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MYDRIASIS IN THE MOUSE: A QUANTITATIVE METHOD OF ESTIMATING PARASYMPATHETIC GANGLION BLOCK

BY

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It has been suggested that pentamethonium and hexamethonium differ in their effects on different ganglia (Paton, 1951), and it is desirable that their relative activities should be determined on several autonomic ganglia. Whereas determinations on the cat's superior cervical ganglion by the nictitating membrane preparation can be made fairly accurately, methods for measuring inhibitory activity on parasympathetic ganglia, for example, by inhibition of the peristaltic reflex of the guinea-pig or rabbit ileum (Feldberg and Lin, 1949), are subject to wider variations.

As a method which would give a reliable estimate of potency on the parasympathetic system, blockage of the ciliary ganglia, producing mydriasis in the mouse, has been selected. The method, which was first described by Pulewka (1932), and used by Ing, Dawes, and Wajda (1945) for studying atropine-like substances, was found by Wien and Mason (1951) to be applicable to ganglion blocking drugs, but they showed that the log. dose-response line obtained with hexamethonium had a curvilinear component, whereas that for atropine was rectilinear.

In the present investigation, the log. dose-response line of hexamethonium has been examined in some detail and it has been found that part of this is sufficiently rectilinear to enable trial assays of this compound to be carried out. From these trial assays suitable conditions for performing assays on other compounds in terms of hexamethonium were found, and the results with a few such compounds are recorded. Although the species (mouse) upon which the present experiments have been performed is one which differs in many of its physiological responses from higher mammals, it has the advantage, from a statistical viewpoint, that large numbers of animals may readily be used in each experiment.

METHODS

Experimental.—The experimental procedure was essentially the same as that described by Ing *et al.* (1945). Inbred albino mice of both sexes, from our own laboratory stock, were used once only. For the main investigation mice of mean body weight 16 g. (15.5 to 16.5) were selected. The mice were placed singly in glass jars upon a white table placed directly beneath a fluorescent strip light (about 4 feet above), in an otherwise dark room. The jars, arranged in groups of five, were covered by wire mesh and the animals were left under these conditions for at least half an hour before the experiment. The pupil was measured, by means of a graduated scale in the eyepiece of a dissecting microscope, in the approximately antero-posterior diameter. After initial readings the animals were injected intraperitoneally with the drug to be examined, the volume of injection being 0.01 ml./g. body weight. Dose levels were separated by a log. interval of 0.17609. Subsequent readings were made at ten minutes after the injection and, in some experiments, also at twenty and thirty minutes.

The compounds investigated were hexamethonium and pentamethonium dibromides; hexamethylene-bis(ethyldimethylammonium) dibromide and tetramethylene-bis(diethylmethylammonium) dibromide (Barber and Gaimster, 1952; Wien and Mason, 1951; Wien, Mason, Edge, and Langston, 1952); tetraethylammonium bromide; and nicotine acid tartrate.

Statistical.—Although a record of each mouse was kept, the results were analysed by taking the total difference in response of each group of five mice. These totals were subjected to variance analysis and estimates of potency and fiducial limits calculated according to Fieller's formulae (1940; 1944).

RESULTS

Examination of the Dose-response Line for Hexamethonium

In view of Wien and Mason's (1951) findings, it was necessary to establish the shape of the log. dose-response line. In their experiments two curves are shown (Exps. 1 and 2); the first of these had a significant quadratic component, the second a significant cubic component. An analysis of a curve, Fig. 1*a*, determined in the present investigation, using groups of five mice on seven different occasions giving thirty-five mice per dose level, was performed.

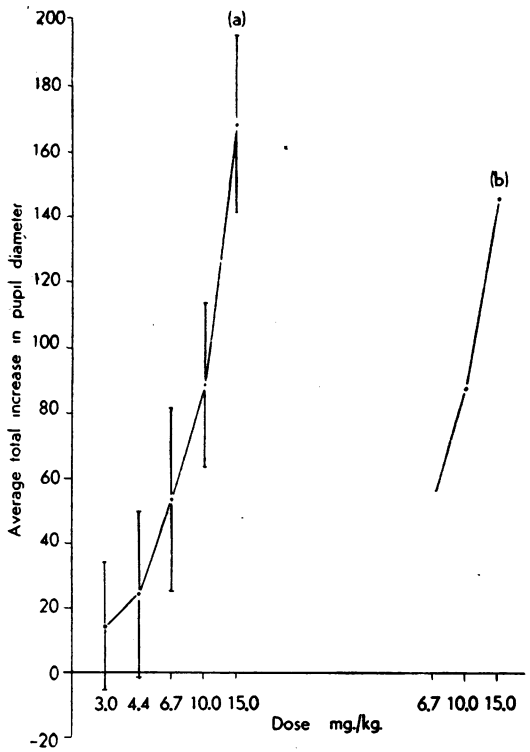

FIG. 1.—Dose-response lines for mydriatic effect in mice receiving graded doses of hexamethonium dibromide. (*a*) Each point represents the mean of seven groups, each of five mice: vertical lines show the standard deviation of each point. (*b*) Each point represents the mean of 27 groups, each of five mice.

