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THE ASSAY OF ANTICHOLINESTERASE DRUGS BY THE CHROMODACRYORRHOEA RESPONSE IN RATS

BY

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With the introduction of new anticholinesterase compounds of the alkyl phosphate group an accurate assessment of their potencies and duration of action relative to such well-established drugs as eserine and neostigmine has become important. It was quickly found that attempts to assess them pharmacologically by various isolated organ preparations were impracticable because the alkyl phosphates act much more slowly than eserine and neostigmine and their effects are irreversible during the time of an assay. It was thought that an assay method in an unanaesthetized animal would overcome many of these difficulties and yield data of more direct value.

The phenomenon of chromodacryorrhoea in rats, first described by Freud (1933), seemed to be a promising basis for a test, if it could be made satisfactorily quantitative. Freud showed that the injection of adequate amounts of muscarinic drugs into rats caused the secretion of opaque reddish-brown tears. Tashiro, Badger, and Younker (1940) showed that the pigment was a chloroform soluble substance giving a strong fluorescence in ultra-violet light, and this material was identified by Figge (1945) spectrographically as consisting mainly of proto-porphyrin with a small amount of coproporphyrin I. These porphyrins are evidently the secretion of the Harderian gland, which was shown by Derrien and Turchini (1924) to have glandular cavities lined by cells whose vacuoles contained a pigment showing the same characters as the red tear pigment. Figge found that, of a large number of animals examined, only rats, Syrian hamsters, and some strains of mice (e.g., C_3H strain but not JK strain) produced this secretion. Tashiro, Smith, Badger, and Kezur (1940) found that eserine reduced the amount of acetylcholine that was required to produce a response very greatly, and Mendel and Hawkins (1943) showed that injection of cholinesterase into rats abolished the response to normal doses of acetylcholine.

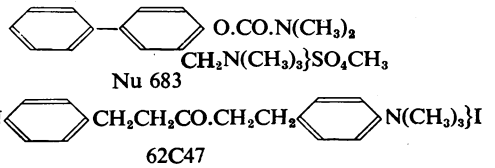
This paper describes how this response may be used as a basis for an accurate and quantitative assay of anticholinesterase drugs.

MATERIALS

Male albino Wistar rats weighing 100–200 g. were found to give the most satisfactory and consistent results. They were divided into groups of six rats whose weights fell within the range of ±10–15 g. from the mean. The standard acetylcholine solution was prepared by dissolving 100 mg. acetylcholine chloride and 100 mg. KH_2PO_4, 12 H_2O in 100 ml. of a 0.8 g./100 ml. solution of NaCl. This solution has a *p*H of 4.1–4.3 and keeps without loss of activity in the refrigerator for a few weeks.

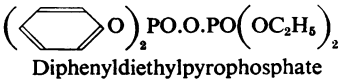
Tetraethylpyrophosphate (TEPP) was the pure thrice-distilled ester (sp. gr. 1.25) which on hydrolysis with NaOH yielded 98–99 per cent of the theoretical acid. "Hexaethyltetraphosphate" (HETP) was a mixture of alkyl polyphosphates prepared by heating 1 mol of P_2O_5 with 2 mols of triethylphosphate at 140° C. for one hour. Both these substances are hydrolysed in aqueous solution and hence fresh aqueous solutions were prepared immediately before use. Di*iso*propyl fluorophosphonate (DFP) was the pure ester kept as a 2 per cent (w/v) stock solution in dry propylene glycol. Fresh aqueous solutions were prepared immediately before use.

The other anticholinesterase drugs studied were: eserine sulphate, neostigmine methylsulphate, the dimethylcarbamic ester of 5-phenyl-2-hydroxybenzyl-trimethylammonium bromide (Nu 683), bis-(4-dimethyl-aminophenylethyl)ketone dimethiodide (62C47 Glock and Mogey, 1948), quinidine sulphate, and diphenyl-diethylpyrophosphate.



