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THE ASSAY OF ANTI-ACETYLCHOLINE AGENTS FOR ANTAGONISM OF PILOCARPINE-INDUCED SALIVATION IN RABBITS

BY

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Experimental conditions affecting tests of atropine-like agents for antagonism of pilocarpine-induced salivation in rabbits have been examined. A simple method of assay of such agents is described. It gave results of fair accuracy and reproducibility, permitted a full statistical analysis, and provided an estimate of the error.

The effect of drugs on salivary flow has been examined in experimental animals by several techniques. Cushny (1920) used dogs with salivary fistulae, and Bülbring and Dawes (1945) measured the flow of saliva from the submaxillary gland of anaesthetized cats by cannulation of Wharton's duct. Issekutz (1917), who collected the saliva which dripped from the mouths of rabbits under urethane, reported that 1.5 to 2.0 mg. atropine stopped pilocarpine-induced salivation. This last method, which requires little technical skill, has been adapted by many workers, but there has been little detailed analysis of the optimal conditions. Furthermore, statistical evaluation of the results has rarely been attempted and the accuracy of the methods is therefore virtually unknown. Brown and Werner (1949), indeed, found such variability between responses of different rabbits that they regarded any quantitative assay as impracticable.

This paper describes conditions that affect the responses and the accuracy of the assay and outlines a simple method which gives results whose accuracy can be readily and fully assessed.

Analysis of Factors Influencing Salivary Flow

Use of Urethane.—Unlike several workers who used unanaesthetized animals (e.g., Fromherz, 1933; Lands, Nash, and Hooper, 1946; Chen, 1954), we could not regularly obtain a suitable amount of saliva from rabbits which had received no urethane. However, a large oral dose of urethane 1 g./kg. or more was undesirable, for the narcotic action lasted some 6 to 10 hr., and the animals lost their cough and swallowing reflexes completely (Brown and Werner, 1949; Brown, Thompson, Klahm, and Werner, 1950; Hoekstra, Tisch, Rakieten, and Dickison, 1954; Tripod, 1949). The dose of urethane is therefore critical.

The variation in the amount of saliva collected in 30 min. with the dose of urethane was determined after various doses of pilocarpine, for one set of eight rabbits (Fig. 1). The drugs were given in random order, over 4½ weeks, each animal receiving every possible combination. At all three doses of pilocarpine, the slopes of the urethane dose-response lines were very steep. Indeed, the urethane dose appeared to control the response more effectively than did the pilocarpine. The urethane probably did not affect the actual rate of salivation, but only increased the efficiency of collection.

An oral dose of 0.5 to 0.625 g./kg. of urethane was effectively sedative to the rabbits without producing loss of consciousness, and a good collection of saliva was possible. Collection was more efficient from rabbits given 1.0 g./kg. urethane, but, owing to the risk of harmful effects to the animals, 0.5 to 0.625 g./kg. was preferred.

The urethane was administered orally in 25 ml. saline to ensure adequate hydration and a good reserve of body water upon which to draw during and after the profuse salivation.

No other anaesthetic was tried in these investigations.

Dose of Pilocarpine.—Doses of pilocarpine used by previous workers in this field have ranged from 0.25 mg./kg. (Fromherz, 1933) to 100 mg./kg. subcutaneously (Graham and Lazarus, 1940). The relation between the dose of pilocarpine nitrate and the salivary response was investigated over the range of 1.25 to 5.0 mg./kg., at three