An analysis of variance showed that the rectilinear and first degree curvilinear components were both very highly significant ($P<0.001$), although the variance ratio for the latter component was very much less than for the former. Neither the cubic nor quartic components were statistically significantly ($P>0.2$). In other words, the log. dose-response line is a smooth parabola. Converting to responses to log. units straightened the line, but this procedure was not an improvement because the standard deviations regressed from 1.03 to 0.07 from the lower to the upper points. It was found that correction by covariance analysis for the initial pupil diameter made no significant alteration in the standard deviation of the experiment as a whole ($P>0.2$), and the values of the mean squares were also practically unaffected; thus the conclusions drawn from the uncorrected variance analysis are in no way altered or modified. Inspection of Fig. 1*a*, however, shows that most of the curvature resides in the lower half of the curve and therefore an analysis of variance and covariance was made using only the top three dose-levels. The results revealed that the rectilinear component was very highly significant ($P<0.001$), whereas the first degree curvilinear component was still significant, although much less so ($P<0.05$). It was found that correction by covariance analysis for the initial pupil diameter made a highly significant improvement ($P<0.01$) in the standard deviation of the experiment as a whole, whereas all factors except rectilinearity, which was still very highly significant ($P<0.001$), became insignificant ($P>0.05$). In Fig. 1*b* is shown the log. dose-response line for hexamethonium dibromide, each point representing the mean response (not corrected for initial pupil diameter) of 27 groups of five mice each. This line has, in fact, a significant quadratic component ($P<0.05,>0.01$), the variance due to groups being insignificant ($P>0.05,\simeq 0.2$); the standard deviation is 24.21. However, in assays carried out on hexamethonium and other ganglionic blocking drugs reported later, in which only three or four groups of animals were used on each of three dose levels, deviations from parallelism and curvilinearity were not encountered, except for tetraethylammonium bromide. Thus for practical purposes this part of the log. dose-response line may be regarded as rectilinear and therefore suitable for carrying out valid potency estimates without correcting the results by covariance analysis. These findings are discussed below.

The extreme upper end of the log. dose-response line has not been studied so thoroughly because the pupil of the mouse can be made to dilate until its area is greater than the visible area of the cornea. With these larger diameters, it becomes necessary for the experimenter to move his own focus appreciably from one end of the scale to the other, and since it is impossible to hold the animal absolutely still, the estimation becomes more inaccurate with increase in pupil diameter.

Although it is not an unusual finding that the lower part of a log. dose-response line is not rectilinear, it was thought possible, in view of the rectilinear line obtained with atropine that hexamethonium was in fact effectively blocking not only the parasympathetic (ciliary) ganglion, controlling miosis, but also the sympathetic (superior cervical) ganglion, impulses from which can produce mydriasis. If this were so a relative miosis might be demonstrated by administering hexamethonium together with a maximal or near maximal dose of atropine sulphate, a method which has been successfully adopted by Grewal (1951) for examining anticholinesterases, muscarine-like compounds, and other miotics. In the

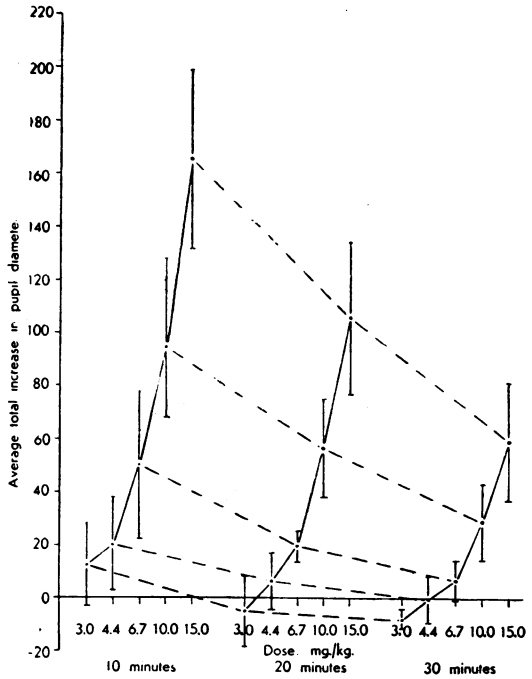

FIG. 2.—Dose-response lines for mydriatic effect in mice, showing response at 10, 20, and 30 minutes after injection, each point representing the mean of four groups of five mice each: vertical lines show the standard deviation of each point. All three curves determined on the same four groups of mice, corresponding doses being joined by broken lines.