Nu 683

62C47



Diphenyldiethylpyrophosphate

METHODS

Injections of acetylcholine were made subcutaneously under the loose skin of the rat's flank. When 300–500 μg. acetylcholine were injected a secretion of reddish-brown opaque tears commenced in 30–60 sec.; this could be tested by inserting a spill of filter paper into the conjunctival sac when the pigment was readily visible on the filter paper; when the secretion was profuse, it welled out of the conjunctival sac and formed an opaque red pool covering the eyeball and smearing the fur covering the eyelids; it could also often be seen at the nostrils. The secretion continued for about 4–6 min. as a rule, but after administration of anticholinesterase drugs it tended to be more prolonged. If the amount of acetylcholine injected was decreased the secretion appeared somewhat later (1½–2 min.), and it was usually preceded by colourless tears (lacrimal gland secretion only) and was less intensely coloured; finally, if the amount of acetylcholine was still further reduced either no secretion or colourless tears only were formed. In different groups of rats tested over the past fifteen months the threshold amount required to produce red tears has been in the range of 80–250 μg. acetylcholine; for a given group of rats the range of sensitivity has usually been narrow, the mean threshold dose varying by only ±25–30 μg. Occasionally rats reacted well outside this range, and they were consequently rejected. The absence of one or both Harderian glands is not a very rare occurrence in rats, and consequently it is essential that both conjunctival sacs be tested. For practical purposes the threshold figure adopted has been that dose at which half the animals gave a positive response rather than the mean of the individual thresholds for each animal, and in what follows the term threshold will be used with this connotation. In a given group of animals the threshold has shown no change over several weeks, and it may be tested as frequently as every 30 min. without exhaustion of the gland's secretory capacity. The presence of secretion has always been tested for 1½ min. after injection and if negative again at 3 min. If an animal shows no secretion at 3 min. it is most unlikely that any secretion will appear. The anticholinesterase drugs tested were administered by intraperitoneal injection.

The inhibition by the drugs of true cholinesterase, from haemolysed human red blood cells, and of pseudo-cholinesterase, from human oxalate plasma, was tested with acetyl-β-methylcholine bromide and benzoylcholine chloride respectively as substrates, as will be described elsewhere (Burgen, 1949).

An actual assay is carried out as follows: the threshold of six animals, in the absence of anticholinesterase drugs, is first determined; after being left in their cage for 30 min. the animals are injected intraperitoneally with the cholinesterase inhibitor. If the time of maximum activity of the drug is known the threshold of the animals need only be tested at this time. If, however, the time course of the drug is unknown two or three groups of six rats are used; these are tested at suitable intervals, such that the same group of rats is never tested more frequently than once in thirty minutes. In this way sufficient values can be obtained to give the complete time-effect curve and thereafter further doses need only be tested at the time of maximum activity. It has been our custom to test the response of every new batch of rats to 0.25 mg. eserine sulphate per kg. both as a test of the constant sensitivity of the rats used and so as to have available figures for both absolute potencies and potencies relative to eserine sulphate.

RESULTS

Effects of eserine

After the injection of 0.2–0.3 mg. eserine sulphate per kg. the threshold to acetylcholine began to fall after 2–3 min., reached a minimum after 18–25 min. (Fig. 1), and then began to return towards

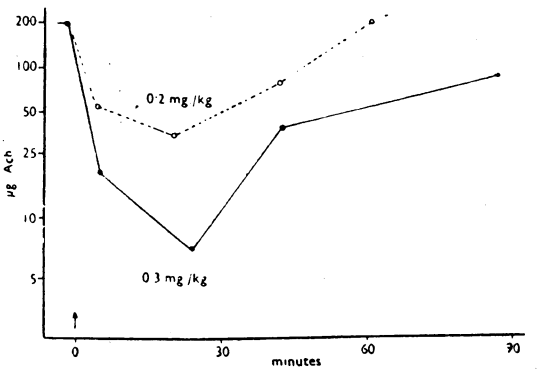


FIG. 1.—Abscissae: time in minutes. Ordinates: amount of acetylcholine needed to produce red tear secretion. At the arrow eserine sulphate was injected intraperitoneally. o----o 0.2 mg./kg; ● —— ● 0.3 mg./kg.

the initial level. It will be seen that after the administration of 0.2 mg. eserine sulphate per kg. the threshold fell from 200 μg. to 35 μg. acetylcholine, whereas with 0.3 mg. eserine sulphate per kg. the threshold was lowered to 7 μg. acetylcholine. Fig. 2 shows the dose-response curve covering the effective range of eserine dosage which is linear when the ordinate is plotted as the

$$\log_{10}\left(\frac{\text{Initial acetylcholine threshold}}{\text{Minimum threshold after the drug}}\right)=\log_{10}\left(\frac{To}{Tm}\right)$$

