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ASSESSMENT OF DEPRESSOR ACTIVITY AND MYDRIATIC ACTIVITY OF HEXAMETHONIUM ANALOGUES

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Most of the techniques used in our laboratory for the pharmacological study of compounds synthesized as possible depressor agents are well known; their merits and demerits have received much attention. There are some, however, which have been used comparatively seldom for studies of this sort. Therefore, in the hope that our experience may be of use to other workers in the field, we are describing certain types of experiment which we perform frequently.

Since most of the compounds which we have tested resemble hexamethonium fairly closely in chemical structure, and since the latter is believed to lower blood pressure mainly—perhaps wholly—by blocking autonomic ganglia, we have been interested in techniques for assessing ganglion-blocking activity. The fact that changes in pupil size can be readily measured with mice by the Pulewka technique has been exploited by several workers (Wien and Mason, 1951; Edge, 1953; Schwarzacher and Stumpf, 1954), who have shown how the mydriatic effect of hexamethonium-like compounds may be used as a measure of their ganglion-blocking activity. Further details about the measurement and comparison of the mydriatic effects of hexamethonium-like compounds are given below.

Although a technique depending essentially upon the measurement of the degree of ganglion-blockade obtained at a particular site may furnish valuable information about the potency of hexamethonium analogues, the possibility that at least some of the latter may lower blood pressure by mechanisms other than ganglion-blockade makes it imperative that their depressor activity should be assessed mainly by a direct method. We have therefore tried to develop a reliable technique for comparing depressor effects in animals under conditions not far removed from those obtaining clinically. The "ear capsule" technique introduced by Grant and Rothschild (1934) for measuring the blood pressure of the unanaesthetized rabbit appealed to us for the following reasons: (1) No complications due to the anaesthetic arise; (2) because measurements are made with the animal upright, a ganglion-blocking agent produces a much greater depressor effect than it would were the animal lying on a table (owing to abolition of the reflexes which oppose gravitational pooling of the blood); (3) it is possible, in order to avoid changes in sensitivity due to cumulation, or to the development of tolerance, to inject compounds at relatively infrequent intervals (this is not readily accomplished in acute non-survival experiments); (4) it is easy to inject drugs intravenously into rabbits, which are comparatively cheap and readily available.

METHODS

Rabbits.—White rabbits of an inbred strain and weighing 1.5–2.0 kg. were used. Their blood pressures were recorded with a modified Grant ear capsule. This capsule differed from that described by Grant and Rothschild (1934) mainly in that the membrane—for which sausage skin is suitable—was transilluminated from a torch bulb mounted within the pressure chamber. The rabbits were placed in an electrically heated box, which was warmed to, and then maintained at, body temperature before any measurements were made. The minimum pressure required to occlude the central artery of the right ear was recorded once or twice a minute until steady readings were obtained. The drug (in 0.3 ml. isotonic solution) was then injected into the marginal vein of the left ear. Injections of potent drugs were made no more often than once every 3 days. Each rabbit was given alternately a standard drug (hexamethonium or pentolinium) and the compound under investigation, and pairs of rabbits were used to improve the comparison of the two compounds by a "cross-over" test.

Mice.—Mydriatic effects were measured by Pulewka's method (Burn, 1950). The mice were kept for at least 30 min. in separate beakers under bright illumination before their pupil diameters were measured with a dissecting microscope containing an arbitrary scale in the eyepiece. To make the illumination as uniform as possible, the beakers containing the mice were placed beneath long low-power fluorescent tubes and on top of glossy white paper. Drug solutions were given intraperitoneally, and

changes in pupil size were measured at fixed times afterwards.

Preliminary experiments were performed with batches of 20 mice divided into groups of 5. One mouse of each group was given a control injection of 0.9% NaCl (0.01 ml./g. body weight); the others were given 20 mg./kg. (2 mice) or 40 mg./kg. (2 mice) of one of the compounds under investigation dissolved in saline. One group of each batch received the same dose of hexamethonium bromide. Later, saline controls were dispensed with, and the compounds were given in distilled water.

