ASSESSMENT OF DIRECT CHOLINESTERASE INHIBITORY ACTIVITY BY PUPILLARY MIOSIS

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Factors affecting the pupillary response to ocular administration of a direct inhibitor of cholinesterase have been investigated. The miotic response is dependent on the volume and concentration of solution and on the direct inhibitory activity of the compound. Measurements of miotic activity of twelve anticholinesterase materials show good agreement with *in vitro* biochemical measurements of their direct inhibitory activity. A rapid and simple method is suggested which reveals ten-fold differences of direct inhibitory activity, and detects activity equivalent to 0·01 μg. of TEPP.

The measurement of direct cholinesterase inhibitory activity by *in vitro* methods, although accurate, demands special biochemical laboratory facilities, and may be adversely influenced by the presence of organic solvents present in commercial preparations. A more rapid semi-quantitative method of assessment would frequently be of value, for example, in the preliminary screening of organic phosphorus insecticides, for detecting the presence of direct inhibitors as impurities in samples of indirect inhibitors (e.g., schradan, parathion or dimefox), and for examining commercial preparations.

Among the known "direct inhibitory" responses in mammals, pupillary constriction (miosis) is perhaps the most definite. It has the advantage of being readily seen and measurable. A method based on this response has already been described for confirming the potency of di*iso*propyl phosphorofluoridate (dyflos), after storage, using the miotic effect upon the rabbit pupil, and comparing the stored material with dyflos standards of known potency[1]. In the rabbit the rate of pupillary constriction depends on the concentration of dyflos administered[2]. Studies on ocular effects accompanying miosis have been made[2,3], and an abrupt dose-response relation has been observed in man[3].

Some organic phosphorus compounds, the indirect inhibitors, become highly potent anticholinesterases only after "conversion" to direct inhibitors within the mammalian body, or by insect or plant tissues[4]. The only mammalian tissue known to produce this conversion to a significant extent is the liver, so that a purely local cholinergic miosis should in theory be produced only by a direct inhibitor. Also, miosis should occur only above a limiting concentration proportional to the direct inhibitory activity of a compound, subject to some modification by its physical properties.

Materials and Methods

The present investigation involved, firstly, a study of some of the factors affecting the production of miosis by a known direct inhibitor,

and thus the development of a standardised technique; tetraethyl pyrophosphate (TEPP) was selected for this purpose. Secondly, the miotic effects of serial dilutions of several organic phosphorus compounds and commercial insecticidal preparations were assessed, and the results compared with the corresponding *in vitro* I 50 concentrations. This is defined as the molar concentration of substance producing 50 per cent inhibition of rat brain cholinesterase on incubation at 37° for 30 minutes.

Young albino guinea pigs were used. The standard inhibitor was a "pure" (96 per cent) sample of TEPP supplied by Messrs. Albright and Wilson Ltd. The solvents were water, propylene glycol, and tetrahydrofurfuryl alcohol containing 10 per cent v/v added water; none of these alone caused miosis, although propylene glycol and tetrahydrofurfuryl alcohol caused very slight transient irritation. The needles used for ocular administration were standard No. 16 hypodermic (0·55 mm. diameter, 24 s.w.g.).

Ocular administration was by allowing slowly-formed drops to fall from the vertically-held needle and syringe on to the centre of the cornea, from a height of 3 or 4 mm. The lower lid was then pulled upwards and outwards and released, to trap the drop and promote even spreading. Periodic observations of pupillary diameter were then made for a suitable period, with special emphasis on the speed of onset and of attainment of maximum response. In most tests, the other pupil was used as a control; alternatively the pupils of a similar but untreated animal were used. Between and during pupillary inspection, all animals were kept under comparable diffuse lighting conditions, sufficiently bright for adequate observation, without causing enough reflex pupillary constriction to mask a partial contraction.

The guinea pig and rabbit pupils are circular, or almost so, at all stages of constriction. However, it was not found possible to standardise lighting or operational conditions sufficiently to measure the pupillary diameter accurately and reproducibly. An arbitrary division of response was adopted, based on visual estimation of the degree of maximal contraction, and the time required to reach this maximum.