presence of atropine, hexamethonium should be effective only on the sympathetic ganglion and should produce constriction of the pupil. This might provide a method for determining the potency of a ganglionic blocking drug on both divisions of the autonomic system, not only in the same species but on the same organ. The results of experiments in which doses of hexamethonium from 3.0 to 30.0 mg./kg. were used did not substantiate this hypothesis. This may be due to the fact that after both parasympathetic and sympathetic control has been inhibited by atropine and hexamethonium respectively, the pupil assumes a position of nearly maximum dilatation, which would make the observation of any changes difficult.

The Effect of Time, Groups, and Body Weights

Three other factors have been investigated. Firstly, responses at varying times after injection are shown in Fig. 2; these graphs show that the variation about the mean for each dose is less when observed at twenty than at ten minutes. However, since Wien and Mason (1951) found a maximal response at ten minutes, this reading only was recorded in all subsequent work. When the top three dose levels only were used, the mydriatic activity at 20 minutes in terms of that at 10 minutes was 71.0% (58.5 to 83.1%) with $P=0.05$. Secondly, the variation between groups of mice was significant ($P<0.01$) only when these were tested on different days and not when tested on the same day (morning and afternoon), ($P>0.2$). This implies that an increase or decrease in the efficiency of the experimenter does not occur during any one day, and consequently eliminates the necessity for randomizing the order of injecting the doses. The third factor investigated was the possible effect of body weight upon the response obtained. It was found that there was no significant difference ($P>0.2$) in the response obtained by using animals of 16 or 20 g. body weight.

Assays of "Unknown" Solutions of Hexamethonium

Assays of "unknown" solutions of hexamethonium dibromide have been carried out, and the analysis of variance of one such four-point assay is shown in Table I. In this particular assay the solution was estimated to have a potency of 129.6% of the standard solution with fiducial limits

TABLE I

VARIANCE ANALYSIS OF 4-POINT ASSAY OF STANDARD AND "UNKNOWN" SOLUTIONS OF HEXAMETHONIUM DIBROMIDE

Variance Due to	Degrees of Freedom	Mean Squares	Variance Ratio	Significance
Groups ..	3	2,549.90	4.25	$P>0.05<0.2$
Treatment:				
(1) Standard and "unknown"	1	5,148.06	8.57	$P<0.05>0.01$
(2) Slope ..	1	12,600.06	20.98	$P<0.01>0.001$
(3) Departure from parallelism	1	1,1`9.06	1.896	$P>0.2$
Error ..	9	$s^2=600.67$	$s=24.51$	
Total ..	15			

TABLE II

ESTIMATED POTENCIES OF "UNKNOWN" SOLUTIONS OF HEXAMETHONIUM

Expt. No.	No. of Groups (5 mice each)	Estimated Potency (%)	Fiducial Limits (P=0.05) (%)	Actual Potency (%)
1	4	129.6	105.9–187.6	120.0
2	3	122.3*	99.8–171.9	112.5
3	3	77.6	52.4– 95.7	75.0
4	3	56.2†	24.1– 74.8	50.0
5‡	4	101.0	90.2–113.3	100.0
	3	99.4	83.1–118.9	
	2	110.0	83.0–151.2	

* Not significantly different from standard. † Difference very highly significant ($P<0.001$). ‡ Six-point assay.

(P=0.05) of 105.9 to 187.6% (Table II); the true value was 120%. The values obtained with other "unknown" solutions are shown in Table II. The last of these (5) was a six-point assay and shows that increasing the number of dose levels increases the accuracy of the potency estimate, whereas estimates obtained on increasing numbers of groups reduces the fiducial limits of the estimate.

TABLE III

MYDRIATIC ACTIVITY OF GANGLIONIC BLOCKING DRUGS COMPARED WITH HEXAMETHONIUM DIBROMIDE (=100)

Compound	Mydriatic Activity	Fiducial Limits
Tetraethylammonium bromide ..	33.2*	27.2–43.8
Pentamethonium dibromide ..	28.4	24.0–34.3
Hexamethylene-bis-(ethyldimethyl-ammonium) dibromide dihydrate	301.4	257.4–349.2
Tetramethylene-bis-(diethylmethyl-ammonium) dibromide	289.7	250.9–343.3

* In this assay there was an opposed curvature.

