THE MYDRIATIC RESPONSE OF MICE TO ATROPINE

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The time after subcutaneous injection at which atropine produces most mydriasis in male mice increases with increased body-weight. If the response is measured when it is greatest, and is expressed as a percentage of the maximum mydriatic response obtainable in mice of the weight-range used, the mydriatic response to a dose of atropine is constant in male mice weighing from 16 to 50 g. The time-courses of the mydriatic response to atropine in mice of widely differing weights are parallel to the time-courses of the atropine concentration in the cardiac blood of the mice of the same weight-range given a high dose of atropine subcutaneously. The mydriatic response to atropine is found to be increased at times of poor ambient lighting (such as dusk): if however the mice are tested under conditions of artificial lighting (supplemented by daylight when present), the mydriatic response to a standard dose of atropine is nearly constant at all times from 8 a.m. to 2 a.m.

Since the dilatation of the pupil was first described as a qualitative test for the presence of the active principle of solanaceous plants by Runge (1824), many workers have used this response to assay atropine and atropine-like substances. The method, adapted for mice, was placed on a quantitative basis by Pulewka (1932), and subsequently slightly modified by Oelkers and Vincke (1935) and by Ing, Dawes and Wajda (1945).

Some factors influencing the accuracy of the bioassay method have been examined by Tonnesen (1948) and Huycke (1957). In general, mice within only a limited weight-range have been used, and doubts have been expressed as to the advisability of using the same mice repeatedly. Since mice over 25 g. are often readily available in the laboratory, being considered unsuitable for many pharmacological tests, and since the mydriatic assay provides a simple and direct means of investigating the relationship between dose, body-weight and biological response, it was decided to examine the mydriatic response to atropine in mice over a wide weight range. During this work, other factors were found which influenced the mydriatic response.

Experimental Methods

Measurement of mydriasis. Male albino mice, T.T. strain, 16–50 g., were used. The diameter of the pupil of the right eye was measured on a graticule set in one eye-piece of a binocular microscope, magnification $\times$ 17·5. During measurement, the mouse's head was held approximately 10 cm. from a 60 W bulb of an Anglepoise lamp which was partly masked to give a light aperture of 4 cm. The mouse was held in position for 8–15 sec., until the pupil size had become constant. Immediately after measurement of the initial pupil diameter, the mouse was injected subcutaneously between the shoulders with atropine sulphate solution. The pupil was remeasured at a selected time after injection, and the

mydriatic response expressed as the arithmetic difference between the two pupil diameters in units of the graticule scale (20 units = 1 mm.). In certain experiments, the diameter of the lens of each mouse's eye was measured also, and taken as the maximum pupil size possible. This ranged from about 38 units in smallest mice to 50 units in the largest mice used. The arithmetic difference between this value and the initial pupil size was taken as the *maximum mydriatic response possible.*

Atropine was calculated as the base, and injections were given in a dose volume of 0·10 ml./10 g. of mouse. Mice were kept during the test in groups of five, each group in a compartment about 12 cm. square.

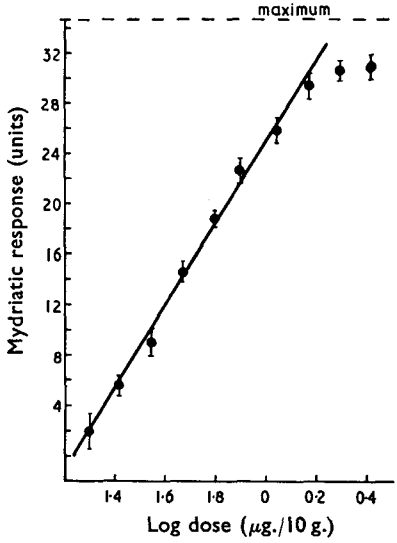

FIG. 1. Mydriatic response to atropine in 20–25 g. mice. Vertical lines represent 2 × s.e.