It was noted that the presence of an incomplete contraction could often be confirmed by exposing the treated and control pupils to bright light for a few seconds. The partially contracted pupil re-expanded less fully and less rapidly than did the normal pupil.

Factors Affecting Response

Dose-response Relations

Two dosage variables had to be considered, the volume and the concentration of solution administered. There was found to be a definite increase in miotic response with increasing volume of solution administered at constant concentration. Hence the dose volume was standardised at one drop of volume 8 to 10 μl. The effect of ten-fold concentration changes was then found to be marked and reproducible. A small amount of individual variation was expected, and could be minimised by using two or more animals at each dose level. On no occasion has a gross

discrepancy been noted, provided the correct technique was used; statistical calculations were not possible.

No difference in response has been detected between the sexes in young guinea pigs. However, fully adult (500 g.) guinea pigs gave a lesser response than young (200 g.) animals. The rabbit eye was found to be about one-tenth as sensitive as the guinea pig eye.

Effect of Solvent

Substitution of propylene gylcol for water as diluent for 1×10^{-5} v/v TEPP produced a slightly more rapid onset of contraction, but no difference in final effect.

Tetrahydrofurfuryl alcohol containing 10 per cent v/v added water, as solvent for 1×10^{-5} v/v TEPP, was not detectably different in effect from propylene glycol; the undiluted alcohol caused slight irritation.

Possibility of False Positives

Some samples of commercial materials which were obviously irritant also caused a transient miosis, occuring within a minute or less, partial in degree, and returning to normal within 5 to 10 minutes. In some animals such a response caused a "sympathetic" miosis in the untreated eye, presumably due to a pain reflex. All such transient effects were accompanied by signs of corneal damage.

The introduction into the eye of a corrosive substance, such as 4 per cent aqueous sodium hydroxide, produced a rapid transient contraction of this type, even if the cornea had been locally anaesthetised with 2 per cent butacaine sulphate.

Miosis due to direct cholinesterase inhibitors never occurred within less than two minutes of application, even after 100 per cent TEPP. This was presumably due to the time taken for diffusion to the site of action, and for acetylcholine accumulation to occur. Also, recovery was delayed, and contraction of the untreated eye was never detected. There was no difficulty in distinguishing between a response produced by a direct inhibitor of cholinesterase and one produced by corneal irritation. No other cause of a "false positive" result has been seen.

Comparison of Miotic and *In Vitro* Results

Serial dilutions of a number of anticholinesterase substances were next examined by the standardised technique. These substances are listed in Table I.

TABLE I

Anticholinesterase substances examined by the standard technique

Schradan	Octamethyl pyrophosphoramide
Dimefox	Bis(dimethylamido)phosphorofluoridate
DDVP	Dimethyl 2:2-dichlorovinyl phosphate
Parathion (technical)	Diethyl *p*-nitrophenylthionophosphate
Tabun	Ethyldimethylamidophosphorocyanidate
Sarin	*O-iso*Propyl methylphosphonofluoridate
"AC 528" (technical 100 per cent)	2:3-Dioxylenebis(*OO*-diethylphosphorodithioate)
"FAC 20" (commercial 20 per cent)	*OO*-Diethyl *iso*propylcarbamoylmethyl dithiophosphate
Methyldemeton (thiolo- and thiono-isomers)	*OO*-Dimethyl (2-ethylthioethyl) thiophosphate
Demeton (thiolo-isomer)	*OO*-Diethyl(2-ethylthioethyl) thiophosphate

The results obtained, and a comparison with those of corresponding *in vitro* biochemical tests, are summarised in Table II.