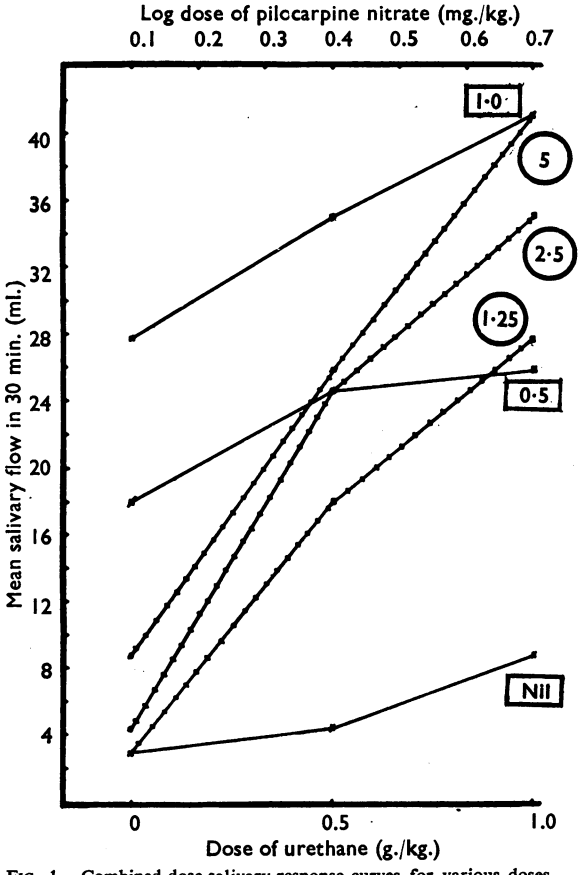

FIG. 1.—Combined dose-salivary response curves for various doses of urethane and pilocarpine, in a set of 8 rabbits. ———— Pilocarpine (log dose on top abscissa) after various doses of urethane (g./kg., in squares). |—|—|—|—| Urethane (bottom abscissa) preceding various doses of pilocarpine (mg./kg., in circles).

different doses of urethane (Fig. 1). The pilocarpine nitrate was injected subcutaneously 30 to 45 min. after the oral dose of urethane. The rabbits were then placed in their stocks and the saliva which dripped from their mouths was collected.

In the absence of urethane, an increase in the dose of pilocarpine from 1.25 to 5.0 mg./kg. had little effect on the small amount of saliva obtained. On the other hand, with 0.5 g./kg. and 1 g./kg. of urethane, the response to even 1.25 mg./kg. pilocarpine was considerably more than that to 5 mg./kg. after no urethane. When 0.5 g./kg. of urethane was given, there was little difference between the amounts of saliva secreted after the injection of 2.5 mg. and 5 mg./kg. pilocarpine. However, the higher dose was used throughout the investigations, since, after it, salivation was of convenient duration (90 min.) and of suitable intensity—not too readily antagonized by drugs, yet susceptible enough to give a sensitive assay.

Choice of Animals.—Rabbits of 1.5 kg. and over were used. Differences in sex and weight did not appear to influence the response to the drugs.

The rabbits showed enormous variability in their sensitivity to atropine. In about half of them the salivary response to pilocarpine was not affected significantly by doses of atropine up to 80 μg./kg., given subcutaneously 15 min. before the pilocarpine. On the other hand the remainder showed about a 50% decrease in flow after 5 to 10 μg./kg., and almost a 100% decrease after 25 μg./kg. This insensitivity to atropine was presumably due to the presence in the blood of an enzyme—atropinesterase (Glick, 1940)—capable of hydrolysing atropine. Serum from several resistant rabbits was able to destroy atropine *in vitro*.

Although both Glick and Glaubach (1941) and Ammon and Savelsberg (1949) have reported widely ranging figures for the effective atropinesterase activity of the blood of those rabbits possessing it, we found that rabbits showed either a large (>50%) reduction in salivary flow after 25 μg./kg. atropine, or else an insignificant reduction (<20%) after double or treble that dose. Consequently, once a consistent salivary response to the pilocarpine alone had been obtained in a rabbit, a single test with 25 μg./kg. atropine given 15 min. before the pilocarpine was sufficient for the assessment of atropine resistance. All the animals showing less than a 50% inhibition of salivation by this dose were rejected.

Interval Between Administration of Atropine and of Pilocarpine.—In order to find the optimal time interval between the injection of atropine, or similar drugs, and of pilocarpine, a random design assay was performed on one set of eight rabbits, in which the antagonism of salivary flow was measured for four different atropine-like drugs given 0, 5, 15, 35, and, on two occasions, 70 min. before the injection of pilocarpine. The drugs used were atropine sulphate, atropine methyl nitrate, benactyzine (β-diethylaminoethyl benzilate HCl) and oxyphenonium (phenylcyclohexyl glycollic acid ester of β-diethylaminoethanol methobromide). The animals received 0.5 to 0.625 g./kg. urethane orally and 5 mg./kg. pilocarpine nitrate subcutaneously.

The mean percentage inhibitions of salivation are given in Table I and the time-response curves are plotted in Fig. 2.