Determination of blood atropine content. Samples of cardiac blood were taken from mice anaesthetised 5 min. previously with pentobarbitone sodium (0·9 mg./10 g., i.p.) at 15, 30, 45, 60 and 120 min. after injection of atropine (1 mg./10 g., s.c.). The thorax was cut open and up to 1 ml. of blood withdrawn from the right ventricle into a syringe containing about 200 units of heparin in 0·05 ml. saline. The contents of the syringe were mixed and immediately reinjected subcutaneously, at a volume of 0·10 ml./10 g. weight of recipient mouse, into 26–30 g. mice, the initial size of whose pupils had been measured shortly beforehand. Usually enough blood could be withdrawn from each donor mouse to inject into 2 or 3 recipient mice, except for samples withdrawn 120 min. after dosage, when it was necessary to inject 0·20 ml./10 g. The pupils of the recipient animals were measured again 45 min. after injection and the mydriatic response used to calculate the concentration of atropine-like substance in the blood injected, by reference to a standard dose-response line. This line was determined in 26–30 g. mice for known amounts of atropine which

had been mixed immediately before injection with blood withdrawn from mice which had received pentobarbitone only. It was found that admixture with blood slightly depressed and delayed the mydriatic response to atropine after subcutaneous injection. A similar displacement of the atropine dose-response curve to the right when atropine was injected mixed with blood was reported by Godeaux and Tonnesen (1949).

Blood samples were withdrawn from 6–11 mice at each time interval after injection; a further 12–30 mice were used to assay these samples.

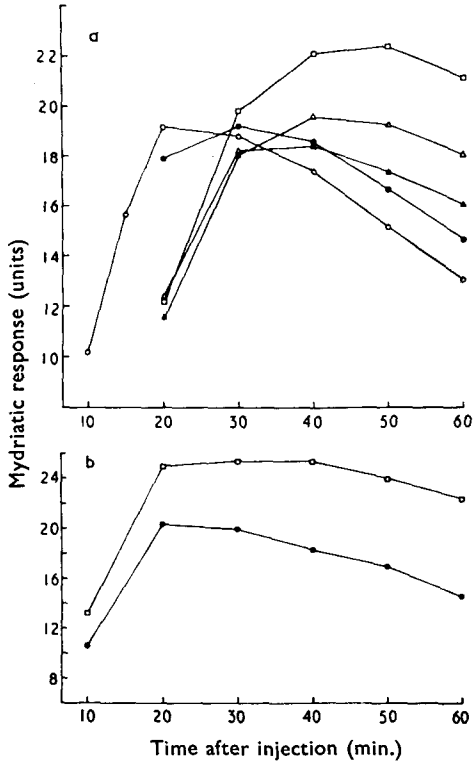

FIG. 2. Time course of the mydriatic response to:—(a) 0·625 μg./10 g. atropine injected subcutaneously in mice of different weights: ○——○ 16–20 g. ●——● 21–25 g. ▲——▲ 26–30 g. △——△ 31–40 g. □——□ 41–50 g. (b) 2 μg./10 g. atropine injected intraperitoneally into mice: ●——● 21–25 g. □——□ 41–50 g.

RESULTS

Dose-response Relationship

Mice of 20–25 g. were injected, in random order, with one of 10 different doses of atropine differing from each other by 0·1250 (log scale). 7–25 mice were injected with each dose, and the mydriatic response measured 30 min. after injection. Mean responses with their standard errors are represented in Fig. 1.

The log dose-response line, weighted to allow for the differing numbers of animals used at each dose-level, was calculated from all responses except those to the two largest doses (to which the mean responses were over 85 per cent of maximum). Deviation from linearity of regression was not significant ($P = 0{\cdot}10$). The slope of the calculated line was $32{\cdot}2 \pm 1{\cdot}4$ (s.e.).

Variation of Response With Body Weight

The mydriatic response to a selected dose of atropine (0·625 μg./10 g.) was measured in mice of various weights from 16 to 50 g. Since the time between injection and peak mydriasis was found to vary markedly with the size of the mice, the mydriatic response for each mouse was determined at 20, 30, 40 50 and 60 min. after injection (and at 10 and 15 min. in the 16–20 g. mice). 58–81 mice were used in each weight group. The time-courses of the responses for the various weight groups of mice are represented in Fig. 2.