TABLE II

COMPARISON OF MIOTIC AND *in vitro* DIRECT INHIBITORY POWERS OF VARIOUS ANTICHOLINESTERASE ORGANIC PHOSPHORUS MATERIALS

Substance(*a*)	Miotic limit (*b*) (Active ingredient)	Molar I 50	Miotic limit / Volume I 50
TEPP	10^{-7}	10^{-8}	100
Schradan	10^{0}	10^{-2}	
Dimefox	10^{0}	10^{-1}	
DDVP	10^{-4}	10^{-6}	1,000
Parathion (technical)	10^{-2}	10^{-3}	100
Tabun	10^{-8}	10^{-9}	100
Sarin	10^{-9}	10^{-10}	100
"AC 528" (technical 100 per cent)	10^{-4}	10^{-5}	100
"FAC 20" (commercial 20 per cent)	10^{-1}	10^{-3}	1,000
Methyldemeton ("Metasystox"):			
thiolo-isomer	10^{-4}	10^{-5}	100
thiono-isomer	10^{-3}	10^{-4}	100
Demeton ("Systox") (thiolo-isomer)	10^{-4}	10^{-6}	1,000

(*a*) Dissolved in propylene glycol
(*b*) Highest non-miotic volume concentration

Pure undiluted schradan and dimefox were found to produce no miosis, and fatal doses of these two materials could be absorbed by the eye without miotic response.

DISCUSSION

It would be expected that the magnitude of miotic response due to a direct cholinesterase inhibitor would be dependent on the amount of the substance reaching the effector end organs of the sphincter pupillae muscle, and hence on the amount of inhibitor administered.

The experimental findings are in agreement with this supposition. The magnitude of miotic response in the young albino guinea pig was dependent on both the volume and the concentration of solution of inhibitor administered. The results proved to be reproducible, and variation between individual animals was small. This test served to detect direct inhibitory activity equivalent to 0·01 μg. of TEPP.

The substitution of propylene glycol or tetrahydrofurfuryl alcohol for water as solvent for TEPP affected only the speed of onset of miosis. This is consistent with the supposition that change of solvent affects the rate of transport and release of inhibitor to the site of action, but not the amount carried. Malathion (*OO*-dimethyl-(1 : 2-dicarbethoxy)-ethyl dithiophosphate), a water-insoluble substance, gave a similar final response whether as aqueous emulsion or a propylene glycol solution.

The twelve substances selected for comparison of miotic and *in vitro* inhibitory activity showed a wide variation in structure and physical properties. In spite of this, the lowest concentration at which miosis could be detected showed a consistent relation to the I 50 concentration. The molar I 50 concentrations obtained by *in vitro* assay can be converted to approximate volume dilutions by multiplying by the molecular weight and dividing by 1000 times the density, giving a factor of about 0·1 for the substances tested, whose molecular weights are about 150 to 300.

Hence, Table II shows that, for most materials, the highest non-miotic concentration was about 100 times the I 50 concentration. This ratio of 100 was found with all but three of the substances tested, DDVP, FAC 20 and the thiolo-isomer of demeton, for which the ratio was 1000. Thus, graphs plotted from the logarithms of the corresponding pairs of concentrations showed a good linear relation. Observations made during these tests indicated that the main effect of differences in physical properties between compounds may be a slight effect upon the rate of development of miosis.

The possibility exists that miosis due to rapidly reversible direct inhibitors may be of detectably shorter duration than with "irreversible" inhibitors. It was observed that the duration of miosis produced by 1×10^{-3} v/v DDVP ($2\frac{1}{2}$ to 3 hours) was much shorter than that of similar miosis produced by 1×10^{-5} v/v TEPP (4 to 20 hours). Also, a rather sharp break-off of miotic effect with dilution was observed with DDVP. This is consistent with the known reversibility of DDVP inhibition[5].

The following method is now used for the estimation of miotic activity.

A series of ten-fold dilutions of the substance is made up in a non-irritant water miscible solvent. A standard volume of each dilution is then administered, usually one drop from a No. 16 needle, or 10 μl. from a micrometer syringe. One eye in a young albino guinea pig is used for each dilution. Comparisons with the control eye are made at regular intervals for one hour under lighting conditions sufficiently bright for adequate observation without producing appreciable reflex pupillary constriction. The results are then interpreted in relation to those obtained with a standard direct inhibitor, such as TEPP. Precision may be improved, by testing two or more eyes with each dilution. A reflex pupillary constriction due to corneal pain or irritation may be distinguished from a true cholinergic miosis by its more rapid and transient nature, often with sympathetic miosis in the other eye.

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