Atropine appeared to exhibit maximal activity when administered about 15 min. before the pilocarpine; its effect after 35 min. was less than after 5 or 15 min. Atropine methyl nitrate had a more prolonged action which reached a maximum when it was given about 35 min. before pilo-

TABLE I

THE EFFECT OF VARYING THE INTERVAL BETWEEN THE INJECTIONS OF AN ATROPINE-LIKE DRUG AND OF PILOCARPINE ON THE INHIBITION OF SALIVARY FLOW

Drug	Dose μg./kg. s.c.	Mean % Inhibition of Salivation at Different Times (min.) Between Injection of Drug and Pilocarpine				
		0	5	15	35	70
Atropine SO_4 ..	5	32·6*	45·4	55·1	36·9*	
Atropine methyl nitrate	2	24·4*	35·6*	49·9	59·3	53·5
Benactyzine	750	36·5	30·5	24·5	22·3	
Oxyphenonium ..	5	48·9*	60·9	63·9	78·5*	78·5*

S.E. of each mean, 7·2.
* Response significantly different from that at 15 min. ($P < 0.05$).

carpine. Even 70 min. after administration, atropine methyl nitrate still showed a greater effect than after 0 to 15 min. Oxyphenonium, the other quaternary compound, gave identical results. With benactyzine, however, maximal activity was obtained when the drug was given at the same time as pilocarpine.

It thus appears that the times at which different atropine-like drugs exert maximal activity after subcutaneous injection vary considerably. In the assay method described subsequently, the time between administration of the atropine-like drug and the pilocarpine was 15 min., in order to standardize conditions. The activity displayed under these conditions may not be maximal, particularly with compounds containing a quaternary nitrogen atom, and the relative potencies in terms of atropine might thus be smaller than could otherwise be obtained.

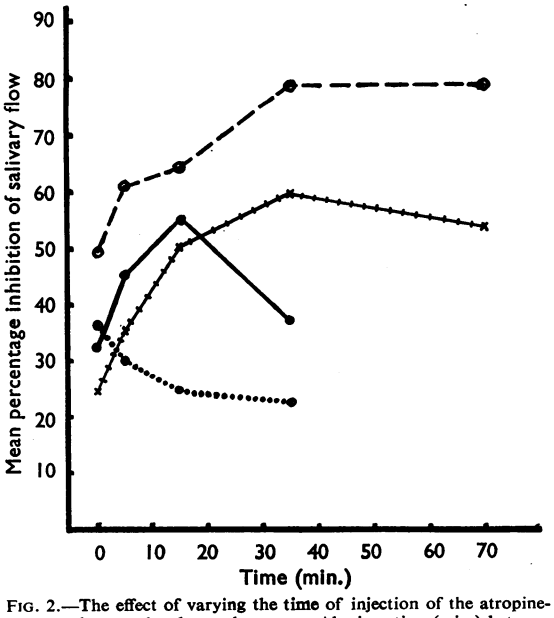

FIG. 2.—The effect of varying the time of injection of the atropine-like drug on the observed potency. Abscissa, time (min.) between injections of the drug and of pilocarpine (5 mg./kg.). Ordinate, mean percentage inhibition of salivary flow, for 8 rabbits. All drugs injected subcutaneously. ●————● Atropine sulphate 5 μg./kg. ×—|—|—|—× Atropine methyl nitrate 2 μg./kg. ●·····● Benactyzine HCl 750 μg./kg. O — — — O Oxyphenonium bromide 5 μg./kg.

ANALYSIS OF RESULTS

Regular gradation of response with dose was shown in atropine-sensitive rabbits—that is, a regular decrease of salivary flow was obtained with increasing doses of atropine. The response of each animal was expressed as the percentage depression of the total volume of saliva collected after a dose of atropine or similar agent, compared with the mean of the volumes obtained from the same animal in two control experiments. The results obtained from each of eight animals in a set were averaged. In practice, control experiments were performed only once in every six to twelve experiments, the responses in all those tests falling between any two controls being expressed with reference to the mean of these two control values. The average standard deviation of such a mean control value, calculated from figures for each of eight rabbits tested over nine months, was 10.9 ml. Collection of saliva was maintained for 90 min. after the rabbits had received pilocarpine and had been placed in their stocks. By the end of this time, the flow had ceased in all except a few control observations where it had fallen to a rate of below 0.2 ml./min.

Eight animals for each experiment gave results of adequate accuracy. A set of animals could be used twice weekly, with only very occasional rests, for at least 15 months without any ill-effects or tolerance.

