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# A test for the assessment of the duration of the action of neostigmine and of the relative potencies of various anticholinesterases using the pupil of the mouse

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The miotic effect of anticholinesterases was used to monitor the duration of their activity and to compare the relative potency of different members of this group. Female albino mice were used and the diameter of their pupils measured with the help of a monocular microscope at a magnification of × 60. After a suitable control period, readings were taken at 30 min intervals. This test was found to be superior to previous tests described for the same purpose.

During the search for a suitable indicator substance for the assessment of a new formulation for the slow release of drugs (Collings & Schneider, 1970), it was decided to use the anticholinesterase, neostigmine. Various methods for monitoring the release of neostigmine proved unsuitable. Eventually a test measuring the effect of neostigmine on the diameter of the mouse pupil proved satisfactory. The success of this test in assessing the duration of the drug effect led to an investigation of its usefulness for the evaluation of the biological potency of different anticholinesterases.

This paper describes details of the method.

## EXPERIMENTAL

In the early experiments albino mice of the Schofield strain of either sex weighing approximately 20·0 g were used. However, female mice were found to be the more sensitive and also gave more consistent results; therefore in the later work only female animals were used. There was no restriction of food or drink before the experiment. The mice, suitably restrained, were placed on the stage of a monocular microscope and their pupils examined through a 10× objective and a 6 × eyepiece which contained a graticule calibrated in arbitrary units. Control readings were taken at 10 min intervals until they became consistent. Thereupon the drug was administered either as an aqueous control solution or in different slow release formulations. Injections (0·1 ml/20 g) were given subcutaneously into the scruff of the neck and readings were taken every 30 min; lengthening of the time intervals produced unduly large pupils. Readings were expressed as a percentage of the control values. To eliminate observer discrepancies, each set of experiments was made by the same observer. Some mice had pupil diameters outside the usual range and were not used, nor were the mice that had continuously changing pupils so that consistent control values could not be obtained. The reason for this behaviour was not ascertained.

Drugs used were: neostigmine methyl sulphate (Prostigmin, Roche); physostigmine sulphate (BDH); pyridostigmine (Mestinon, Roche); atropine sulphate (Burroughs Wellcome); edrophonium chloride (Tensilon, Roche).

The slow release formulations used are described by Collings & Schneider (1970).

## RESULTS

The use of the mouse pupil for the estimation of the slow release of drugs is illustrated in Fig. 1. The effect of an aqueous solution given in a dose of 1·25 $\mu$g of neostigmine/20 g, Fig. 1b, was compared with that of 5·0 $\mu$g of neostigmine/20 g contained in two types of slow release formulation (A and B), Fig. 1c,d. An experiment with an aqueous solution containing the same dose of neostigmine (5·0 $\mu$g/20 g), Fig. 1a, had to be abandoned, as all the animals involved showed violent convulsions. None of the animals that had received the drug at the same level in the slow release formulation convulsed; all survived. The duration of the effect was prolonged from 150 min or under, to 370 min or more than 440 min respectively with the two slow release formulations.

