cu.m. phosgene

No. of days mice killed after pregassing	No. of mice in group	Histological examination of lungs
1 day	5	All show varying degrees of oedema, some with patches of leucocytic infiltration. Bronchial and bronchiolitic epithelia seem intact or in process of being lifted up by oedema. A few leucocytes.
2 days	5	Oedema seems more severe and extensive. Infiltrated with leucocytes and monocytes. Bronchial and bronchiolitic epithelia intact. Collapse.
3 days	5	Varying amounts of oedema in two cases. Severe in others. Very patchy with areas of collapse showing numerous leucocytes. Bronchial and bronchiolitic epithelia intact.
4 days	5	Two still with extensive oedema and collapsed infiltrated alveoli. Remainder with hardly any oedema. Bronchial and bronchiolitic epithelia intact; normal.
5 days	5	Two show extensive patchy oedema and collapse with emphysema. Remainder show slightly patchy oedema resolving. Bronchial and bronchiolitic epithelia intact and normal in all.
7 days	4	Three normal. One shows small patches of collapse and alveolitis. Bronchial and bronchiolitic epithelia normal.
10 days	4	All normal (one shows much recent haemorrhage. Traumatic).
14 days	4	All normal.

Differences in respiration between control and pregassed animals

When toxic substances are injected into animals the variation in response is largely due to the difference in susceptibility between them; but when animals are exposed to a gas the dose which each animal inhales is partly determined by the respiration of that animal. Experiments were made in order to find out whether the pregassing effect could be explained by differences in respiration in the pregassed and control groups during the terminal gassing. Ideally it would be useful to measure the total air breathed, the oxygen uptake, the CO_2 output, and the respiration rate; in view of the practical difficulties involved only the last two were measured.

Respiration rate

Two groups of rats were taken at random and one group exposed to the pregassing concentration. Five days later all the rats were exposed in the chamber two at a time, one rat being taken from the pregassed and one from the control group. They were placed in small wire cages close to a glass window in the gassing chamber, and their respirations counted by two observers, possible bias in the observer being eliminated by tossing a coin to decide which observer should count a particular rat.

1. In the first experiment 9 pairs of rats were exposed. Respirations were counted for 15-second periods every two minutes during the 10-min. exposure. Some animals struggled and others held their breath, resulting in great variation between animals and difficulty in counting.

2. In a second experiment the rats were lightly anaesthetized with 0.5 c.c. per kg. of nembutal solution. (This would itself affect the respiration rate, but it was thought that any systematic involuntary difference would still be apparent.) 13 pairs of rats were used and respirations counted for 1/2-min. periods every minute during the 5-min. exposure. The results for these two experiments are plotted in Fig. 3. It is clear that the animals cut

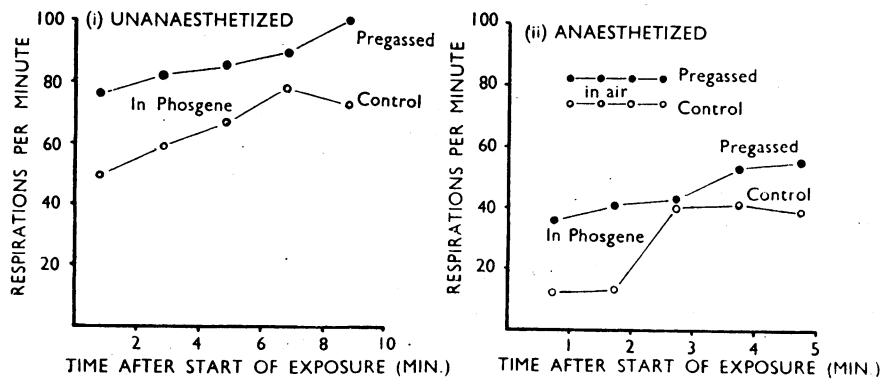

FIG. 3.—The average respiration rates (respiration per min.) of control and pregassed rats during exposure to phosgene. (i) Unanaesthetized. $C=500$ mg./cu.m.; $t=10$ min. (ii) Anaesthetized. $C=700$ mg./cu.m.; $t=5$ min.

down their respiratory rate in phosgene very markedly at first (even when anaesthetized), but breath-holding lessens as the exposure proceeds. (Compare Boyland *et al.*, 1946.)

TABLE II

RESPIRATION RATES PER MINUTE OF ANAESTHETIZED AND UNANAESTHETIZED RATS IN AIR AND AIR–PHOSGENE MIXTURES

	(i) Not anaesthetized Ct = 5,000 t = 10		(ii) Anaesthetized Ct = 3,500 t = 5		(iii) Anaesthetized Ct = 5,900 t = 10	
	Control	Pregassed	Control	Pregassed	Control	Pregassed
Average respiration rate in air	160*	—	74	82	—	—
Average respiration rate in $COCl_2$	66	86	32	46	41	49
Mortality	9/9	9/9	†4/13	2/13	10/10	4/8
Percentage increase in respiratory rate in pregassed group	30		44		19	
t_f = "Students" ratio for f degrees of freedom	t_{16} = 2·10 P = 0·052		t_{24} = 3·31 P = 0·003		t_{16} = 1·33 P = 0·200	
	Combined P < 0·005					

*Rate obtained from Fig. 54 given by Gaddum (1940).

†In the anaesthetized groups the mortalities were lower than would be expected normally, presumably because of the reduction in breathing rate caused by anaesthesia.