A large number of drugs could therefore be compared for their activity in antagonizing pilocarpine-induced salivation, on the same set of eight rabbits.

The time course of the salivation obtained from a set of eight rabbits in control experiments and in tests with various doses of atropine is given in Fig. 3. Where no atropine was given, the salivation response to pilocarpine appeared to fall away exponentially with time. If moderate to strong doses of atropine were given 15 min. before the pilocarpine, however, the time-log response lines appeared to curve. This might indicate that atropine was less readily eliminated or removed from the receptors than pilocarpine.

The dose-response relation for atropine was investigated several times, on two different sets of rabbits. Good approximations to a linear log dose-response line were obtained over the 20 to

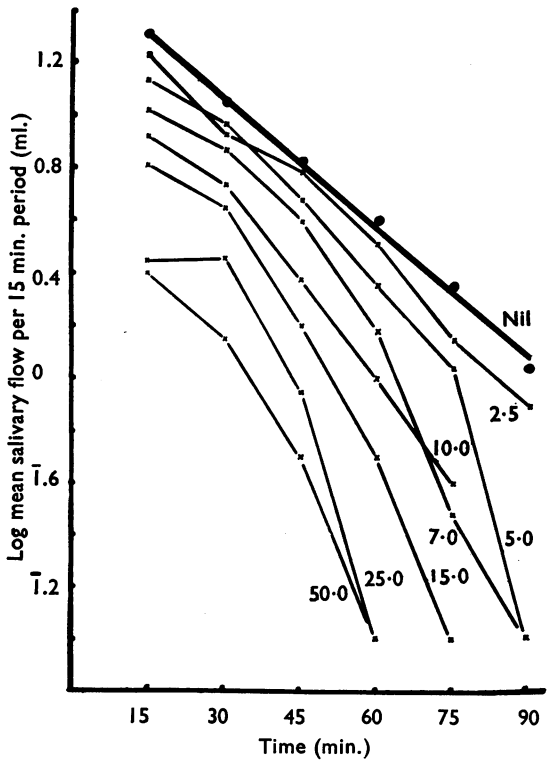

FIG. 3.—Time-salivary flow curves for a set of 8 rabbits, after 5 mg./kg. pilocarpine nitrate, preceded by various doses of atropine. Dose of atropine in μg./kg. is given alongside the appropriate line. Abscissa, time (min.) after injection of pilocarpine. Ordinate, mean log volume (ml.) of saliva for each rabbit, measured for 15 min. periods.

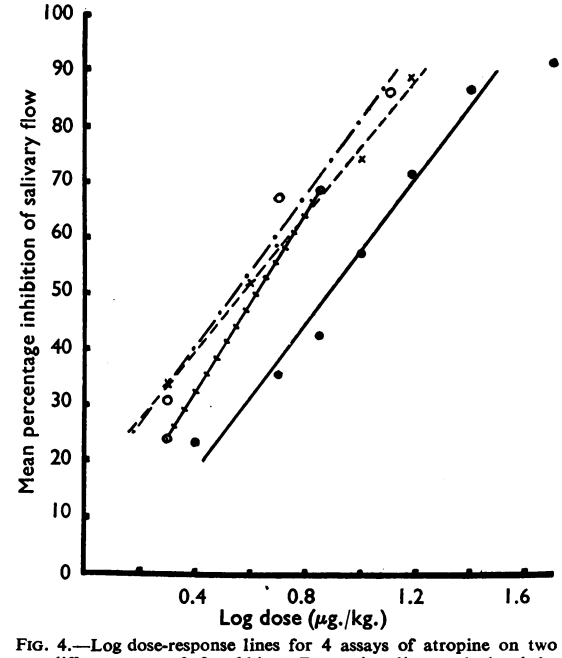

FIG. 4.—Log dose-response lines for 4 assays of atropine on two different sets of 8 rabbits. Regression lines calculated by method of least squares. ●———● Assay 5/55 on set A rabbits, × — — — × Assay 8/55, O — · — O Assay 2/56, ⊙—|—|—⊙ Assay 2/56 on set B rabbits.

90% maximal response range, and regression lines were fitted by the method of least squares (Fig. 4). The variance due to deviation from linearity was never statistically significant (i.e., $P<0.05$). Results were analysed by the standard analysis of variance method; one set of results is given in Table II. Although atropine sulphate was used, the doses were always calculated in terms of the base.