In further experiments with the more active compounds, 10 of a group of 20 mice were given hexamethonium bromide at two different dose levels and 10 the compound under investigation, also at two different dose levels. The estimate of equipotent doses thus obtained was used as the basis of a second similar experiment designed to give a better comparison.

Compounds.—Most of the compounds referred to by code name in Table II are of structure N–X–N, 2Br, where X is a hydrocarbon chain usually containing 5–7 carbon atoms joining the two substituted ammonium groups. Structural details of such compounds are given in Table I. "Ecolid" (Ciba Su-3088) is ethylene-1-trimethylammonium-2-(*N*-methyl-4′5′6′7′-tetrachloro)-*iso*indolinium dichloride. The

TABLE I
STRUCTURAL DETAILS OF COMPOUNDS

Code Name	Hydrocarbon Residue	Cationic Groups
Pentolinium	Pentamethylene	*N*-Methyl-pyrrolidinium
"Gaplegin"	Hexamethylene	Ethyl-dimethylammonium
NP328 ..	,,	*N*-Methyl-morpholinium
NP73 ..	Pentamethylene	2-Hydroxyethyl-dimethylammonium
Hexamethonium	Hexamethylene	Trimethylammonium
NP325	,,	2-Hydroxyethyl-dimethylammonium
"Pendiomide"	$-CH_2CH_2N(CH_3)CH_2CH_2-$	Ethyl-dimethylammonium
NP266 ..	Pentamethylene	*N*-Methyl-morpholinium
NP263	,,	*N*-Ethyl-piperidinium
NP336 ..	Hexamethylene	*N,N*-Diethyl-anilinium
NP15 ..	Ethylene	2-Hydroxyethyl-dimethylammonium
NP270 ..	Pentamethylene	*n*-Propyl-dimethylammonium
NP277 ..	Tetramethylene	2-Hydroxyethyl-diethylammonium
NP251 ..	*p*-Xylylene	Trimethylammonium
NP253 ..	,,	2-Hydroxyethyl-dimethylammonium
NP255 ..	,,	*N*-Methyl-morpholinium