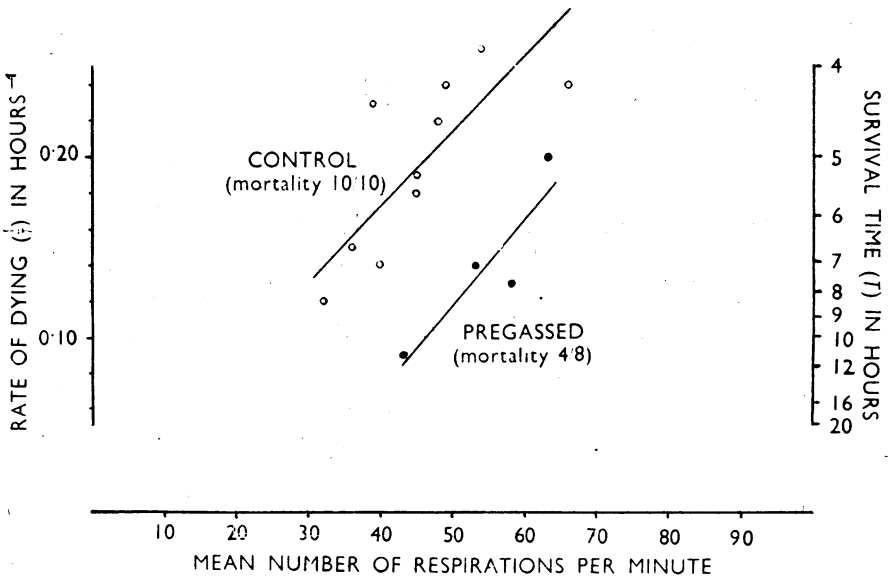

FIG. 4.—A comparison of the respiration rates of anaesthetized rats during exposure to phosgene and their subsequent survival times, in control and pregassed groups. C=590 mg./cu.m.; t=10 min.

3. In a third experiment an exposure time of 10 min. was used; otherwise it was similar in detail to the second experiment. There were originally 10 rats in each group, but 2 died in the pregassing group under anaesthesia.

A summary of the results from these three experiments is given in Table II. The analysis shows that there is little reason for doubt that when exposed to phosgene the pregassed rats breathe faster than the controls.

In the third experiment each rat was marked and its survival time recorded; the results are given in Table III and are plotted together with the lines of best fit in Fig. 4.

TABLE III

AVERAGE NUMBER OF RESPIRATIONS PER MINUTE IN PHOSGENE AND SUBSEQUENT SURVIVAL TIMES OF CONTROL AND PREGASSED RATS

Control group		Pregassed group	
Average No. of respirations per min. in $COCl_2$*	Survival time (hours)	Average No. of respirations per min. in $COCl_2$*	Survival time (hours)
32	8·4	18	Survived
36	6·8	40	Survived
39	4·4	43	11·7
40	7·2	53	7·0
45	5·3	54	Survived
45	5·4	58	7·8
48	4·6	63	5·0
49	4·2	64	Survived
54	3·8		
66	3·9		
Mortality 10/10		Mortality 4/8	

*In order of magnitude to facilitate reference to Fig. 4.

Clearly there is a very strong correlation in the groups between survival time and respiration rate ($r=0.83$, $P<0.001$). The regression equation for the control group suggests a relationship of the type $\frac{1}{T}=KX$, where T is the survival time and X the respiration rate. Analysis of the transformed variate ($100 \times \text{hours}^{-1}$) gives:

Analysis of transformed variate ($100 \times \text{hours}^{-1}$)

Source	Degrees of freedom	Sum of squares	Mean square	F
About lines	10	88·4	8·8	25
Between lines { slope ..	1	0·4		
Between lines { position ..	1	225·0	225·0	

It is clear that there is a highly significant difference in position between the lines ($P<0.001$).

CO_2 output during gassing

The method used was similar to that described by Gaddum and Hetherington (1931). The apparatus is sketched in Fig. 5.

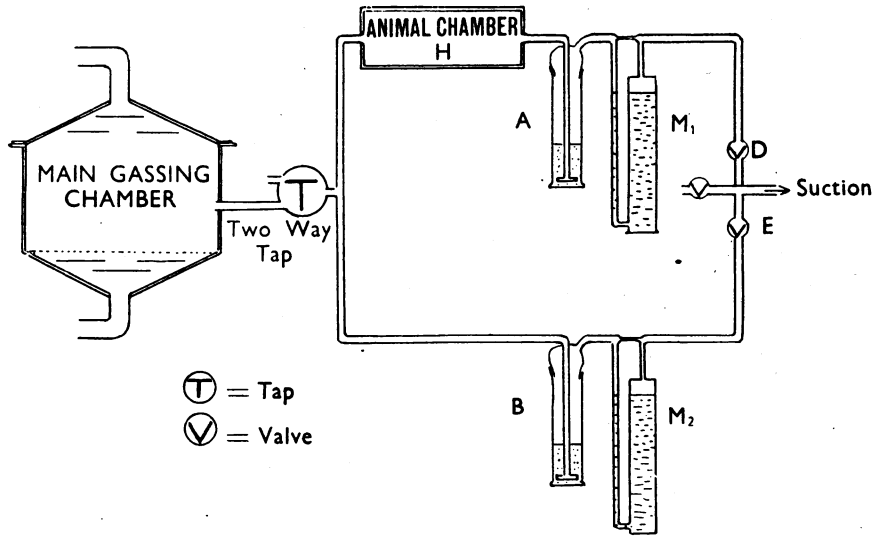

FIG. 5.—Apparatus for measuring CO_2 output of small animals during gassing. The flow of air along tubes E and D was adjusted to 3 litres per min. by means of the valves and the flowmeters M_1 and M_2. H was a chamber sufficiently large to accommodate the animals or animal, without too much dead space. A two-way tap allowed circuit to be opened to outside air or to a main gassing chamber. Bubbler A sampled the atmosphere together with expired air from animals, bubbler B sampled atmosphere only.

The amount of CO_2 produced by the rat was calculated by deducting the CO_2 contained in the air—measured by bubbler B—from that produced by the rat plus the amount contained in the air—measured by bubbler A.

In order to estimate the actual concentrations of phosgene to which the animals were exposed (and not that in the large chamber), a method was devised whereby CO_2 and phosgene could be absorbed and estimated in the same bubbler. Our methods of overcoming certain problems arising in these measurements are discussed in the Appendix.

It was first of all necessary to know whether the CO_2 output during exposure to $COCl_2$ and the dose of phosgene breathed were related. An experiment was therefore carried out in which normal rats were exposed to lethal concentrations of phosgene one at a time. The CO_2 output and phosgene concentrations were estimated and the survival time of each animal recorded. The results are given in Table IV.