Table III gives data from five different assays, four on one set of rabbits and one on another. There is a close similarity of the results in Set B. The difference in the estimates of ED50 for atropine between the two sets of rabbits is largely an indication of differences encountered in the sensitivity of individual rabbits to atropine—even those apparently entirely lacking atropinesterase. The differences in response of these two sets of rabbits emphasize the need to compare anti-acetylcholine agents in the same group of rabbits. Many different compounds were so tested and

TABLE II

DOSE-INHIBITION OF SALIVATION DATA FOR ATROPINE ON ONE SET OF 8 RABBITS

Rabbit	% Decrease in Salivary Flow 15 min. after Atropine s.c.				Total
	Dose of Atropine (μg./kg.)				
	2	4	10	15	
1	−34	41	30	77	114
2	67	97	82	91	337
3	6	32	57	77	172
4	51	33	86	94	264
5	70	100	95	100	365
6	16	38	72	77	203
7	28	46	71	97	242
8	69	29	100	98	296
Total ..	273	416	593	711	1,993
Mean ..	34·1	52·0	74·1	88·9	

Slope $b=61{\cdot}1$. ED50 = 3·6 μg./kg.

ANALYSIS OF VARIANCE

Source of Variance	Sum of Squares	d.f.	Mean Square	F	P
Regression	13,903	1	13,903	39·2	<0·01
Deviation from linearity	65	2	32·5	10·9	>0·05
Between doses	13,968	3			
Between animals ..	12,488	7	1,784	5·0	0·01–0·1
Residual error	7,440	21	354·3		
Total	33,896	31			

$\lambda=0{\cdot}31$ where $\lambda=\sqrt{\frac{\text{Mean Square Error}}{b}}$ S.E. of $b=9{\cdot}7$.

TABLE III

RESULTS OF VARIOUS ASSAYS OF ATROPINE FOR INHIBITION OF SALIVATION

Rabbits:	Set A	Set B			
Assay No.	1	1	2	3*	4*
Date of Assay	5/55	8/55	2/56	2/56	4/56
No. of dose-levels used	6	4	3	2	2
Slope (b)	65·7	61·1	68·0	81·3	60·3
S.E.b	11·5	9·7	7·1	17·9	12·4
ED50 (μg./kg.)	7·5	3·6	3·4	4·2	5·1
Limits of error (P=0·05) of ED50	5·8–9·9	2·9–4·8	2·8–4·2	3·5–4·9	4·2–6·1
λ	0·40	0·31	0·20	0·30	0·22

* Part of a randomized design assay incorporating two other compounds.

their potency referred constantly to that of the standard (atropine), whose effective potency appeared to stay reasonably constant for at least nine months.

Results for Other Atropine-like Agents

In addition to atropine, several other anti-acetylcholine agents were tested by this method for their antagonism of induced salivation. Atropine methyl nitrate, oxyphenonium, and propantheline were tested on rabbits of Set B at about the same time as the second atropine assay (Table III); several doses were used in each assay. For all these compounds, doses were calculated in terms of the salt. The calculated regression lines are shown in Fig. 5, and the analysis of the results given in Table IV. The variance due to linear regression was highly significant ($P<0.01$) and that due to deviation from linear regression not significant ($P>0.2$).

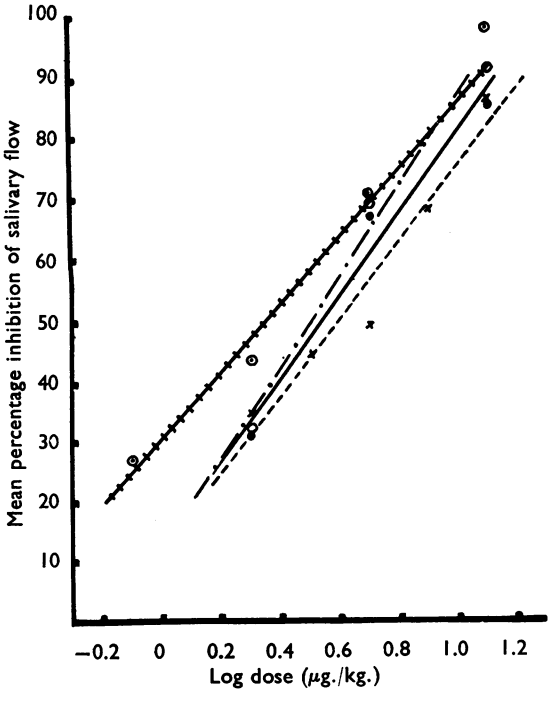

FIG. 5.—Log dose-response lines for: ●———● Atropine sulphate. ⊙—!—!—⊙ Atropine methyl nitrate. ×— — —× Oxyphenonium. O — · — O Propantheline. All results obtained on same set of 8 rabbits.