The response curve is steep, so that a change of 10 per cent in the dose of eserine corresponds to a change in acetylcholine threshold of c. 150 per cent. Repeated tests on the same group of rats have shown that the standard deviation of the mean of a potency determination on eserine is c. ± 3 per cent. It was found that with doses of an anticholinesterase drug which reduced the threshold below 5 μg. acetylcholine the end point became difficult to determine as the tears were very watery and poorly pigmented. Evidently in this range of dosage the anticholinesterase drugs

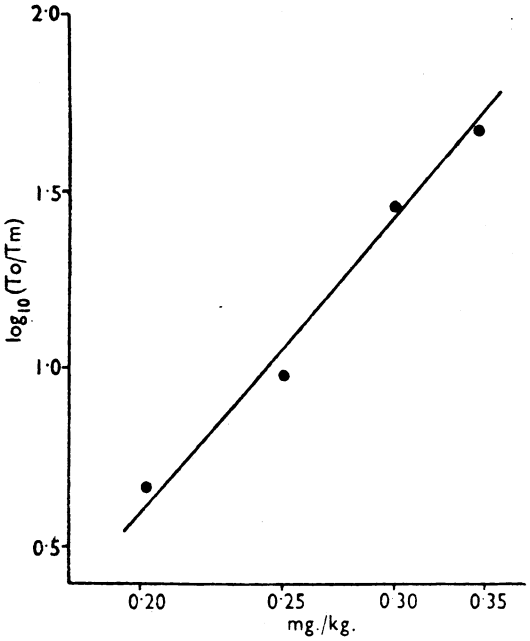


FIG. 2.—Abscissae: dose of eserine sulphate mg./kg. Ordinates: $\log_{10}\frac{To}{Tm}$, where To = initial red tear threshold to acetylcholine and Tm = minimum threshold reached after administration of eserine.

produce a relatively greater sensitization of the lacrimal gland than of the Harderian gland.

Eventually if the amount of anticholinesterase drug was increased sufficiently a spontaneous secretion occurred without the injection of acetylcholine, presumably owing to the acetylcholine released at the cholinergic nerve endings in the gland.

Comparisons with other drugs

Other anticholinesterase drugs have given dose-response curves with slopes similar to that of eserine, but displaced along the abscissae according to the relative potencies. Fig. 3 shows the curves obtained for four other drugs compared with that of eserine. A line has been drawn at the dose level corresponding to a tenfold potentiation of acetylcholine—i.e., the point at which $\log_{10}\left(\frac{To}{Tm}\right)=1.0$. The negative logarithm of the molar concentration of drug at which this potentiation was attained is called the pT10 and has been found to be a convenient measure of relative potencies.

The Table gives potency figures determined in this way for a number of known anticholinesterase drugs; data for DFP are not included because it was not found possible to produce a tenfold potentiation of

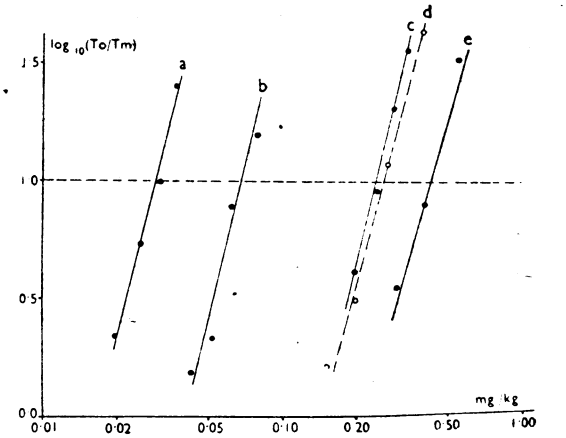


FIG. 3.—Abscissae: dose of drugs in mg./kg. Ordinates: $\log_{10}\frac{To}{Tm}$. a = neostigmine methylsulphate, b = "62C47," c = eserine sulphate, d = TEPP, and e = "Nu 683." The horizontal line is the tenfold potentiation level.

the response to acetylcholine by a single intraperitoneal dose of this drug; a twofold potentiation of the response was obtained with 0.6 mg./kg., but 0.8 mg./kg. was lethal to all the animals of the group studied. This illustrates a point in the series studied; with "62C47" the LD50 was about a hundred times as large as the tenfold potentiating dose; with eserine, neostigmine, and Nu 683 between ten and twentyfold; but with TEPP and HETP only about twice as great; whilst with DFP the tenfold potentiating dose could not be attained. With the short-acting drugs tests of potency could be performed on successive days, but with TEPP and HETP the animals responded normally to eserine only after four to seven days; in order to eliminate any error from this cause they were not used again for assay purposes in under fourteen days.