It might be expected that most simply the dose breathed would be proportional to the CO_2 output (R). The dose breathed cannot be measured, but we have found (Box and Cullumbine, 1947) that the median mortality time and Ct are related by the type of expression $\frac{1}{T}=K\ (Ct)$ where T is the survival time,

TABLE IV

CO_2 OUTPUT OF RATS IN PHOSGENE AND THEIR SUBSEQUENT SURVIVAL TIMES

(1)	(2)	(3)	(4)	(5)	(6)
Mean concentration C in mg./cu.m. phosgene	C.c. of CO_2 produced per kg. body wt. in phosgene in 10 min.* (R)	Fractional CO_2 output of average: $R \div 199$	Survival time (hours)	Reciprocal survival time (hours^{-1})	(1) × (3) "C" when differences in R are allowed for
458	87	0·44	about 24	0·042	202
436	108	0·54	survived	—	235
427	127	0·64	14·5	0·069	273
496	138	0·69	19·0	0·053	342
499	143	0·72	about 24	0·042	359
442	154	0·77	11·0	0·091	340
475	155	0·78	about 24	0·042	370
442	182	0·92	9·3	0·107	407
541	189	0·95	6·6	0·152	514
386	193	0·97	survived	—	374
449	198	0·99	19·5	0·051	445
894	212	1·07	12·2	0·082	957
446	217	1·09	9·7	0·103	486
449	228	1·15	5·3	0·189	516
479	284	1·43	5·2	0·192	685
464	360	1·81	7·0	0·143	840
522	411	2·06	4·4	0·227	1075
	Mean of R = 199				

*Rats in order of CO_2 output.

C is the concentration to which the animal is exposed for time t, and K is constant. Hence the type of expression which might be expected would be:

$$\frac{1}{T} = KCR \quad \text{(i)}$$

where R = c.c. of CO_2 produced per kg. body weight during the period of gassing.

If this were true, converting to logs we should have:

$$\log \frac{1}{T} = K + b_1 \log C + b_2 \log R \quad \text{(ii)}$$

and b_1 and b_2 would both be equal to unity.

To test this, the values of $\frac{1}{T}$, C, and R were converted to logarithms. The best fitting equation obtained by the method of least squares was:

$$\log \frac{1}{T} = -5{\cdot}13 + 0{\cdot}56 \log C + 1{\cdot}13 \log R.$$

The values for b_1 and b_2 and their standard errors are

$0{\cdot}56 \pm 0{\cdot}58$ and $1{\cdot}13 \pm 0{\cdot}26$ respectively.

The range of values of C here is very small and consequently the coefficient of log C (0.56) is very unreliable and not significantly different from zero or from unity. But our previous work leads us to expect that this part of equation (ii) is well founded and the true value of the coefficient b_1 will not be far from the expected value, i.e., unity. The coefficient $b_2 = 1.13$ is highly significantly

different from zero and not significantly different from one. Hence there are grounds for believing that the relationship is of type (i).

The experiment leaves no doubt as to the correlation between CO_2 output and reciprocal survival time ($r=0.79$). As the dosage (Ct) was not always constant, the partial correlation coefficient between reciprocal survival time and CO_2 output with the effect of C eliminated was worked out; this is still 0.79 correct to two places of decimals, a value which is highly significant.

It is clear, therefore, that the CO_2 output during exposure to phosgene gives a good indication of the amount of gas breathed. The experiment shows that there is an enormous difference in the amount of gas breathed by different animals, presumably owing to the variations in breath-holding and in the degree of activity of the animals in the concentration. In fact, over half the variation ($R^2=0.65$) is attributable to factors involved in differences of respiration and concentration of the gas. The remaining variation ($1-R^2=0.35$), or less than half of it, is attributable to other factors such as differences of susceptibility. In order to test the hypothesis that CR represented the dose breathed, columns (1) and (3) of Table IV were multiplied to give a product which might be considered as

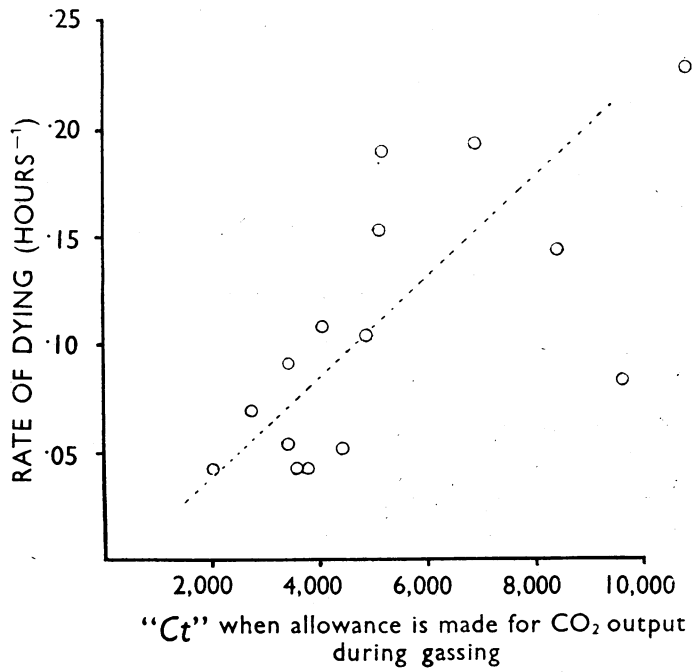

FIG. 6.—The points represent the survival times (plotted as reciprocals) for corresponding values of "Ct" for individual animals when allowance is made for differences in CO_2 output during the gassing. The line is that obtained in a previous investigation for median survival time at various dosages.

representing better the "dosage" to which the lungs of animals were exposed. In Fig. 6 reciprocal survival time is plotted against this product multiplied by t (10 min.) and the points are compared with the relationship between Ct and survival time found before (Box and Cullumbine, 1947). It can be seen that the hypothesis is in reasonable agreement with the facts.

CO_2 output of control and pregassed rats during gassing

Twenty rats, which had been submitted to the pregassing concentration 5 days previously, and 20 controls were exposed to phosgene in the apparatus in groups of 4 at a time. The CO_2 production was first determined during a 10-min. period while the animals were breathing air and later during a 10-min. period while they were breathing an air–phosgene mixture of mean $Ct=1450$ ($t=10$ min.). The results were as follows:

Control				Pregassed			
No. of rats	Average weight (g.)	Average rate of CO_2 production (c.c./min./kg.)		No. of rats	Average weight (g.)	Average rate of CO_2 production (c.c./min./kg.)	
		In air	In $COCl_2$			In air	In $COCl_2$
4	197	32·4	16·0	4	207	22·6	16·0
4	195	24·3	15·6	4	202	29·5	17·8
4	197	26·8	16·2	4	210	31·0	20·3
4	200	25·4	14·9	4	190	18·3	16·7
4	197	32·4	18·9	4	190	30·9	24·1
	Means	28·2	16·3		Means	26·4	19·0
	Mortality 1/20				Mortality 0/20		

The apparent increase in CO_2 output in phosgene (16.3 to 19.0 c.c. CO_2/min./kg.) is not statistically significant; $t=1.64$ for 8 degrees of freedom and P is between 0.2 and 0.1.