TABLE IV

ASSAYS OF ATROPINE-LIKE DRUGS FOR INHIBITION OF SALIVATION

Compound	Atropine (Sulphate)	Atropine Methyl Nitrate	Oxyphenonium	Propantheline
No. of dose-levels tested	3	4†	5	3
Slope (b)	68·0	55·3	63·7	74·3
S.E.b	7·1	15·2	9·4	10·2
ED50 (μg./kg.)	3·4	2·2	3·9	3·2
Limits of error of ED50 (P=0·05)	2·8–4·2	1·4–3·6	3·2–4·8	2·6–4·0
Approx. relative potency*	1·0	1·5	0·9	1·1
λ	0·20	0·41	0·25	0·21

* $= \frac{\text{ED50 Atropine}}{\text{ED50 Compound}}$

† Only 3 used in the analysis, the fourth giving a response over 90% of maximal.

The relative potencies of the three compounds in terms of atropine are expressed as the ratios of their ED50s (Table IV). There was little difference in the activity of these four agents, although atropine methyl nitrate appeared slightly more potent than the tertiary base.

(2+2) Assay

The assessment of the relative potency of one drug in terms of another from dose-response data obtained at different times must, however, inevitably be liable to more error than that derived from tests carried out under identical conditions for both drugs. An assay method incorporating randomized doses was therefore adopted, in which, at any one time, the four pairs in a set of eight rabbits received different doses. Two doses of each compound giving respectively about a 35% and 70% response were administered

TABLE V

COMPARISON OF ACTIVITIES OF ATROPINE-LIKE DRUGS WITH THAT OF ATROPINE

	λ	Log Relative Potency (M)	S.E. of M	Rel. Potency	Limits of Error of Rel. Potency (P=0·95)
Assay 1: Combined b=65·6	0·30				
Atropine x atropine $MeNO_3$		0·357	0·104	2·28	1·40–3·70
x homatropine HBr		−2·039	0·109	0·0091	0·0055–0·0152
Assay 2: Combined b=59·8	0·22				
Atropine x tricyclamol Cl		−0·211	0·086	0·62	0·41–0·93

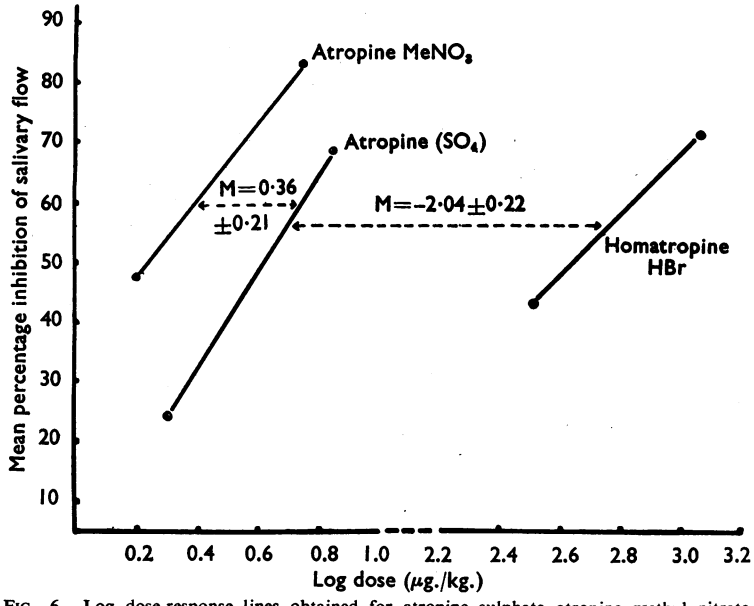

FIG. 6.—Log dose-response lines obtained for atropine sulphate, atropine methyl nitrate, and homatropine HBr in a 2 + 2 + 2 assay on a set of 8 rabbits. Values for M (log relative potency) ± its limits of error ($P=0.95$) are given, with atropine taken as standard.