TABLE

Name and molecular weight	Potency (eserine = 100)		pT10
	By weight	Molar	
Eserine sulphate (324) ..	100	100	6.11
Neostigmine methyl sulphate (322)	860	860	7.05
Nu 683 (393)	59	71	5.96
TEPP (290)	100	89	6.10
HETP (—)	24	—	—
62C47 (610)	360	670	6.95
Quinidine sulphate (391)	<1	<1	<4.00
Diphenyldiethylpyrophosphate (386)	<1	<1	<4.00

Duration of effects

As previously noted the effect of eserine wore off in two to three hours, depending on the dose administered; neostigmine and "Nu 683" had similar

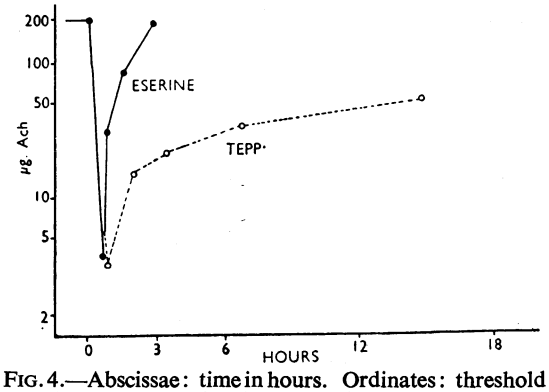


FIG. 4.—Abscissae: time in hours. Ordinates: threshold doses of acetylcholine. Anticholinesterase drug injected at zero time. ●——● eserine sulphate 0.32 mg./kg ; o----o TEPP 0.3 mg./kg.

durations, and "62C47" a briefer effect lasting only one to two hours TEPP and HETP, however, reached their peak action only after forty to fifty minutes, and their effect declined more slowly (Fig. 4), so that after twelve hours a marked potentiation was still present and even after thirty-six to forty-eight hours a small degree of potentiation was still present. The relatively slow development of the action of TEPP appears to be the result of a slow reaction between TEPP and cholinesterase and not to delayed absorption (Jansen, Nutting, and Balls, 1948; Burgen, 1949). Such effects as were obtainable with DFP reached a maximum in twenty-four to forty-eight hours and were still detectable seven days later.

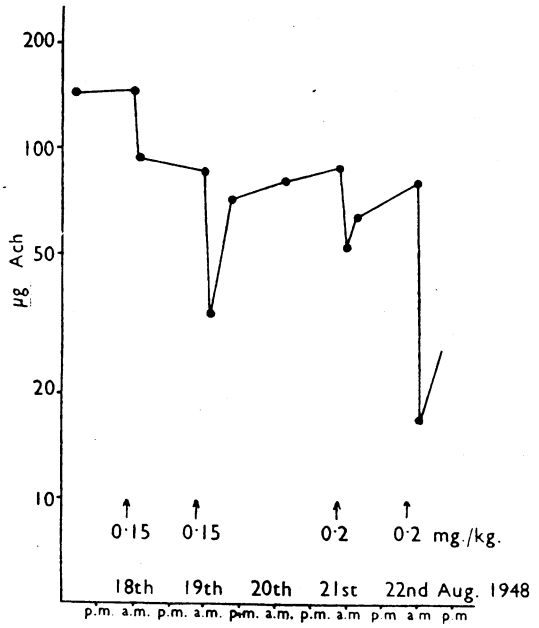


FIG. 5.—Abscissae: date. Ordinates: acetylcholine threshold. At arrows 0.15 mg./kg. and 0.2 mg./kg. TEPP injected intraperitoneally.

Cumulative effect of TEPP

Attempts to demonstrate a cumulative effect of TEPP by this assay have not been easy to interpret, but Fig. 5 shows some data obtained at levels of 0.15 mg./kg. and 0.20 mg./kg.; it will be seen that at both levels when the dose of TEPP was repeated after twenty-four hours a larger effect was produced than after the first dose.