Burroughs Wellcome compound 288C53 has the structure

$$(C_6H_5)_2C=CH-(CH_2)_4-N(CH_3)_2-CH_2CH_2-N(CH_3)_2CH_2CH_3,\ 2I$$

Compound SC-1919, from G. D. Searle & Co., is *n*-

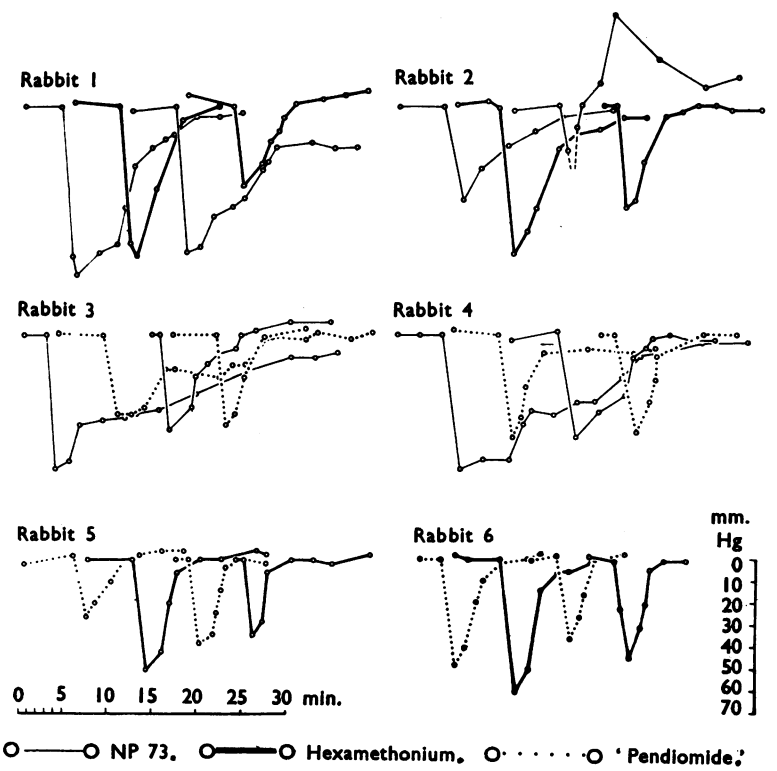

FIG. 1.—Effects on the blood pressure of unanaesthetized rabbits of quaternary ammonium compounds each given intravenously in a dose of 0.3 ml. of a 0.085M solution. The 4 injections given to each of the 6 rabbits were made at intervals of 3 days. The results of these cross-over experiments indicate that hexamethonium and NP73 (hexamethylene bis-2-hydroxyethyldimethylammonium-bromide) are almost equally active in lowering the blood pressure and distinctly more active than "Pendiomide" on a molar basis.

butyl-triethylammonium bromide. Compounds of the "NP" series were synthesized in the department.

RESULTS

Experiments on Rabbits

After a few days' experience of the procedure most rabbits gave blood-pressure readings which did not vary by more than 5–10 mm. Hg from day to day, or when measured repeatedly over 30–60 min. A few rabbits which continued to give much more variable readings were rejected.

The reliability of each observer's results was checked by testing him with "unknown" solutions, some of which were inactive. No serious difficulty was experienced in detecting the active compounds, even when the observer was relatively unpractised in the technique, so long as the rabbits had been accustomed to the procedure.

A typical experiment with 6 rabbits gave the results illustrated in Fig. 1. Batches of 6 rabbits were generally employed, each rabbit being given 2 different compounds twice in 10 days. A semi-quantitative comparison of the depressor activities of a number of compounds was made by (1) judging from pilot experiments what doses are approximately as potent as the standard dose of hexamethonium, and (2) comparing the effects of these doses by a cross-over experiment of the type illustrated in Fig. 1. Typical results are given in Table II.

TABLE II

DEPRESSOR AND MYDRIATIC ACTIVITIES OF VARIOUS QUATERNARY AMMONIUM COMPOUNDS

Equidepressor doses in the rabbit were estimated as shown in Fig. 1. Equidepressor doses in man were calculated from clinical data (Smirk, 1952, and unpublished observations). Compounds for which no rabbit dose is given had negligible depressor activity when 25 μM were given intravenously

Code Name	Equidepressor Doses (μM)		Depressor Activity[1]	Mydriatic Activity[2]
	Rabbit	Man		
"Ecolid" ..	2·5	4	+++++	+++++
Pentolinium ..	5	6	++++	++++
288C53	10	40	+++	+++++
"Gaplegin" ..	20	25	++	++
NP328	23	—	++	+++
NP73	23	50	++	+++
Hexamethonium	25		++	++
NP325	30	—	++	++
"Pendiomide" ..	30	50	++	++
NP266 ..	30		++	+++
SC-1919(=NP179)	50		+	++
NP263	50		+	+++
NP336			−	++
NP15			−	++(+)
NP270			−	++(−)
NP277			−	+
NP251			−	−
NP253			−	−
NP255			−	−
Choline[3] ..			−	−
Betaine[3]			−	−

[1] Based upon experiments on rabbits.

[2] Based upon preliminary experiments on mice. When subsequent experiments with larger numbers of mice altered the previous estimate of activity, the new estimate is given in parentheses.

[3] Used as controls in experiments with "unknown" solutions.

Although some experiments extended over several weeks no significant tolerance developed. But if hexamethonium was injected frequently (0.3 ml. of a 0.085M solution given subcutaneously twice a day), rabbits did develop some tolerance to it; within a fortnight the test dose of hexamethonium given intravenously would produce little or no fall of blood pressure.

Experiments on Mice

Variation in Control Readings.—Spontaneous variation in pupil size was small over one period of observations. The pupil diameters of the mice which received injections of saline alone remained practically constant. No change was observed with 140 out of 180 mice. Of the remaining 40, 37 showed only a slight (less than 20%) increase in pupil size and 3 an equally slight decrease. In view of these results, saline controls were dispensed with in later experiments.