The dosage used in the previous experiment was rather low, so the experiment was repeated using higher concentrations. Eight pregassed and 8 control rats were used in the experiment and they were exposed in pairs.

Control				Pregassed			
No. of rats	Average wt. (g.)	Ct(mg./min./cu.m.)	c.c. CO_2/min./kg.	No. of rats	Average wt. (g.)	Ct(mg./min./cu.m.)	c.c. CO_2/min./kg.
2	185	1,940	23·4	2	182	2,270	32·4
2	185	3,360	20·4	2	195	3,360	23·1
2	210	2,610	23·4	2	190	2,390	20·5
2	190	3,700	23·3	2	185	3,690	20·3
Means	192	2,900	22·6	Means	188	2,780	24·1
	Mortality 6/8				Mortality 3/8		

As before the apparent increase in CO_2 production (22.6 to 24.1 c.c. CO_2/min./kg.) is not statistically significant; $t=0.5$ for 6 degrees of freedom and $P=0.6$.

It must be concluded that there is no significant difference in CO_2 production of pregassed and control rats during subsequent exposure to phosgene.

The respiration experiments suggest that after pregassing and during the period when oedema has subsided but visible lung damage persists (i.e., from about the 4th–7th day), the animals breathe more rapidly but less deeply. It seems likely that this would result in the effect demonstrated, since damage in the second gassing would probably be more superficial with this type of breathing.

Summary and Conclusions

1. When rats and mice are exposed to preliminary non-lethal doses of phosgene a transitory effect (lasting from about the 3rd to the 7th day in mice) is produced, resulting in an apparent increase of resistance to phosgene.

2. In order to produce this effect it is necessary to produce lung damage.

3. Repeated exposures do not produce a cumulative effect.

4. The respiration rate is related to the dose breathed.

5. Rats which have been exposed to a pregassing dose breathe more rapidly in phosgene, but take longer to die.

6. The CO_2 output is related to the dose breathed.

7. There is no significant difference between the CO_2 outputs of pregassed and control rats during exposure to phosgene 5 days later.

It seems likely that the effect can be explained by the more rapid and shallower type of breathing of the pregassed rats in phosgene caused by lung damage in the first exposure.

Appendix

Problems arising in the measurement of CO_2 production of rats and mice during exposure to phosgene (or other toxic agents)

General

The normal amount of CO_2 present in laboratory air is about 0.05 per cent or 0.5 c.c. per litre. The CO_2 output of an average size rat is about 4 c.c. per min. Hence in the type of apparatus described the greater the air flow used the greater will be the proportion of CO_2 absorbed from the air and the less the proportion due to the rat. Hence the air flow must not be too high, otherwise atmospheric CO_2 will tend to swamp the CO_2 expired by the rat and reduce the accuracy of the method. On the other hand, in order that the air already in the rat chamber may be washed out rapidly, the dead space must be cut to a minimum and flow kept as high as possible. Further, the type of bubbler with sintered glass diffuser plates cannot be used at flows much above 3 litres/min. without frothing.

Bearing these considerations in mind, the following design was adopted:

1. Not more than 1 litre of chamber space per rat (dead space about 750 c.c.).
2. Flow of 3 litres/min.
3. Phosgene and CO_2 were sampled in the same bubbler.

With this arrangement the chamber would be completely washed out in a quarter of a minute and the error would be negligible in 10-min. exposures. Further, for every 4 c.c.

of CO_2 produced by the rat only about 1.5 c.c. would pass through the bubbler from the air. The estimation of CO_2 and phosgene in the same bubbler (1) ensures that the actual atmosphere breathed by the rat is sampled, (2) gives double the rate of flow possible with two bubblers using the same rate through the chamber, and (3) avoids duplication of solutions, bubblers, flowmeters, and air lines.

The absorbent was a mixture of 25 c.c. of $N/1$ NaOH and 50 c.c. of 6 per cent (w/v) hexamine. The bubblers were Dreschel bottles with sintered glass diffuser plates.

Method

The contents of the bubbler were washed out into a beaker and 50 c.c. of 66 per cent (w/v) solution of barium nitrate added to precipitate the carbonate. The volume was made up to 170 c.c. and a few drops of phenolphthalein added. The residual alkali was titrated with $N/1$ nitric acid. The endpoint (red-colourless) is extremely sharp, as the precipitated barium carbonate acts as a white background for the colour change. The hydrogen ion concentration was then adjusted to *p*H 2 by adding sufficient $N/1$ HNO_3 to make the total volume added 48.5 c.c.

The solution remaining was colourless and the chloride was estimated by titrating with $M/50$ mercuric nitrate, using 1.5 c.c. of 1 per cent diphenylcarbazone in alcohol as indicator (Roberts, 1936). The large amount of excess acid necessary was due to the buffering action of the hexamine.

It was found by using a *p*H meter that the addition of the amounts of reagents described always resulted in the correct *p*H. Blanks were carried out for both titrations using all the reagents. It was necessary to make a correction for the CO_2 produced by the breakdown of phosgene ; this correction was in practice very small, and is described below.

The efficiency of absorption of phosgene

As the procedure described above and the concentration of the reactants were not quite the same as we normally used for sampling and analysing phosgene concentrations, tests were carried out as follows: a phosgene–air mixture in which the $COCl_2$ was about 1,000 mg./cu.m. (which is higher than any concentration used in the experiment) was sucked through two bubblers in series, using the absorbent mixture described ; in a set of three experiments no chloride was detected in the second bubbler.