Correlation with in vitro *inhibition of cholinesterase*

Some of the reasons why any close agreement between *in vitro* and *in vivo* potencies of anticholinesterase drugs of diverse modes of action is unlikely will be considered elsewhere (Burgen, 1949). Fig. 6, however, does show a general correspondence

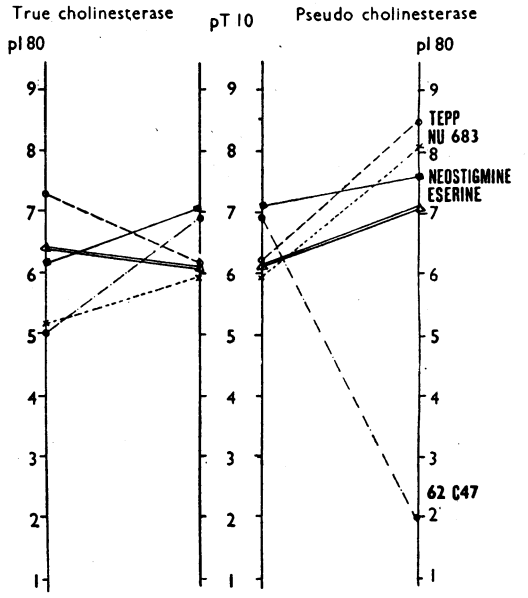


FIG. 6.—Figures in centre column pT10 determined by chromodacryorrhoea (see Table). Left-hand column pI80 for true cholinesterase. Right-hand column pI80 for pseudo-cholinesterase. △==△ eserine sulphate; ●——● neostigmine methylsulphate; ●—·—● "62C47"; ×·····× "Nu 683"; o----o TEPP.

between the potencies found by the chromodacryorrhoea method and those found *in vitro*. In this figure the pT10, determined as described earlier, is compared with the negative logarithm of the molar concentration of inhibitor required to inhibit true and pseudo cholinesterases *in vitro* to the extent of 80 per cent (pI80). An inhibitory level of 80 per cent was selected for comparison because there was evidence (Gunter and Mendel, 1945; Hawkins and Gunter, 1946; Koelle and Gilman, 1946; Hawkins and Mendel, 1947) to suggest that this is approximately the level at which cholinesterase inhibition becomes physiologically effective. These figures should therefore be numerically comparable; theoretically the lines joining the points should be nearly horizontal and this is in fact approximately true with the marked exception of the pI80 for pseudocholinesterase inhibition by "62C47."

Effect of atropine on chromodacryorrhoea response

Atropine in as small a dose as 20 μg./kg. raises the threshold to acetylcholine. In consequence the chromodacryorrhoea response must be regarded as a very sensitive test for atropine.

Discussion

It has been shown that the chromodacryorrhoea response is a sensitive quantitative indicator of the activity of anticholinesterase substances *in vivo*. It is clear that the protection of acetylcholine against hydrolytic destruction tested in this way can be divided into protection (1) during transport in the blood stream to the Harderian gland and (2) in the gland substance itself. There can be little doubt that destruction in the blood is the more important of the two factors, and consequently it becomes of importance to inquire as to the nature of rat serum esterase. In male rats most of the serum cholinesterase is true cholinesterase (Hawkins and Mendel, 1947), although in female rats a considerable amount of pseudocholinesterase occurs (Brauer and Root, 1947), so that effects in our tests would be expected to correlate better with inhibition of the true cholinesterase than of the pseudo-cholinesterase. This has been true, and it is illustrated not only by the weak action of DFP and by the lack of action of quinidine (see Table), both selective pseudo-cholinesterase inhibitors, but also by the powerful action of "62C47," a selective inhibitor of the true cholinesterase. It would seem therefore that the chromodacryorrhoea response in male rats is a test mainly of the ability of the drugs to inhibit true cholinesterase. Another important aspect of the test is that, with drugs that show some other marked pharmacological property besides anticholinesterase activity, it allows an assessment of the contribution of this activity in the whole animal.

Summary

The potency and the duration of action of anticholinesterase drugs have been measured in the unanaesthetized rat by the chromodacryorrhoea response. An approximate correspondence was obtained with their potencies as inhibitors of true cholinesterase *in vitro*.

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