Variation from mouse to mouse was not much greater. For more than 80% of the mice the initial pupil diameter was 3–5 (arbitrary) units, and all readings were within the range 2–7. Even when an ordinary bench lamp was used to keep the mice under bright illumination and the lighting was far from even, the variation from mouse to mouse was not obviously more than when the illumination was provided by long fluorescent tubes; but it was important to have as uniform illumination of the pupil as possible at the time of measurement. Less variation was obtained when an auxiliary light was attached to the microscope. To ensure a minimum initial value of the pupil diameter it was important to accustom mice to the bright illumination for 30–45 min. before taking the initial reading.

Mydriatic Effect of Hexamethonium.—Doses of the order of 10 mg./kg. of hexamethonium bromide produce considerable mydriasis in mice (Wien and Mason, 1951; Edge, 1953). Our mice were approximately as sensitive to hexamethonium as the mice used by these workers. We noted a 2–3-fold increase in pupil size with 10 mg./kg., and a 3–4-fold increase with 20 mg./kg., of an aqueous solution of hexamethonium bromide injected intraperitoneally. For economy, we used young mice (6–8 weeks old and weighing approximately 20 g.) of homogeneous stock; but older mice were about as sensitive to hexamethonium on a weight basis.

Observational Errors.—With the eyepiece scale used, the possible error in each reading was about half to one unit. Since the value of an increase in pupil diameter involves the difference between two readings, the possible observational error in the estimate would range from about 7% to as much as 50% according as the difference was as large as 15 units (equal to 1.1 mm.) or as small as 4 units (0.3 mm.). This suggests that measuring the total pupil diameter only would give a better estimate of the degree of mydriasis, provided that the initial pupil diameter did not vary in one direction. However, analysis of such results as those given in Table IV shows no appreciable difference whether the increase in pupil size or the total diameter of the pupil was used.

The only serious discrepancy noticed in the measurements of 4 different observers working on different days lay in the time at which the maximum mydriatic effect was obtained; one observer noted it much later (30–40 min.) than the others (10–20 min.). It was then found that the former had been injecting hexamethonium bromide dissolved in 0.9% NaCl, the latter hexamethonium bromide dissolved in distilled water. The mydriatic effects of the two solutions were therefore compared simultaneously on batches of 20 mice. These experiments showed that the maximum response was obtained less rapidly when hexamethonium was given in saline (Fig. 2); nevertheless the difference was smaller than that expected from the difference between observers.

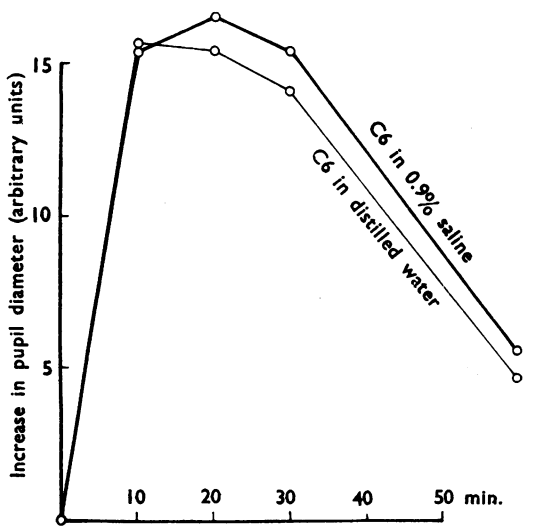

FIG. 2.—Mydriatic effect of hexamethonium bromide (20 mg./kg.) in mice at different times after its intraperitoneal injection. The upper (heavy) curve gives the mean increase for 10 mice given 0.2% hexamethonium dissolved in 0.9% NaCl; the lower curve gives the mean increase for 10 mice given hexamethonium dissolved in distilled water.