Assays of Other Ganglion Blocking Drugs

The compounds examined are listed in Table III, the results being expressed in terms of the activity of hexamethonium dibromide, which was used as a standard throughout. For each compound the estimation of potency and fiducial limits was made by carrying out a six-point assay repeated on four different occasions, giving twenty animals on each of three dose-levels. In only one of these assays, namely that in which tetraethylammonium bromide was compared with hexamethonium dibromide, was there a significant deviation from parallelism ($P<0.01, \geqslant 0.001$), and in this assay only there was a significant opposed curvature ($P<0.05, \geqslant 0.01$). Tetraethylammonium bromide was one-third as active as hexamethonium dibromide although its ganglionic blocking effect is known to be complicated by other effects which might account for the opposed curvature of the regression lines. In all the other assays, the log. dose-response lines were without significant deviation from parallelism and without combined or opposed curvature ($P>0.05$). It will be seen from Table III that the estimated potency of pentamethonium dibromide was 28.4%, a figure which may be compared with 33.3% found by Paton and Zaimis (1949) on the rabbit ileum, and 75.0% found by Wien, Mason, Edge, and Langston (1952) on the guinea-pig ileum. The bis-ethyldimethylammonium homologue of hexamethonium dibromide was 301.4% as potent as hexamethonium dibromide in its mydriatic effect, which may be compared with Wien and Mason's (1951) figure of 200% on the guinea-pig ileum. The compound, tetramethylene-bis-(diethylmethylammonium) dibromide (Wien *et al.*, 1952) possessed a mydriatic activity of 289.7%, whereas Wien *et al.* found it to have a ganglion blocking activity of only 25% on the guinea-pig ileum and 50% on the cardiac vagus of the cat.

It was not possible to estimate nicotine acid tartrate, since it was found that maximum dilatation of the pupil occurred at two to four minutes after injection, and that in order to obtain mydriasis at 10 minutes the doses required were within the toxic range.

DISCUSSION

The log. dose-response line for mydriasis in the mouse produced by hexamethonium dibromide, unlike that for atropine sulphate (Ing *et al.*, 1945; Wien and Mason, 1951), is parabolic. The reason for this still remains obscure, although Wien and Mason (1951) have suggested it is probably due to a different mode of action. For a certain range of dose levels, i.e., from 6.6 to 15.0 mg./kg. of hexamethonium dibromide, the curvature arises from the variation encountered in the initial pupil diameters. However, from a statistical viewpoint, provided that the two log. dose-response lines in an assay are parallel the curvature does not invalidate an estimate of potency, since the two lines are separated by a constant horizontal distance. Moreover, in the assays performed, with the exception of that in which tetraethylammonium bromide, which is known to have mixed pharmacological actions, was compared with hexamethonium dibromide, neither deviations from parallelism nor curvature were encountered. It would thus seem that one may safely make an estimate of potency, with its fiducial limits, without correcting for initial pupil diameter, although if this correction was found to reduce the standard deviation of the experiment appreciably, the fiducial limits of the estimate might also be reduced. Moreover, converting the responses to log. units had little effect on the potency estimate and consistently increased the fiducial limits.

The result obtained with tetramethylene-bis-(diethylmethylammonium) dibromide seems particularly worthy of comment, since by this method its activity, in terms of that of hexamethonium, is very different from that found when tested on other preparations. This may represent a true differential action of this compound, not only with respect to the parasympathetic as opposed to the sympathetic system, but also within the parasympathetic system itself; or it may merely represent a difference due to species variation.

Summary

1. It has been shown that the log. dose-response line obtained with hexamethonium dibromide in producing mydriasis in the mouse can be used for quantitative comparisons of similar compounds, since part of this line can be regarded as rectilinear. The variation encountered between groups of mice receiving the same dosage is greater when performed on different days than on the same day.

2. Although the final regression line obtained with hexamethonium dibromide has a slight curvature, assays performed with " unknown " solutions of hexamethonium and other closely related drugs are without curvature and do not deviate from parallelism. By using five mice per dose-level in a six-point assay, repeated on four different occasions, a reliable estimate of potency with fairly close fiducial limits may be obtained.

3. The mydriatic activities of the following compounds have been compared with hexamethonium dibromide: (*a*) tetraethylammonium bromide was about one-third as active, although this result may have been partly influenced by a direct stimulating action; (*b*) pentamethonium dibromide was about one-quarter as active; (*c*) the bis-ethyldimethylammonium homologue of hexamethonium and the bis-diethylmethyl homologue of tetramethonium, were both about three times more potent. These results were discussed in the light of the different effects of these compounds on other ganglia.

4. It was not possible to estimate nicotine acid tartrate by this method, mainly due to the very rapid onset and decline in its action.

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