The efficiency of absorption of CO_2

The first experiments were carried out with ordinary bead bubblers, but the efficiency of absorption of CO_2 with 15 c.c. in each bubbler and rates of flow of 3–5 litres/min. was in no case greater than 50 per cent. In order to increase the efficiency larger amounts of the reagents and Dreschel bottles with sintered glass diffuser plates were used. With a total of 75 c.c. of reagents, efficiencies of over 80 per cent with a rate of flow of 3 litres/min. were obtained.

It was found that owing to small physical differences the efficiencies of the bubblers varied. Each bubbler had therefore to be standardized and its efficiency measured.

This can be done (1) if the exact concentration of CO_2 bubbled through is known or (2) by using a long train of bubblers so that the leak at the end is negligible. However, the exact determination of the CO_2 without the use of bubblers is not an easy task, and the use of a long train of bubblers is clumsy and involves a large number of titrations ; also the back pressure set up by these bubblers is high, and this causes added complications. In view of the above the following device was used.

If the efficiency of absorption is independent of the CO_2 concentration, imagine two bubblers of unequal efficiency, arranged in series.

Let the fractional leak of the first bubbler be L_1 and that of the second L_2. Then if the concentration of CO_2 entering the first bubbler is c, cL_1 leaves the first bubbler and enters the second and cL_1L_2 leaves the second.

$\therefore$ Concentration absorbed by the first bubbler $= c(1-L_1)$ which is proportional to T_1
and " " " " second " $= cL_1(1-L_2)$ which is proportional to T_2
where T_1 and T_2 are the titrations in bubbler 1 and 2.

$$\text{Then } \frac{T_2}{T_1} = L_1\frac{(1-L_2)}{1-L_1} = \text{say } R_1$$

if the order of the bubblers is reversed we have

$$\frac{T_2}{T_1} = L_2\frac{(1-L_1)}{1-L_2} = \text{say } R_2$$

Then by simple algebra

$$L_2 = R_2\frac{(1+R_1)}{1+R_2} \text{ and } E_2 = (1-L_2)$$

$$L_1 = R_1\frac{(1+R_2)}{1+R_1} \text{ and } E_1 = (1-L_1)$$

Hence the efficiencies (E_1 and E_2) of the bubblers may be found without knowing the actual concentration of gas passing. All that is required is the ratio R_1 of the titrations when arranged in a given order and the ratio R_2 when that order is reversed.

Independence of efficiency and CO_2 concentration

The above assumes that the efficiency is independent of the CO_2 concentration. This was tested in the following manner.

Dreschel bottles 1 and 2 were set up in series, each bottle containing 25 c.c. of $N/1$ NaOH and 50 c.c. of 6 per cent (w/v) hexamine as before. Air was drawn through the chamber containing the rats and then through the bubblers at a rate of 3 litres/min.; four experiments were carried out and the concentration of CO_2 varied by altering the number of rats. Each run lasted 10 min., and in each case a duplicate run was made with the order of the bubblers reversed.

The results were:

Expt.	Order	No. of rats	Blank minus titration		R_1	R_2	Efficiency per cent	
			Bubbler	Bubbler			Bubbler	Bubbler
			(1)	(2)			(1)	(2)
1	1–2	1	4·00	0·55	0·137		86	
	2–1		4·30	0·80		0·186		82
2	1–2	2	7·25	1·05	0·149		85	
	2–1		7·60	1·40		0·184		82
3	1–2	3	10·40	1·70	0·163		84	
	2–1		10·50	1·95		0·185		82
4	1–2	4	16·65	2·45	0·147		85	
	2–1		11·95	1·95		0·163		83

Mean efficiencies: (1) 85 and (2) 82 per cent.

Hence it would appear that the efficiency of absorption of the bubblers is independent of the CO_2 concentration over the range actually used during the experiment.

The calculation of the CO_2 output of the rats in a concentration of phosgene

The bubblers were standardized by the method described and their efficiencies recorded.

An example is given to show the calculations involved: 4 rats in the chamber. Air flow through each bubbler 3 litres/min. Efficiency of bubblers: (1) 85 and (2) 82 per cent.

Bubbler (1) was connected in position A (see Fig. 5) and absorbed CO_2 from air and

rats, bubbler (2) in position B absorbed CO_2 from the air only. Expected phosgene concentration 500 mg./cu.m. Duration of run 10 min.

Bubbler A (rats and air)		Bubbler B (air only)	
$COCl_2$ titration	CO_2 titration	$COCl_2$ titration	CO_2 titration
c.c. of $M/50$ $Hg(NO_3)_2$	c.c. of $N.HNO_3$	c.c. of $M/50$ $Hg(NO_3)_2$	c.c. of $N.HNO_3$
Blank .. 0·10	24·80	0·10	24·80
Test .. 14·50	12·65	15·80	23·50
Difference 14·40	12·15	15·70	1·30

$COCl_2$: 1 ml. of $M/50$ $Hg(NO_3)_2 \equiv 0{\cdot}99$ mg.$COCl_2$

average titration $\frac{A+B}{2} = 15{\cdot}05$. $\therefore$ Concentration $= \frac{15{\cdot}05 \times 0{\cdot}99 \times 1000}{30} = 497$ mg./cu.m.

Hence $Ct = 4{,}970$ mg.min./cu.m.

CO_2 correction

In the hydrolysis of $COCl_2$, CO_2 will be formed. Correction is made for this here, but as may be seen the effect of this correction is normally so small that it may be ignored.

If x is the $COCl_2$ titration (c.c. of $M/50$ $Hg(NO_3)_2$) then

correction of CO_2 will be simply $\frac{x}{50}$ c.c. *N.* acid.

CO_2 : 1 c.c.*N.*acid $\equiv 11{\cdot}2$ c.c. CO_2

Total CO_2 through bubbler A $\equiv \frac{12{\cdot}15}{0{\cdot}85} = 14{\cdot}30$ c.c.*N.*acid

Correction for $COCl_2$ $\equiv \frac{14{\cdot}4}{50} = 0{\cdot}28$ c.c.*N.*acid

Difference (CO_2 from rat and air) $\equiv 14{\cdot}02$ c.c.*N.*acid

Total CO_2 through bubbler B $\equiv \frac{1{\cdot}30}{0{\cdot}82} = 1{\cdot}58$ c.c.*N.*acid

Correction for $COCl_2$ $\equiv \frac{15{\cdot}7}{50} = 0{\cdot}31$ c.c.*N.*acid

Difference (CO_2 from air only) $\equiv 1{\cdot}27$ c.c.*N.*acid

Hence CO_2 from 4 rats $\equiv 14{\cdot}02 - 1{\cdot}27 = 12{\cdot}75$ c.c.*N.*acid

and this is equivalent to 143 c.c. CO_2.