Tolerance to Hexamethonium.—As Mohanty (1955) has shown, mice develop considerable tolerance to the mydriatic action of hexamethonium. We have performed experiments similar to his, taking the mean figures for groups of 15 mice given hexamethonium daily (Table III), and some in which the mice were individually identified (Table IV). The latter showed that even by the third day there was a statistically significant

TABLE III

DEVELOPMENT OF TOLERANCE TO THE MYDRIATIC ACTION OF HEXAMETHONIUM

Fifteen mice were used for each experiment. The mice were given one test dose (5 mg./kg.) and two larger doses (each 20 mg./kg.) of hexamethonium bromide each day for 5 days. The mean increases in pupil size given below were measured 15 min. after the intraperitoneal injection of the test dose

Day	Increase in Pupil Size (Arbitrary Units)		Mean Initial Pupil Diameter (Arbitrary Units)
	Mean	S.D.	
1	4·33	2·28	4·33
2	3·87	2·64	4·46
3	2·67	2·41	4·67
4	2·20	1·65	4·60
5	2·00	1·73	4·46
1	4·40	2·30	4·20
2	3·34	1·56	4·93
3	2·20	1·59	4·40
4	1·87	1·31	4·74
5	1·33	1·20	4·60

TABLE IV

DEVELOPMENT OF TOLERANCE TO THE MYDRIATIC ACTION OF HEXAMETHONIUM IN INDIVIDUAL MICE

Hexamethonium bromide (20–40 mg./kg.) was given by intraperitoneal injection 3 times each day for 5 days to 36 individually marked mice from 6 litters. On days 1, 3, and 5, a test dose (5 mg./kg.) was given intraperitoneally before the first large dose of the day. The increase in pupil diameter was recorded 18 min. after the injection of the test dose

Litter No.	Day of Observation	Increase in Pupil Diameter (Arbitrary Units)	
		Bucks	Does
1	1	4 6 5 5 4	3
	3	4 4 3 7 4	1
	5	3 3 3 4 3	1
2	1	3 6 9 3	4 4
	3	2 3 6 2	5 7
	5	1 2 2 2	1 1
3	1	2 3 4	3 4 6
	3	0 3 3	1 3 1
	5	2 0 1	1 2 1
6	1	5 2 11	3 3 7
	3	3 3 8	1 3 2
	5	4 3 7	3 0 1
5	1	2 6	4 5 4 5
	3	2 3	2 6 5 3
	5	0 1	2 1 3 2
4	1	2	2 2 2 4 4
	3	2	0 2 1 1 3
	5	1	0 1 0 1 3

reduction in the response to hexamethonium. As tolerance developed, the decrease in mydriatic effect was roughly proportional to the initial response to hexamethonium, and few mice did not appear to develop tolerance under these conditions (in which the mice were given two loading doses as well as the smaller test dose of hexamethonium each day). On the other hand, the test dose of hexamethonium bromide (10 mg./kg.) could be given several times without the development of a significant degree of tolerance so long as there was an interval of several days between successive doses.

Selection of Mice.—Since it proved difficult to obtain a reliable comparison of different ganglion-blocking agents with small numbers of mice, because of the large variation in the mydriatic response of individual mice, experiments were performed to see whether the variation could be reduced by using litter-mates. An analysis of variance of the results obtained with 36 mice (from 6 litters, each of 6 albino mice, all born within 3 days of one another of parents from a homogeneous population, and containing 18 mice of each sex) showed the within-litter variation to be significantly less ($0.01>P>0.001$) than the between-litter variation. There was also a significant ($P<0.001$) sex difference: the bucks were more sensitive to hexamethonium than were the does. Thus it seems advisable to use mice of the same sex for experiments whose results are to be compared.

In a further experiment, these 36 mice were divided into two equal groups. Each group was treated in turn with hexamethonium at different times on the same day and under the same lighting conditions. Analysis of variance of the results showed both a litter effect and a sex effect significant at the 5% level, and, unexpectedly, an effect due to division of the mice into two groups, significant at the 5% level.

The difficulty of evaluating the factors responsible for variation in the response to hexamethonium became even more evident when an attempt was made to group mice on the basis of their apparent sensitivity to the drug. Three groups of 15–20 mice were treated successively with hexamethonium bromide (20 mg./kg. intraperitoneally), and all those (actually 43%) for which the increase in pupil size differed from the mean by more than 3 units were rejected. When the remaining 30 mice were given a second dose of hexamethonium, the standard deviation was as large as before selection of the mice.