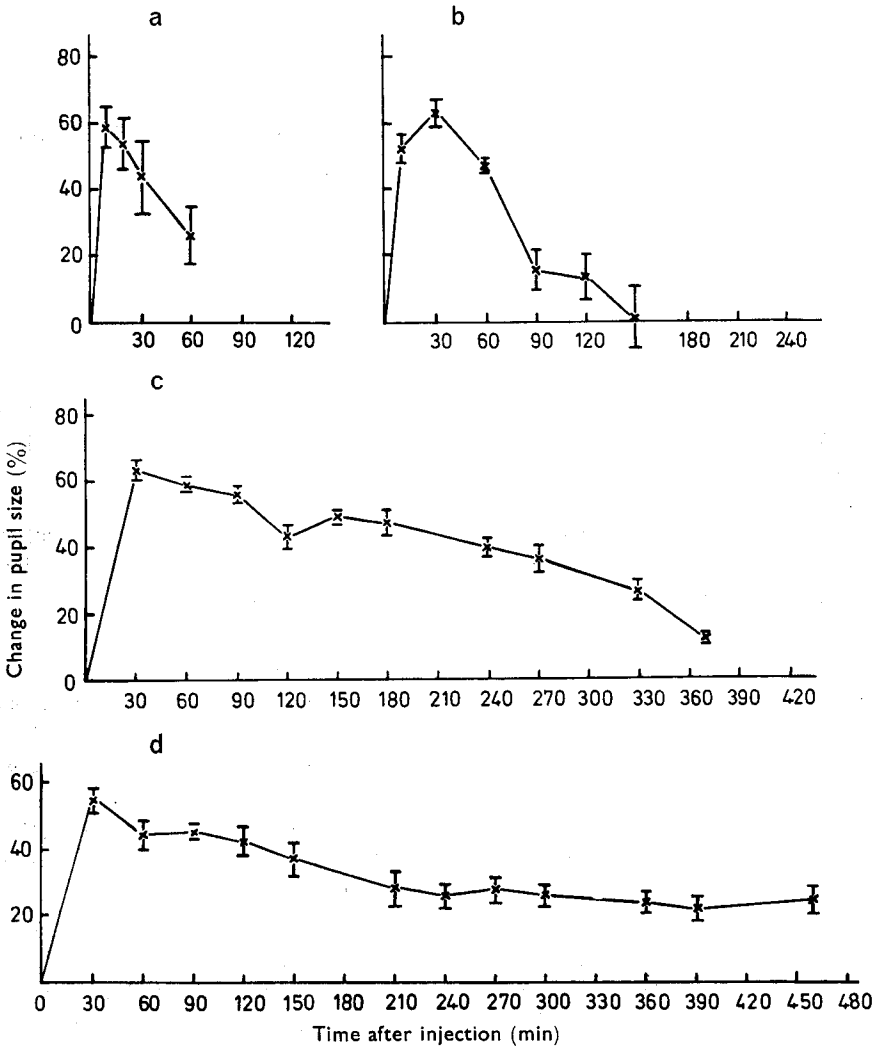


FIG. 1. Duration-action curves for neostigmine, using the mouse pupil test. (a) 5·0 $\mu$g/20 g in aqueous solution; (b) 1·25 $\mu$g/20 g in aqueous solution; (c) 5·0 $\mu$g/20 g in slow release formulation A; (d) 5·0 $\mu$g/20 g in slow release formulation B. Each point on the graph represents the mean value ($\pm$ s.e.) of 10 assays. Readings represent maximum change of pupil size (30 min after injection).

Fig. 2a shows the dose-response curves for the four anticholinesterases, neostigmine, physostigmine, pyridostigmine and edrophonium, the corresponding correlation coefficients for which were 0·80, 0·74, 0·88 and 0·82 respectively. The lines in Fig. 2 are calculated regression lines. In all cases there was a significant correlation between the dose of the drug and its effect on pupil size.