Hence CO_2 output per rat was 3·57 c.c./min.

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THE CHOLINESTERASE INHIBITING ACTION OF FLUOROPHOSPHONATES

BY

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From the Physiological Laboratory, Cambridge

(Received November 18, 1946)

Recently the cholinesterase inhibiting action of fluorophosphonates has been used for physiological experiments and in clinical trials. Our knowledge that these compounds had such an action dates back to experiments carried out in this laboratory during the early part of the war, but the results could not be published at that time. The first compound tested was the dimethyl fluorophosphonate, and its cholinesterase inhibiting action was found in 1941 (Adrian, Feldberg, Kilby, and Kilby). In 1942 the more toxic di*iso*propyl fluorophosphonate was prepared by McCombie and Saunders and found to have the same action (Barrett, Feldberg, Kilby, and Kilby). Since then various workers have examined in detail the cholinesterase inhibiting activity of this and other related fluorophosphonic esters.

The pharmacological action of the fluorophosphonates when injected into animals or inhaled by men or animals suggested the possibility of such mode of action. The pharmacological effects of these compounds will be described shortly (Kilby and Kilby). The most conspicuous effect in man on exposure to the vapours of these substances was a long-lasting miosis associated with a spasm of accommodation. The effect started a few minutes after the exposure and lasted for a few days, or even up to a week in the case of the di*iso*propyl ester. In the absence of other central effects in man on exposure to these compounds in low concentrations, it seemed unlikely that the effects were due to a central action. They suggested rather a local effect on the eye by absorption through the mucous membrane, and this was shown by the fact that if one eye was protected from the vapour, the pupil of that eye did not constrict. A peripherally produced miosis of such long duration at once suggested the possibility that these compounds might act not directly on the smooth muscles in the eye, but indirectly, like eserine, by inhibiting the action of cholinesterase.

To test this possibility we first examined the effect of fluorophosphonates on the isolated rabbit's intestine. On such a preparation the action of drugs which, like acetylcholine, act directly on the muscle differs characteristically from that of those which, like eserine, act by inhibition of cholinesterase activity. The

directly acting drugs produce an immediate contraction which proceeds rapidly to a maximum, and after the drug has been washed out the muscle again quickly relaxes. The contraction produced by cholinesterase inhibiting drugs, such as eserine, is characterized by its long latency, its slow development, and its very gradual disappearance when the drug is washed out. The fluorophosphonates when tested on the isolated rabbit's intestine produced a contraction resembling in its details that produced by eserine and not that produced by acetylcholine. The contraction produced by the di*iso*propyl ester, for instance, persisted for hours after washing out the drug. We therefore proceeded to test the effects of these compounds on the activity of plasma cholinesterase.

METHODS

The cholinesterase inhibiting activity of the fluorophosphonates was compared quantitatively with that of eserine sulphate, the procedure being as follows. To 0.2 c.c. of heparinized human plasma was added 0.5 c.c. of a solution containing either eserine or the fluorophosphonate in varying concentrations; then the mixture was kept at room temperature for 10 minutes before 1 μg. acetylcholine in 1 c.c. saline solution was added. After 5 minutes standing at room temperature, the mixture was made up to 10 c.c. with frog saline containing eserine 1 in 100,000, which at once stops the action of any cholinesterase not yet inactivated. The solution was then assayed for acetylcholine on the frog rectus muscle preparation.

RESULTS

Both the dimethyl and di*iso*propyl ester were found to inhibit the cholinesterase activity of human plasma, and their action was stronger than that of eserine tested in a similar way. Of the two esters, the di*iso*propyl had a more powerful cholinesterase inhibiting action than the dimethyl ester. An accurate quantitative comparison was made of the action of the di*iso*propyl ester with that of eserine sulphate. It was found that this ester in a concentration of 1 in 80 millions had a more powerful action than eserine 1 in 15 millions, but a weaker action than eserine 1 in 14 millions. Therefore, under the conditions of our experiment, the ester at 1 in 80 millions had about the same cholinesterase inhibiting action as eserine sulphate at 1 in 14½ millions, i.e., the di*iso*propyl ester was about 5½ times as active as eserine sulphate when compared weight for weight, which is about 3 times as active when compared in molar solutions.

DISCUSSION

Our results show that the fluorophosphonates have a cholinesterase inhibiting action which is stronger than that of eserine. The pharmacological actions of these compounds as far as they resemble those of acetylcholine, therefore, can be explained by the inhibition of cholinesterase and an accumulation of acetylcholine.

Dixon *et al.* (1942, 1944), when later on comparing the cholinesterase inhibiting action of the di*iso*propyl ester with that of eserine, found that the difference

between the activity of the two was much greater than in our experiments. This discrepancy may well be due to the difference in the procedure adopted by Dixon *et al.*, because the cholinesterase preparations were exposed to the action of the drugs for longer periods than in our experiments. We know, however, that eserine is slowly destroyed by cholinesterase, so that its action would diminish over longer periods. Fluorophosphonates apparently cause an irreversible inhibition, as shown by Dixon *et al.*

SUMMARY

The dimethyl and di*iso*propyl fluorophosphonates have a strong cholinesterase inhibiting action. This action explains the acetylcholine-like effects of these substances. The dimethyl ester was the first to be investigated (1941). Subsequent work on other compounds of the series has confirmed their action.

We are indebted to the Director-General of Scientific Research (Defence) for permission to publish the above, and to Dr. H. McCombie, Dr. B. C. Saunders, and their research team for supplying the di*iso*propyl fluorophosphonate used.