It can be seen from the figure that the heavier the mice, the slower was the onset of mydriatic effect and the more delayed the peak response, to a standard dose of atropine. Mice of 16–20 g., for instance, showed peak mydriasis 20 min. after injection, at a time when the mydriatic response of 41–50 g. mice was only about half of that at the time of their peak effect. This peak occurred 50 min. after injection, by which time the response of the 16–20 g. mice had decreased by about 25 per cent.

The response appeared to be slightly greater in larger mice than in small. However, when this was expressed as a percentage of the maximal mydriatic response possible (by which means allowance was made for the slightly greater size of the eyes of larger mice), it was evident that the response was constant for all weight ranges of mice from 16–50 g. (Table I).

TABLE I

MYDRIATIC RESPONSE OF MICE TO ATROPINE (0·625 μg./10 g.)

Weight-range (g.)	16–20	21–25	26–30	31–40	41–50
(i) Maximum mydriatic response possible (units)	33·7	34·2	36·1	35·8	39·2
(ii) Time of peak effect (min. after injection)	20	30	40	40	50
(iii) Peak mydriatic response:					
(*a*) in units	19·2	19·3	18·6	19·7	22·4
(95 per cent limits)	(18·0–20·4)	(18·1–20·5)	(17·4–19·8)	(18·7–20·7)	(21·2–23·6)
(*b*) as per cent of (i)	57	56	51	55	57
(95 per cent limits)	(53–61)	(53–60)	(48–55)	(52–58)	(54–60)

In this experiment, the volume of injection was kept constant at 0·10 ml./10 g. of mouse. It was possible therefore that differences in the time-courses of mydriatic response in the various weight-ranges of mice could be at least partly attributable to differences in the volume of the injections. This was tested by injecting two groups of 26 mice (31–40 g.) with atropine, 0·625 μg./10 g., one group receiving the dose in a volume of 0·10 ml./10 g. and the other in 0·05 ml./10 g. The time-courses of their mydriatic responses are plotted in Fig. 3.

The time-courses of the mydriatic response for these two groups of mice were parallel, and their mean responses did not differ significantly ($P > 0{\cdot}2$). Furthermore, there was a marked difference between the time-course in these 31–40 g. mice given atropine in an injection volume of 0·05 ml./10 g. and that in the 16–20 g. mice which were given the same dose in an identical total volume of injection (Fig. 2a). It was evident therefore that the shape of the time-course curves for the mydriatic response to atropine was dependent upon the weight-range of the mice used and not upon the volume or concentration of the injection.

To confirm that the slopes of the regression lines for atropine mydriasis did not differ between mice of different weights, the mydriatic responses to a low dose (0·35 μg./10 g.) and a high dose (1·11 μg./10 g.) were determined in mice of two different weight-ranges, 21–25 g. and 41–50 g. These doses produced responses approximately 25 and 75 per cent of maximum. 10 mice were tested in each group. The slopes of the regression lines so obtained were virtually identical in both weight-ranges ($b = 32{\cdot}2$ and 33·2 respectively).

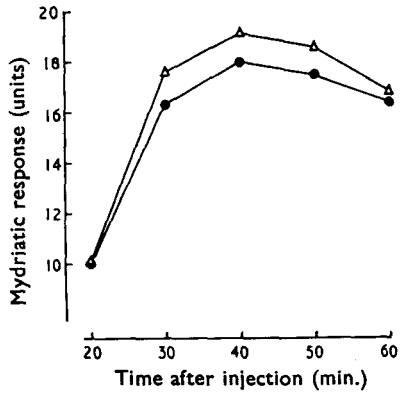

FIG. 3. Time course of the mydriatic response to atropine (0·625 μg./10 g.) in 31–40 g. mice: ●——● Injection 0·10 ml./10 g. △——△ Injection 0·05 ml./10 g.

Since many workers who have investigated the assay of atropine by its mydriatic response in mice have used the intraperitoneal route of injection (Ing, Dawes, and Wajda, 1945; Kroneberg, 1955; Huycke, 1957), the time-course of the mydriatic response to atropine given by this route was determined in mice of two different weight ranges, 21–25 g. and 41–50 g. The dose used for subcutaneous administration (0·625 μg./10 g.) was found to produce a barely significant mydriasis when injected intraperitoneally; therefore a dose of 2 μg./10 g. was used (Fig. 2b).