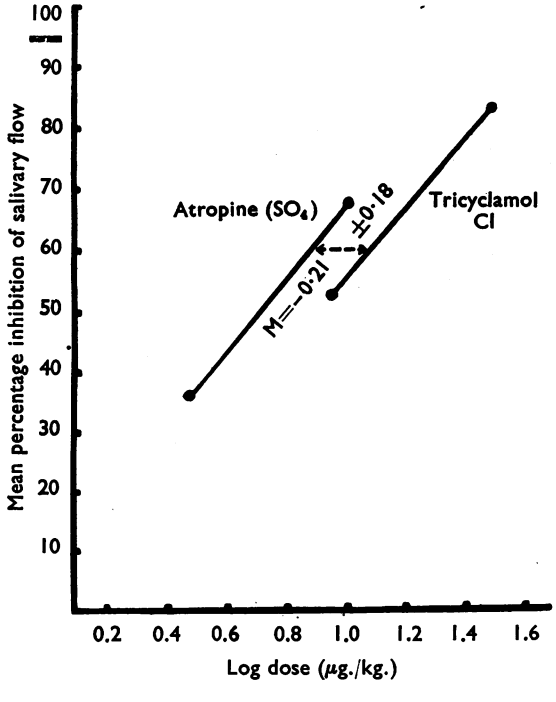

FIG. 7.—Log dose-response lines obtained for atropine sulphate and tricyclamol Cl in a 2 + 2 assay on a set of 8 rabbits. Log relative potency (M) ± its limits of error ($P=0.95$) are given, with atropine taken as standard.

subcutaneously. The ratio of the two doses was the same for all compounds in an assay. In the first assay, three compounds were tested—atropine sulphate, atropine methyl nitrate, and homatropine HBr. The doses were given in random order over six experiments. The assay results were compared with the mean result of two control experiments performed just before and just after the assay. In a second assay, tricyclamol chloride was tested against atropine in a similar manner. Analysis of results followed the same lines as before, with adaptations of the Schild (1942) and Holton (1948) methods of evaluating 2+2 assays.

The dose-response lines obtained in these assays can be seen in Figs. 6 and 7, and the relevant data tabulated in Table V. Variance due to regression was always highly significant ($P<0.01$), whereas that due to deviation from parallelism was not ($P>0.2$).

The relative potencies of atropine methyl nitrate, homatropine HBr, and tricyclamol in terms of atropine were 2.28, 0.0091, and 0.62 respectively; the difference in activity between the test compound and atropine was statistically significant with all the drugs.

DISCUSSION

Rabbits fully sensitive to atropine are essential for a reliable assay, since the dose of atropine producing 50% inhibition of salivation in about half the animals tested was more than ten times that necessary in the other half. Serum from the former rabbits destroyed atropine *in vitro*, whereas serum from the atropine-sensitive rabbits did not. Other atropine-like drugs are also susceptible to destruction in the serum of certain rabbits; homatropine, for example, is almost as readily attacked as atropine (Ammon and Savelsberg, 1949). On the other hand, Trasentin (Glick, 1942) and Trasentin 6H and Lachesine (Blaschko, Chou, and Wajda, 1947) have been reported to be unaffected by atropinesterase, and it would be possible, therefore, to compare these compounds on atropine-resistant rabbits. Other more recently synthesized atropine-like compounds

do not appear to have been investigated for their susceptibility to destruction by atropinesterase.

The incidence and transmission of atropine resistance in rabbits have been investigated by Sawin and Glick (1943) and others; it seems to be at least partly controlled by hereditary factors. Consequently, it would be expected that for any one strain the atropine-resistance factor would be present to a fairly consistent degree, but that there would be a variation between different strains. About 90% of our "English" rabbits, bred locally, were atropine-sensitive, whereas roughly a similar percentage of various blue "Beveren" and black rabbits obtained from another dealer were resistant. According to Sawin and Glick, there may be a relation between the presence of atropinesterase in the blood and the extent of black pigment in the rabbit's coat.

Several authors testing anti-acetylcholine agents for salivary blockade in rabbits have reported a wide variability in sensitivity to atropine, but none has related this to the presence of an enzymic mechanism for destroying atropine. Brown and Werner (1949), for instance, reported that 0.075 to 0.3 mg./kg. atropine intramuscularly "markedly inhibited salivary secretion in some animals, but had little effect in others." They therefore used a quantal response, and reported that the minimal dose of atropine which would reduce the salivary flow to below 20 ml. in 2 hr. in five out of six rabbits tested was 600 μg./kg. At least some of the animals used by these workers probably possessed atropinesterase in their blood.