Effects of Other Compounds.—Our chief aim in performing the following experiments was to discover whether the testing of hexamethonium-like compounds for mydriatic activity, which can be done rapidly and cheaply with mice, is likely to give a useful indication as to which compounds possess strong depressor activity. Of the 270 quaternary ammonium compounds tested, 150 were of the type $N—(CH_2)_n—N$, where n = 2, 3, 4, 5, 6, 10, or 12, and the other groups attached to the nitrogen atoms are alkyl or hydroxyalkyl chains or aromatic groups. In another 60 compounds the two nitrogens were linked by such groups as

$—CH_2CH_2OCH_2CH_2—$ and $—CH_2C_6H_4CH_2—$.

The remaining compounds had but one quaternary nitrogen: 22 were of structure $X—N(CH_3)_3$, and the rest differed from hexamethonium still more.

Most of the compounds dilated the mouse pupil to a detectable extent when they were injected intraperitoneally in a dose of 20 or 40 mg./kg., but fewer than twenty possessed activity comparable to that of hexamethonium. With compounds of the type $(CH_2)_n$ $[NRR'R'']_2$ we found high activity only when n was 5 or 6 and the groups R, R′ and R″ together contained no more than 6 carbon atoms—the best cationic heads were such groups as the trimethylammonium, 2-hydroxyethyldimethylammonium, *N* - methylmorpholinium, and *N*-methyl-pyrrolidinium. None of the compounds with a single quaternary ammonium group possessed striking activity. As our main findings agree with those of other workers, such as Wien and his colleagues (1952, 1954), we shall only describe those that indicate defects in this method of assessing ganglion-blocking activity.

Firstly, we should mention that a number of compounds produced mydriasis by mechanisms other than ganglion-blockade. A few were subsequently shown to be atropine-like. With certain other compounds, such as *n*-amyl-trimethylammonium and its near homologues, dilatation of the pupil shortly preceded the death of the animal and seemed to be due, like the accompanying convulsions, to a nicotine-like action. Even when mydriatic activity was apparently due to ganglion-blockade, we sometimes found that the compound had much less depressor activity in the unanaesthetized rabbit than was expected from a large effect on the mouse's pupil (Table II). An outstanding example of this behaviour was provided by the compound 288C53.

Secondly, it is possible that compounds which have little or no mydriatic activity may yet have strong depressor activity. For example, a ganglion-blocking compound which acted almost exclusively on the sympathetic division of the autonomic system would behave in this manner, because the mydriasis produced by hexamethonium-like compounds depends upon blockade of the ciliary ganglion, which lies in the parasympathetic division. We therefore tested a selection of the compounds which had little effect on the mouse pupil on the unanaesthetized rabbit. None of the compounds tested was strongly depressor. However, not much weight can be given to negative results of this type.

Discussion

The important theoretical advantages of determining effects on blood pressure by the Grant ear-capsule technique in rabbits have already been mentioned. In practice, the results obtained with this method have been in good agreement with the results obtained by clinical bio-assay (Table II). The method requires neither expensive apparatus nor much experimental skill. On the other hand, few observations can be made in a 2-hr. session and a satisfactory cross-over experiment takes more than a week to perform. Apart from giving a good indication of the effect of a compound on blood pressure and heart rate, the method enables certain other useful information to be obtained. For example, the fact that the sharp falls of blood pressure obtained with ethoxyethyltrimethylammonium bromide were accompanied by such parasympathomimetic effects as profuse salivation, pupillary constriction, urination and defaecation suggested that this compound is muscarine-like in action—a conclusion reached long ago by Dale (1914). Again, certain of the close chemical relatives of hexamethonium which might otherwise have been of interest because of their depressor activity caused a flaccid paralysis with arrest of respiratory movements. It was noted that when these compounds, and a variety of others, were tested on Bülbring's rat diaphragm preparation, certain of them acted like decamethonium, though more weakly; but this form of activity did not appear to be closely correlated with ability to produce a flaccid paralysis in the rabbit. This is not surprising, for decamethonium and certain related compounds are known to produce paralysis by at least two distinct mechanisms (Zaimis, 1953).