The onset of mydriasis was more rapid after intraperitoneal than after subcutaneous injection, especially in the heavier mice, in which the peak response was constant from 20 to 40 min. after administration. The 21–25 g. mice showed peak mydriasis at 20 min. At this time, the mydriatic responses, when expressed as percentages of the maximal possible in the respective weight-ranges, were not significantly different.

Comparison Between Responses in Mice Used Once or Repeatedly

The time-courses of the mydriatic response to atropine (0·625 μg./10 g.) of mice which had been used some 5–10 times previously during the preceding months were compared with those of hitherto unused mice. Mice of two weight-ranges were used (31–40 g. and 41–50 g.). A total of 242 mice were tested. The time-courses for the responses in the four groups of mice are plotted in Fig. 4.

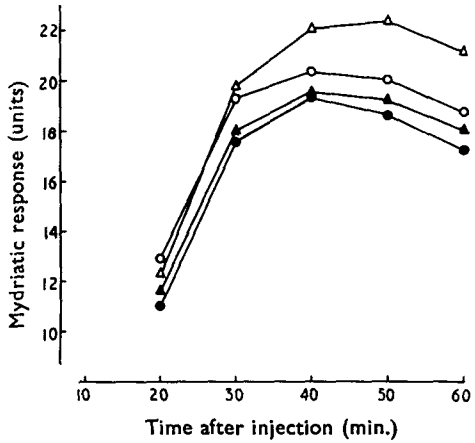

FIG. 4. Time-course of the mydriatic response to atropine (0·625 μg./10 g.) in mice of 2 weight-ranges which have or have not been tested beforehand:

	31–40 g.	41–50 g.
Hitherto-unused	▲——▲	△——△
Used frequently	●——●	○——○

Mice in the 31–40 g. weight-range showed nearly identical mydriatic responses whether or not they had been used repeatedly beforehand. This was not so with the larger mice. Hitherto unused mice of 41–50 g. body weight showed a significantly larger response ($P = 0{\cdot}01$–$0{\cdot}02$) than mice of a similar weight which had been used many times previously. This difference held true whether the responses were expressed directly as units or converted to percentages of the maximal mydriatic response possible in each group.

Determination of Atropine Content of Cardiac Blood in Mice at Various Times after Injection

To confirm that the difference in the time-courses of mydriatic response between mice of differing weight arose from differing rates of absorption of atropine from the subcutaneous site of injection (which seemed likely, since such differences were not evident after intraperitoneal injection), the time-course of the atropine content of cardiac blood was examined in mice of two weight ranges, 21–25 g. and 41–50 g., after subcutaneous injection of the alkaloid. To obtain assayable amounts of atropine in the blood

a dose of 1 mg./10 g. was given. This dose produced complete dilatation of the pupil, urination and slight "jumpiness", but no toxic symptoms.

The blood concentrations of atropine in the two groups of mice at the various times after injection are represented in Fig. 5. Comparison of these curves with those obtained for the time-course of the mydriatic response to atropine in mice of the same two weight-ranges reveals close parallelism. In both tests, the peak effect in the 21–25 g. mice was obtained about 30 min. after injection, whereas that in the 41–50 g. mice occurred at about 45 min. But there was a marked difference in the

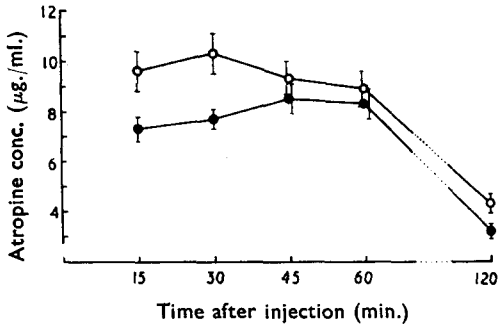

FIG. 5. Atropine concentration in the cardiac blood of mice injected subcutaneously with 1 mg./10 kg. atropine, withdrawn at various times after injection. Vertical lines represent 2 × s.e. ○——○ 21–25 g. mice. ●——● 41–50 g. mice.

relative size of the effects. In the mydriatic response the larger mice displayed a greater effect than the smaller, although there was no significant difference between the mean responses when they were expressed as percentages of the maximal mydriatic response possible in the two groups of mice. With cardiac blood concentration, however, the larger mice showed *less* atropine than the smaller mice at all times. The difference between the mean values for peak blood concentration of the two groups was not statistically significant ($P = 0{\cdot}05$–$0{\cdot}10$) but when values for all times were analysed, the difference in overall concentration between the two-weight ranges of mice was highly significant ($P < 0{\cdot}001$).