Cahen and Tvede (1952), in a quantal assay of salivary blockade in rabbits, showed that the percentage (probit) of rabbits with at least a 50% inhibition of pilocarpine-induced salivation was linearly related to the log dose of atropine. The ED50 for atropine by this method was 62 μg./kg., whereas assessment by graded response in the same 40 animals gave an ED50 of 110 μg./kg. Such a difference between ED50 values might be expected if it is assumed that certain of these 40 rabbits (but less than half of the total) possessed atropinesterase. Thus, a dose of atropine just sufficient to cause a 50% inhibition of salivation in the sensitive animals—giving an ED50 in quantal response—would produce a mean inhibition of considerably less than 50% on the graded response scale, when the almost negligible responses of the atropine-resistant animals are taken into account.

Cahen and Tvede eventually regarded the rabbit as an unsuitable test animal "because of the variability of the individual responses and its unusual tolerance to atropine." We have found, however, that rabbits without atropinesterase are very sensitive to atropine. In the rabbit the dose of atropine causing a 50% reduction in pilocarpine-induced salivation (3–7 μg./kg. s.c.) is considerably less than that needed in the mouse for a 50% maximal increase in pupil size (100 μg./kg. s.c.) and similar to that causing a 50% inhibition of the hypotensive response to a just-maximal dose of acetylcholine in the cat (1 μg./kg. i.v.) (Brown, Quinton, Acred, Bainbridge, and Turner—unpublished observation). Lands *et al.* (1946) also quote a low dose (1 μg./kg. i.v.) for a 70% inhibition of pilocarpine-induced salivation in rabbits.

Certain workers have administered atropine intravenously (Lands *et al.*, 1946; Tripod, 1949; Chen, 1954; Luduena and Lands, 1954). Atropinesterase may not have time seriously to affect the action of atropine given in this way, particularly when, as in the technique of Luduena and Lands, the salivary flow is measured for two periods of only 10 min. each, immediately before and after the injection of atropine. This point was therefore investigated in two sets of eight rabbits. In the first set, composed entirely of atropine-sensitive animals, the mean salivary response in the 10 min. after an intravenous injection of 10 μg./kg. atropine was $6.0 \pm 2.0\%$ (S.E.) expressed as a percentage of the amount of saliva obtained in the preceding 10 min. In the other set of animals, all of which possessed atropinesterase in their blood, the corresponding figures were $63.6 \pm 12.3\%$ (S.E.). Pilocarpine nitrate (1 mg./kg.) was given to both groups 15 min. before the atropine. The difference between the mean salivary responses for the two groups is highly significant. It appears therefore that even with intravenous administration of atropine it is still necessary to use atropine-sensitive rabbits, since the atropinesterase affects the activity of a low dose of atropine even within 10 min. of intravenous injection.

In order to minimize variation between different rabbits, drugs were compared in the same animals. By the use of a (2+2) or (2+2+2) design whereby each of a set of eight rabbits received each of two dose-levels of the drugs tested, the relative potency in terms of a standard has been obtained with its limits of error ($P=0.95$). The values for λ (the standard deviation of a single log dose) ranged from 0.20 to 0.40. We found the relative potencies of atropine methyl nitrate and homatropine in terms of atropine to be 2.3 and 0.009 respectively. Issekutz (1917) reported that atropine methyl nitrate was 3 to 4 times, and homatropine about one fifth, as potent as atropine,

whereas according to Luduena and Lands (1954) the methyl nitrate was eight times as potent as atropine on intravenous administration. It is possible that slowness of absorption of the quaternary compound after subcutaneous injection may partly account for the lower potency obtained by us.

We have not found any reports on the activity of tricyclamol, oxyphenonium, and propantheline in antagonizing pilocarpine-induced salivation in rabbits, although Drill (1954) quotes from some unpublished data supplied by Clark and co-workers that " the intrinsic anti-salivary activity (of oxyphenonium) assayed in rabbits is 1.85 times greater than that of atropine." We have found the relative potency of tricyclamol to be 0.62. The approximate relative potencies for oxyphenonium and propantheline, derived by comparing ED50 values from responses in the same rabbits but not at the same times, were 0.9 and 1.1 respectively.

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