If, as is widely believed, hexamethonium-like compounds lower blood pressure solely by blocking ganglia, the order of activity found in the experiments on rabbits should be roughly the same as that obtained with techniques for measuring ganglion-blocking activity. Some of the discrepancies observed can be seen in Table II. Possibly these may arise through compounds blocking the various ganglia to different degrees. For example, the disparity between the mydriatic and depressor effects of 288C53 may be due to its acting far more powerfully on parasympathetic ganglia than on sympathetic ganglia. However, the recent work of Dontas and Nickerson (1955) suggests that, even with hexamethonium itself, the possibility that the fall of blood pressure is brought about by mechanisms other than ganglion-blockade cannot be ignored. This is one of several reasons why we place especial reliance on the comparison of the depressor effects obtained in the unanaesthetized animal.

There are theoretical objections to using the increase in pupil size as a measure of the potency of hexamethonium-like compounds. Thus mydriasis can be produced by several distinct mechanisms. And even if the action of a compound is confined to ganglia, its action on the ciliary ganglion could conceivably be opposed by an action on the corresponding sympathetic (superior cervical) ganglion. The possibility that blocking actions at these two sites might be distinguished through the use of atropine was investigated by Edge (1953), who gave hexamethonium to atropinized mice to see whether it might produce in them a relative miosis. However, under the conditions of his experiment, hexamethonium had no effect on pupil size. Thirdly, there is the possibility—suggested by much experimental evidence—that the relative blocking activity of hexamethonium analogues varies from one type of ganglion to another.

In view of these objections, we hoped no more of the method than that it would be of value as a "sieving" technique—we wished to discover quickly which of a large number of compounds being tested for useful ganglion-blocking activity should have the earliest attention. From this standpoint, the results obtained were fairly satisfactory. The proportion of compounds which appeared to have considerable ganglion-blocking activity, as judged from the change in pupil size, but which were found on further examination to have little or no activity, was not unduly high—no more than about 1 in 10. All the compounds which other techniques showed to be potent ganglion-blocking agents also dilated the mouse pupil under the conditions described.

The limitations of the Pulewka technique for assessing ganglion-blocking activity are mainly practical. Maximum mydriasis may not occur at the time of measurement. Whereas there is only a moderate variation in the response to a fixed dose of the drug in the one mouse from day to day, there is a considerable variation from one mouse to another. Many observations can be made quickly and easily; one observer can use up to 30 mice at a time—with larger numbers too much time elapses between successive observations on each animal. The accuracy of the method can certainly be increased by using large numbers of mice; but when this is done the expenditure of time is such that the chief practical advantage of the method is lost.

We might add that the constant rate perfusion of the vascularly isolated cat's superior cervical ganglion with a blood substitute (Mohanty, 1955) is our method of choice for accurate comparison of ganglion-blocking compounds.

Summary

1. Practical details are given of two methods used for assessing the depressor activity of compounds tested on account of their chemical resemblance to known ganglion-blocking agents. Theoretical advantages and disadvantages of the methods are discussed.

2. Depressor effects of 38 quaternary ammonium compounds have been compared in chronic cross-over experiments on unanaesthetized rabbits, the blood pressures of which were measured by the Grant ear-capsule technique. The following were estimated to be equipotent intravenous doses of the better-known active compounds: "Ecolid" (2.5 μM), pentolinium (5 μM), Burroughs Wellcome 288C53 (10 μM), "Gaplegin" (20 μM), hexamethonium (25 μM), "Pendiomide" (30 μM).

3. The ganglion-blocking effects of some 300 compounds have been assessed by determining the increase in size of the mouse pupil after intraperitoneal injection of the compound. The order of potency indicated by this technique is appreciably, but not greatly, different from that indicated by the experiments on rabbits.

We are indebted to the following commercial organizations for the supply of numerous compounds and unpublished experimental data: Messrs. May & Baker, Ciba, Burroughs Wellcome, G. D. Searle, Irwin Neisler, the Wm. S. Merrell Co., and the Osterreichische Stickstoffwerke. The expenses of this research were defrayed in part by the Medical Research Council of New Zealand.

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