Variation of Mydriatic Response to Atropine with Time of Day

During the course of the investigations, it became apparent that variations occurred in the mydriatic response to a standard dose of atropine at different times of day. In an attempt to plot such variations, the mydriatic response to atropine (0·625 μg./10 g.) was determined in 21–25 g. mice at intervals of 3 hr. from 8 a.m. to 2 a.m. Since it was thought that these variations might be linked with the state of activity of the animals, this in turn being governed to some extent by the degree of ambient lighting, half the mice were tested under conditions of natural lighting (supplemented at night by the minimal amount of artificial lighting necessary to perform the test) and the other half under artificial light (plus daylight when present). The illumination of the mouse pupils by

shielded electric light during examination was kept constant throughout. A total of 653 mice were tested.

The mean values for the initial and the final pupil diameters and the net mydriatic response to atropine (i.e., the difference between the final and initial pupil diameters) at various times of the day, under natural and artificial lighting, are represented in Fig. 6.

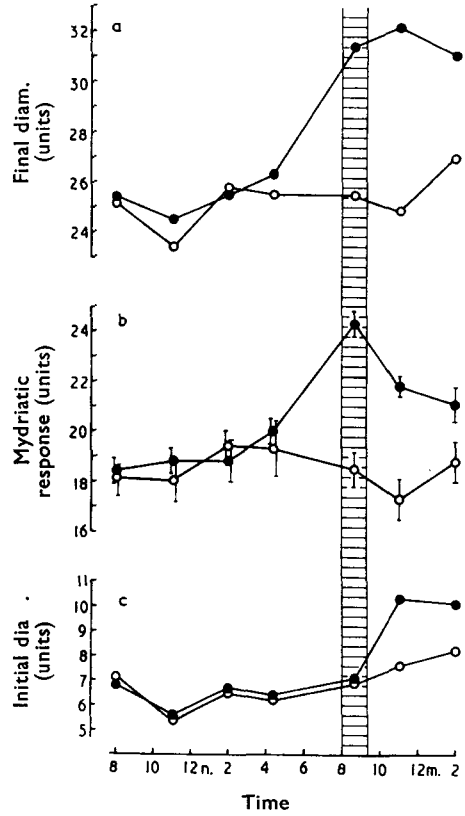

FIG. 6. (a) Final pupil diameter. (b) Net mydriatic response. (c) Initial pupil diameter of 21–25 g. mice given atropine (0·625 μg./10 g.) at various times of the day and night. Vertical lines indicate 2 × s.e. Hatched area represents dusk. ○——○ under artificial lighting (plus daylight when present). ●——● under natural lighting.

Under natural lighting conditions, the initial pupil diameter of the mice was fairly constant throughout the hours of daylight, apart from a slight fall at 11 a.m. It began to rise at dusk (8–9 p.m.). During the hours of darkness, the pupils were significantly larger than during the daytime. Similarly the final pupil size after atropine was constant from 8 a.m. to 5 p.m., but this rose at dusk. Since at this time the initial pupil size had increased little above daylight levels, the net mydriatic response to atropine

was significantly increased. For the readings during the hours of night (11 p.m. and 2 a.m.), the mean value for the final pupil size was the same as that at dusk; since the initial pupil size had now increased considerably, however, the net mydriatic response was reduced from its high value at dusk, but it was still significantly above mean daytime levels.

Under conditions of artificial light (supplemented by daylight when present), the mean values for initial pupil size agreed closely with those for mice under natural lighting, during the hours of daylight (8 a.m. to 8 p.m.), showing not only exactly the same overall mean (6·5 units) but also the same high values at 8 a.m. and 8 p.m., with a nadir at 11 a.m. During the hours of night, however, although the initial pupil size in mice under artificial lighting increased to a just significant degree, the increase was considerably less than that in mice under natural lighting conditions. The final pupil size measured after atropine under artificial lighting conditions changed little between 8 a.m. and 11 p.m., apart from a fall at 11 a.m.; the mean mydriatic response accordingly remained fairly constant throughout this time. At 2 a.m. the final pupil size rose in parallel to the increase in initial pupil size; the mean mydriatic response was therefore virtually unchanged.