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THE POSSIBILITY OF TOXIC EFFECTS FROM 2:3-DIMERCAPTOPROPANOL IN CONDITIONS OF IMPAIRED RENAL OR HEPATIC FUNCTION

BY

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(Received November 21, 1946)

If 2:3-dimercaptopropanol (BAL) is to be adopted as a remedy for arsenical and other forms of poisoning, it is necessary that hazards associated with its use be understood. Toxic effects from overdosage with BAL have already been defined (Peters, Stocken, and Thompson, 1945; Waters and Stock, 1945), and the minimal lethal dose of numerous samples determined. Appreciation of such risks has led to the dose for human use being placed well below the fatal level. But there remains a problem which, so far as we know, has not been considered: the possibility of harmful effects in individuals who are suffering from impaired function of vital organs. Two such instances at once come to mind: damage to the kidneys and the liver. Both organs are concerned in the elimination and detoxication of drugs and poisons, and though their action on BAL is not clear it is likely they are important therapeutic safeguards. We have therefore carried out experiments in which the toxicity of BAL for normal animals has been compared with that for animals suffering from severe renal and hepatic damage. Such complications are frequent with arsenical poisoning and the conditions against which arsenic is employed therapeutically; in these disturbances BAL should be of value.

METHODS

Fully grown male and female rabbits and rats were used. The former averaged 2 kg. body weight, the latter 200 g. Pregnant females were avoided. All were kept on the standard laboratory diet throughout the experimental period and were given water. Severe renal disease in both rabbits and rats was produced by subcutaneous injection of 5 mg./kg. uranium acetate in aqueous solution, hepatic damage in rabbits by subcutaneous injection of 1 c.c./kg. redistilled carbon tetrachloride and in rats by injection of 6 c.c./kg. of this compound. Preliminary studies showed that the uranium acetate induced massive destruction of renal tubules and evidence of serious renal functional impairment in 48 hours, which proved fatal in 5–9 days (one example is given in Table I). Carbon tetrachloride led to severe liver destruction within 24 hours, which resulted in the death of about 20 per cent

of the animals in a week after injection. Uranium acetate thus produces serious renal disease and hardly any liver damage, carbon tetrachloride gives extensive liver destruction but scarcely any renal change.

TABLE I

BODY WEIGHT, BLOOD UREA, AND URINARY FINDINGS IN A RABBIT WITH URANIUM ACETATE INTOXICATION. NO TREATMENT

Time interval	Body weight (kg.)	Blood urea (mg. per cent)	Urine				
			Amount excreted in 24 hours (c.c.)	Total urea in 24 hours (g.)	Specific gravity	Albumin	Microscopic findings
Normal period							
1st day ..	2·75	28·5	125	2·3	1022	Nil	Nil
2nd day ..	3·10	27·0	143	2·02	1020	Nil	Nil
3rd day ..	2·90	23·5	246	3·1	1018	Nil	Nil
4th day ..	2·82	34·0	370	3·5	1016	Nil	Nil
5th day ..	2·82	34·0	175	1·74	1020	Nil	Nil
6th day ..	2·80	34·5	160	2·17	1022	Nil	Nil
Uranium acetate (5 mg./kg.) given on 6th day							
7th day ..	2·90	37·0	185	2·87	1022	Present	Nil
8th day ..	2·80	73·0	215	2·34	1024	Present	Red blood cells
9th day ..	2·70	159·0	38	0·37	1030	Present ++	Red blood cells casts
10th day ..	2·70	300·0	Nil	0·37	1030	Present ++	Red blood cells casts
11th day ..	2·60	348·0	Nil	0·37	1030	Present ++	Red blood cells casts
13th day ..	2·20	288·0	5	0·005	1030	Present ++	Granular casts
14th day ..	2·30	376·0	4	0·14	1030	Present	Granular casts
15th day ..	Dead						

Two, three, and four days after the injection of uranium acetate and one day after carbon tetrachloride, BAL (in the form of BAL 4 g., benzylbenzoate 8 c.c., peanut oil to 50 c.c.) was injected intramuscularly into groups of 10 or 15 such animals, in doses varying from 60 to 120 mg./kg. Equal groups of normal animals were injected in parallel with similar doses of BAL. Since it is a matter of experience that deaths from BAL poisoning occur within a few hours of administration we have accepted a time limit of 24 hours and attributed all deaths taking place during that period to BAL. In most animals death resulted within 4 hours, which is the experience of other workers. Toxic symptoms were carefully noted. Post-mortem examination of fatal cases and microscopic examination of the chief organs, especially of liver and kidneys, were carried out on most of the animals. Material was fixed in 10 per cent formol alcohol, embedded in paraffin and sections stained with Ehrlich's acid haematoxylin and eosin, Weigert's iron haematoxylin and Van Gieson, Heidenhain's azan method for kidneys. Frozen sections were also prepared in some cases and stained for fats.

RESULTS

Tables II and III summarize the experiments.

1. Toxicity of BAL in animals with severe renal damage

Table II shows that there is no difference in reaction to BAL between rabbits and rats with severe renal damage developing two days after injection of uranium acetate and normal animals. Mortality rates are closely in agreement in both series. Toxic symptoms are similar and seldom occur in animals other than those dying from BAL intoxication. In other words, the presence of serious renal disturbance

TABLE II

TOXICITY OF BAL IN ANIMALS WITH RENAL IMPAIRMENT 2, 3, AND 4 DAYS AFTER ADMINISTRATION OF URANIUM ACETATE. DEATHS DURING THE 24-HOUR PERIOD FOLLOWING INJECTION OF BAL

Dose of BAL (mg./kg.)	No. of animals	No. dying	Per cent mortality	No. showing toxic symptoms (percentage in brackets)	Dose of BAL (mg./kg.)	No. of animals	No. dying	Per cent mortality	No. showing toxic symptoms (percentage in brackets)
	"Renal damage" group					*Normal group*			
RABBITS									
(i) Two days after subcutaneous injection of uranium acetate (5 mg./kg.)									
100	15	14	93	15 (100)	100	15	13	87	15 (100)
80	15	8	53	9 (60)	80	15	8	53	9 (60)
60	15	3	20	3 (20)	60	15	2	13	3 (20)
40	15	0	0	0 (0)	40	15	0	0	0 (0)
(ii) Three days after subcutaneous injection of uranium acetate (5 mg./kg.)									
100	10	10	100	10 (100)					
80	10	10	100	10 (100)					
60	10	5	50	5 (50)					
40	10	1	10	1 (10)					
(iii) Four days after subcutaneous injection of uranium acetate (5 mg./kg.)									
100	10	10	100	10 (100)					
80	10	9	90	10 (100)					
60	10	5	50	5 (50)					
40	10	1	10	1 (10)					
RATS									
Two days after subcutaneous injection of uranium acetate (5 mg./kg.)									
140	10	10	100	10 (100)	140	10	8	80	10 (100)
120	10	8	80	10 (100)	120	10	5	50	5 (50)
100	10	4	40	4 (40)	100	10	2	20	2 (20)
80	10	0	0	0 (0)	80	10	0	0	0 (0)

does not alter the LD50 or lead to unexpected signs of BAL intoxication. So far as we can judge, renal upset is not increased by BAL, nor can we find any microscopical evidence of more severe renal structural changes in that group. Since few animals survived the experiment, we have not attempted an analysis of the influence of BAL on the survival time of nephrotic subjects.