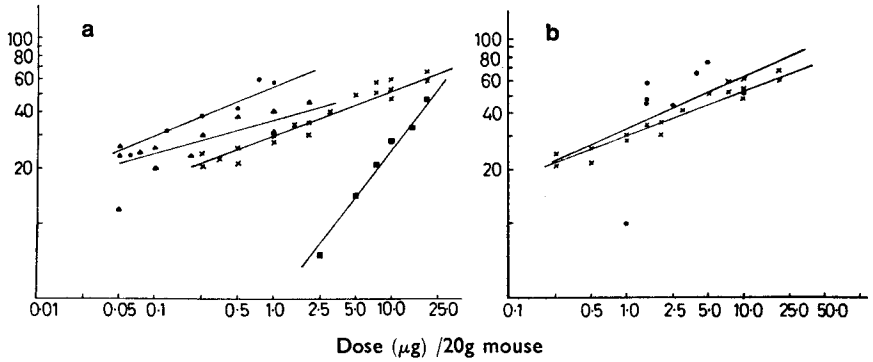


FIG. 2. Concentration-action curves for 4 "reversible" anticholinesterases. (a) Using the mouse pupil test without a mydriatic: (● neostigmine; ▲ physostigmine; × pyridostigmine; ■ edrophonium). (b) Using a similar test with the simultaneous administration of atropine 1·5 μg/20 g (× pyridostigmine; ● pyridostigmine + atropine). Each point on the graph represents the mean value (± s.e.) of 10 assays. Readings represent maximum change of pupil diameter (30 min after injection for neostigmine; 20 min for physostigmine, pyridostigmine and edrophonium. For the test with pyridostigmine + atropine, a set time of 15 min was used) (Grewal, 1951). Ordinate: Change in pupil size (%, log scale).

Fig. 2b illustrates the difference between the test using the antagonistic effect of the miotic pyridostigmine to atropine-induced mydriasis (Grewal, 1951) and the present test using a single drug only. It can be seen that the scatter in the test with atropine is much greater than in the present one, and the values for the correlation coefficients were 0·32 and 0·88 respectively. Further, the threshold value for the pyridostigmine induced miosis without atropine was much lower (125 ng) than that for the test including atropine (1 μg) which made it possible to extend the dose range.

## DISCUSSION

*In vitro* techniques for the measurement of the effect of neostigmine on the serum cholinesterases are regarded as unsatisfactory. Various factors may invalidate the results of such methods, the most important of these being the dilution effect which leads to dissociation of the enzyme-inhibitor system and thereby to unduly high values for enzyme-activity compared with those of the undiluted serum (Krayer, Goldstein & Plachte, 1944). It was therefore decided to use a method which would measure directly a biological effect of the injected anticholinesterase.

The reason why the red tear test (Burgen, 1949) was not successful in our hands remains obscure. It consists in the stimulation of porphyrin secretion from the Harderian glands of the rat by the injection of graded doses of acetylcholine. After determining the threshold value for each rat, a non-toxic effective dose of neostigmine that would lower the threshold value of acetylcholine was given. Acetylcholine injections were then repeated at half-hourly intervals until the control values were regained. In spite of many repeated experiments with various slow release preparations we were unable to demonstrate any delay in the release of neostigmine with this

method. Control levels were invariably regained within 3 h whether aqueous solutions or test emulsions were used. Furthermore the response of individual rats varied from 50–200 $\mu$g of acetylcholine from day to day. Even greater variations were found between groups of rats tested at different periods. In August 1966, no response was obtained in some rats with doses exceeding 1000 $\mu$g, whereas threshold values were as low as 3–12·5 $\mu$g of acetylcholine in January 1967.

No definite reason for these discrepancies was found. The cause may possibly have been environmental, as all the rats were from the same basic stock.

A method for testing the miotic effect of certain drugs after intraperitoneal injection was described by Grewal (1951). This was based on Pulewka's (1932) method of testing mydriatics on mice. The method consisted in assessing the antagonistic effect of the miotic against the mydriatic effect of atropine. With such a test it is impossible to assess unusual responses to either drug and the chances of error are increased. Furthermore it is not possible to use the individual animals as their own controls and to express the results as percentage of the control values. They could only be compared with a separate control group tested for atropine response at a different time. Also the correlation between dose and response became less significant than when only a single drug was used (Fig. 2b).

The test described can be used to assess the relative potencies of anticholinesterases and compared favourably with older tests designed for this purpose. It proved superior to the chromodacryorrhoea test (Burgen, 1949) inasmuch as it was not subject to the tremendous fluctuations in response that made that test unmanageable. It also compared favourably with the test for miotics using their antagonism to atropine mydriasis (Grewal, 1951) in the example studied (pyridostigmine). In this case there was a much closer correlation between dose and response with the present test than with the older one. Indeed the correlation coefficient was not significant (0·32) with the older test, whereas it was highly significant (0·88) with the present one.

The most important advantage however, lay in the fact that the new test proved useful for the assessment of the prolongation of the effect of drugs in a slow release formulation (Collings & Schneider, 1970). Many formulations were tested and the methods proved reliable if the precautions mentioned were observed.

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