Thus the net mydriatic response to a standard dose of atropine remained nearly constant between 8 a.m. and 2 a.m. if the animals were tested under conditions of artificial lighting (plus natural daylight when present). If however only natural lighting was used, the mydriatic response showed a marked increase at dusk, even though at this time lighting conditions appeared adequate to the experimenter. This increase at dusk was actually greater than that obtained during the hours of darkness, when the test would not normally be performed under the conditions of "natural" (i.e. minimum) lighting used here.

Discussion

The linearity of the regression of log dose with net response (in arithmetical units of increase of pupil diameter) has been confirmed here in the bioassay of atropine by its mydriatic effect in mice. Although Pulewka (1932) used a geometric ordinate scale, later workers have usually found the regression of log dose with arithmetic response to be reasonably linear (Oelkers and Vincke, 1935; Ing and others, 1945; Kroneberg, 1955; Huycke, 1957). In this work such a regression has been shown to be linear for responses from about 5 to 85 per cent of the maximum possible.

That the ordinate scale of mydriatic response can be expressed in terms simply of change in pupil diameter implies that the circular muscle of the iris sphincter may be considered to act pharmacodynamically in essentially a similar manner to a piece of longitudinal muscle, such as in a segment of isolated intestine. In such a tissue, equal log increments of atropine added to the organ bath produce equal degrees of depression of the contractile response of the muscle to acetylcholine. Similarly in the mouse eye *in vivo*, equal log increments of atropine injected into the animal may be considered to cause equal degrees of relaxation of the circular iris muscle (which is presumably contracted by endogenous acetylcholine)

since the length of the muscle is equivalent to the circumference, and hence related to the diameter, of the pupil.

Although exceptions have been reported (Rall and North, 1953; Gaddum, 1953; Angelakos, 1960; Russell, Emery and Bowers, 1960), adjustment of dose by a direct proportionality with the body weight of animals to be injected is generally considered adequate to ensure comparable blood levels and comparable pharmacological responses to drugs in animals of different weight, at least within fairly narrow ranges. It is not often however that the validity of this proportionality can be tested in large numbers of animals over wide weight ranges. The mydriatic response to a low dose of atropine in mice offers a simple means of doing so, and has been used here. In this test, providing the mydriatic response is measured at its time of peak effect (which varies with the body weight) and is expressed as a percentage of the maximal mydriatic response possible in the mice tested (to allow for slight differences in size of the eye in mice of grossly different body-weight), the response to a dose of atropine, given in terms of body-weight, is constant in male mice of all weights between 16 and 50 g.

The weight of the animals used by previous workers examining the mydriatic response of atropine in mice has been between 15 and 23 g. (Pulewka, 1932; Tonnesen, 1948; Ing and others, 1945; Huycke, 1957). Grewal (1951) and the last two mentioned workers all stated that mice of larger weight were less sensitive to the mydriatic action of atropine. They did not apparently increase the dose of atropine in proportion to the increased weight of the mice. In this work, where the dose has always been calculated in terms of body weight, no such diminution in response in larger mice has been noted.

That a pharmacological response—here mydriasis—is greater after administration of a drug by the subcutaneous than by the intraperitoneal route is uncommon, in view of the more rapid and complete absorption by the latter route. Presumably after intraperitoneal injection most of the atropine is absorbed into the portal circulation and passes directly to the liver (Werner and Schmidt, 1959) which is the major detoxicating organ for atropine in the mouse (Evertsbusch and Geiling, 1956). After subcutaneous injection, however, the alkaloid passes slowly into the systemic circulation and some reaches the eye without passing through the liver. The rate of onset of mydriasis after doses of atropine producing roughly similar peak responses is more rapid after intraperitoneal than after subcutaneous injection, and peak effects are obtained by 20 min. after injection, in larger as well as in smaller mice.