Rabbits given BAL three and four days after injection of uranium acetate show somewhat decreased tolerance for BAL (Table II). Thus 50 per cent of animals in both groups died after 60 mg./kg. BAL as against a fatal level of 80 mg./kg. with normal animals. The LD50 has thus been decreased a little by the damage to the kidneys. At this stage the animals were very ill, their renal function being grossly impaired as indicated by the very small amount of urine excreted, the high blood urea, and the severe destruction of renal structure shown microscopically. Reference to Table I brings out these features. On the fourth day of uranium poisoning there is often complete suppression of renal function and the animals may be moribund. It is therefore reassuring that, despite such severe renal disturbance, the toxicity of BAL is not greatly increased.

TABLE III

TOXICITY OF BAL IN ANIMALS WITH HEPATIC IMPAIRMENT PRODUCED BY INJECTION OF CARBON TETRACHLORIDE. DEATHS DURING THE 24-HOUR PERIOD FOLLOWING INJECTION OF BAL

Dose of BAL (mg./kg.)	No. of animals	No. dying	Per cent mortality	No. showing toxic symptoms	Per cent showing toxic symptoms	Dose of BAL (mg./kg.)	No. of animals	No. dying	Per cent mortality	No. showing toxic symptoms	Per cent showing toxic symptoms
RABBITS		"*Liver damage*" *group*						*Normal group*			
		24 hours after carbon tetrachloride (1 *c.c./kg.*)									
80	10	9	90	10	100	80	15	8	53	9	60
60	10	2	20	9	90	60	15	2	13	3	20
40	10	1	10	8	80	40	15	0	0	0	0
RATS											
		24 hours after carbon tetrachloride (6 *c.c./kg.*)									
						140*	5	4	80	5	100
120	10	6	60	10	100	120	15	4	27	4	27
100	10	6	60	10	100	100	15	0	0	3	20
80	10	1	10	8	80	80	15	0	0	0	0
60	10	0	0	6	60	60	15	0	0	0	0

2. Toxicity of BAL in animals with severe liver damage

Table III shows an increased mortality (90 per cent) in the rabbits with liver damage during the 24 hours after administration of 80 mg./kg. BAL as compared with the normal group (53 per cent). This difference is statistically significant. A difference is present too in the experiment with rats. In the "hepatic damage" group, 6 of 10 rats (60 per cent) receiving 120 mg./kg. BAL died during the 24-hour period as against 4 of 15 rats (27 per cent) in the normal group; with 100 mg./kg. BAL 6 of 10 of the hepatic damage group died as against none of 15 normal animals. An even more striking feature of the hepatic damage group

is the development of toxic symptoms in the animals receiving low doses of BAL, the normal controls with the same dose of BAL showing no such symptoms. No microscopic evidence of an effect on the structural damage in the liver, attributable to BAL, was obtained ; but the deaths occurred in so short a time after giving BAL that this is not surprising. It seems from these experiments that a somewhat increased toxic activity may be expected when BAL is administered to animals suffering from serious liver damage. It may be conjectured that the normal liver plays a part in detoxication of BAL.

Conclusions

By means of experiments on rabbits and rats we have tried to find out whether untoward effects may arise from the use of BAL in conditions of impaired function of the kidneys and liver. Renal destruction was produced with uranium acetate (Boycott and Douglas, 1914–15) in doses sufficient to damage severely or destroy the greater part of the tubular system, the glomeruli apparently escaping. Carbon tetrachloride was used to damage the liver in quantities which destroyed at least one half, and more often two thirds, of the liver cells (Cameron and Karunaratne, 1936). In both instances the injurious actions were confined to the organ specified, though there is some evidence that other tissues are also damaged in these intoxications ; our dosages apparently succeeded in checking such additional effects. When BAL was injected into animals with severe renal disease no evidence of more severe toxic effects than in normal animals was obtained until the animals were suffering from complete suppression of renal function. Even then the effect was not striking. With hepatic disturbance, however, there was enhanced toxicity of BAL, though here again lowered resistance was not pronounced. It was shown by the occurrence of toxic symptoms with low doses of BAL (40 mg./kg. in rabbits and 60 mg./kg. in rats), though seldom of death. Clearly, our experiments suggest that BAL should not give rise to anxiety when used in conditions of renal impairment unless this is very severe, but that caution should be exercised if there is reason to suspect hepatic insufficiency. Nevertheless, the latter hazard need not contraindicate the careful use of BAL, since tolerance is not unduly diminished in conditions of severe liver damage. We would venture the opinion that BAL might have an unfavourable action on the course of hepatic diseases if used light-heartedly without careful attention to the patient's condition. We gather from Professor R. A. Peters that he has formed a similar impression from his study of the use of BAL in the treatment of infectious hepatitis.

Summary

1. The toxicity of BAL for animals with severe renal or hepatic damage has been compared with that in normal animals.

2. Animals with renal damage differed in no way from normal controls in their response to large doses of BAL. With complete or almost complete failure

of renal function there was a lowered tolerance to BAL, but this was not pronounced.

3. Animals with hepatic damage experienced toxic symptoms and some died after doses of BAL well below the fatal level for normal animals.

4. It is suggested that care should be exercised when BAL is given to patients suspected of impaired liver function. The presence of severe renal disease does not appear to be a contraindication to BAL.

We are indebted to the Director-General of Scientific Research (Defence) for permission to publish this paper, and to Prof. R. A. Peters, F.R.S., for much assistance.

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