That the rate of pupil dilatation after a standard subcutaneous dose of atropine is markedly slower in larger than in smaller mice may be explained in terms of slower peripheral blood flow or slower absorption from the subcutaneous site of injection or both, as a result of fat deposition and increased fibrous tissue in older mice. Such an explanation receives support from the atropine blood concentrations at various times after subcutaneous injection of a large dose of the alkaloid into two groups of mice of differing weight. The time-courses of the blood concentrations

ran parallel to those of the mydriatic effect in the two groups of mice, the larger animals showing peak concentrations some 15–30 min. later than the smaller. The atropine levels in the blood of the larger animals were however slightly but significantly lower than those in the smaller mice. This may imply that, possibly because of its slower absorption, atropine is more rapidly metabolised in older mice, but, since mydriatic responses were at least as great in these animals as in the younger mice, presumably some compensatory mechanism is occurring also—such as increased binding of the alkaloid in sites within the eye, or increased sensitivity to its action as the result, say, of diminished central parasympathetic tone in older animals.

The finding that the time-course of the mydriatic response was the same whether the volume of solution injected was 0·05 or 0·10 ml./10 g. weight, confirmed the statement by Pulewka (1932) that no change in mydriatic response was caused by altering the volume of injection within the range of 0·3 to 0·8 ml.

Pulewka (1932) found no significant change in the mydriatic response to atropine in mice injected with 0·4 or 0·8 μg./10 g. daily, for 13 times in 15 days. Even less variation in response was obtained when the intervals between use were at least 2–3 days. Several other workers have used their mice several times, at 2–4 day intervals (Huycke, 1957; Ing and others, 1945; Oelkers and Vincke, 1935; Tonnesen, 1948; Nyman, 1949). The first two mentioned workers however limited the total number of tests to which any mouse was submitted to 8 and 3 respectively, stating that after that the mice became less sensitive (presumably because these workers found that larger mice were less sensitive than smaller). Here no significant difference could be found in the mydriatic response to atropine in mice of 31–40 g. between those used for the first time and those used many times over the previous few months. In 41–50 g. mice, however, the used animals were slightly less sensitive than the fresh mice. This may have been caused by poor absorption from the site of injection (back of the neck) as the result of formation of scar tissue produced by repeated injections in the same site. The larger mice, being older, would have received more injections. It was noted also that mice used many times before had slightly but significantly larger pupils before atropine injection than had unused mice.

The conclusions reached here from the investigations into variations in mydriatic response to atropine occurring at various times of the day and night may be of considerable practicable importance. In short, it would appear that, provided artificial lighting is always used (in addition to any natural light present), the net mydriatic response should be nearly constant at all times of the day and night (8 a.m. to 2 a.m.). If, however, only natural lighting is used, at times such as dusk (8–9 p.m. here) when such lighting is poor, the mydriatic response may be abnormally high. Normally assays would not be performed in the minimum lighting conditions used here to simulate "natural" lighting at night (when again high responses were obtained), but since absence of extraneous light facilitates observation of the mouse pupils, assays may often be performed in partly

darkened rooms. It is therefore essential that these lighting conditions are kept strictly constant, if reproducible results are to be obtained. No experiments were performed here to discover whether the mydriatic response is constant at all times of the day and night in standard conditions of reduced light. Pulewka (1932), who is the only other worker who appears to have examined this source of variation in the mydriatic response to atropine, worked in a darkened room, and chose the hours of 10.30 a.m. to 5 p.m. only, since he found that the mydriasis induced by atropine was greater in the evening than in the daytime. He took considerable precautions to exclude noise and excitement, since he found that any mice which were disturbed or excited by, for instance, hunger or "being on heat" gave abnormal results. It was noted in the present work that variations, especially in initial pupil size, sometimes occurred between groups of 5 mice apparently otherwise identical but kept in different sections of a "toxicity box". This was ascribed to a state of irritation or excitement engendered in all the animals in one group by one awkward individual in their number. Ing and others (1945) however, stated that initial pupil size was unaffected by excitement. Oelkers and Vincke (1935) agreed with Pulewka about the disturbing effect of noise, etc., and also mentioned a seasonal variation in response encountered. This latter phenomenon could not be confirmed by Tonnesen (1948).

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