Cholinesterase Inhibition *in vitro* by *OO*-Diethyl *O*-*p*-nitrophenyl Thiophosphate (Parathion, E 605)

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OO-Diethyl *O*-*p*-nitrophenyl thiophosphate (I), generally known as parathion or E 605, is an insecticide which is of value against a variety of crop pests. Its mammalian toxicity is usually ascribed to its power of inhibiting cholinesterase, and this is also possibly its mechanism of insecticidal action. The *in vitro* inhibition of the enzyme was first studied by DuBois, Doull, Salerno & Coon (1949), who found that with their experimental conditions a concentration of 1.2×10^{-6} M was necessary to produce 50 % inhibition of the enzyme; they therefore regarded it as a strong *in vitro* inhibitor. Later, Aldridge (1950) showed that a concentration of the same order was required to inhibit red-cell cholinesterase.

Hestrin (1949) has described a chemical method for the estimation of acetylcholine, which has been used recently by Wilson & Bergman (1950) to determine cholinesterase activity. We have employed a modification of this method to assess the inhibition produced by samples of parathion and analogous compounds from different sources, and the wide variation in the results obtained has been related to the purity of the samples.

of this solution were diluted to 100 ml. with phosphate saline and brought to 37° in a constant temperature bath.

Alkaline hydroxylamine reagent. A 20 % (w/v) solution of hydroxylamine hydrochloride was prepared and stored in the refrigerator; it was discarded after 2 weeks. When the reagent was required the hydroxylamine hydrochloride solution was mixed with an equal volume of 20 % (w/v) NaOH.

Diluted HCl. Conc. HCl diluted with an equal volume of distilled water.

FeCl₃ reagent. 10 % (w/v) Hydrated FeCl₃ in 0.1 N-HCl.

All the above solutions, with the exception of the acetylcholine solution, were prepared with analytical quality reagents.

The determination of cholinesterase activity

A 1 % rat-brain homogenate in phosphate saline was used as a source of specific cholinesterase; it was found to maintain its original activity for at least a week when stored in a refrigerator. Into a stoppered tube, graduated at 15 ml. and maintained at 37° in a water bath, were pipetted 2 ml. of the brain homogenate and 1 ml. phosphate saline, followed by 2 ml. acetylcholine solution. After 15 min. the reaction was stopped by the addition of 4 ml. alkaline hydroxylamine reagent. After at least 1 min., 2 ml. diluted HCl and 3 ml. FeCl₃ reagent were added and the contents made up to 15 ml.

(I)

(II)

(III)

(IV)

EXPERIMENTAL

Reagents

Phosphate saline. This corresponds to a Krebs-Ringer phosphate solution without calcium.

Acetylcholine solution. A stock 0.01 M solution of acetylcholine chloride was prepared in 0.001 M-acetate buffer (pH 4.5) and stored in the refrigerator. When required, 3 ml.

and the solution filtered. The solution was transferred to a 1 in. cylindrical cell and the optical density read at 540 mμ. in a Unicam DG spectrophotometer. A slight fading was noted during the first 30 min. after mixing, which was largely accounted for by a similar fading in a blank determination on 5 ml. phosphate saline submitted to the above procedure; to standardize the procedure, readings were taken 30 min. after the addition of FeCl₃ reagent.

A standard curve was constructed with a series of dilutions of acetylcholine; this was linear over the range of concentrations encountered in this investigation. In order to estimate the acetylcholine remaining after the enzymic hydrolysis, the measured optical density, less the blank value, was applied to the standard curve. Fig. 1 shows that the extent of hydrolysis, plotted as $\log C_0/C$, where C_0 and C are the initial and final concentrations of substrate, is proportional to the time of hydrolysis until the reaction has proceeded about half-way to completion, after which the rate slows down. (All logarithms in this paper are to base 10.) This is consistent with a first-order reaction, and Fig. 2 shows the expected proportionality between $\log C_0/C$ and the concentration of enzyme when the time is kept constant at 15 min.

Fig. 1. Variation in extent of hydrolysis of acetylcholine ($\log C_0/C$) with time, the concentration of rat brain being 4 mg./ml.

Fig. 2. Variation in the extent of hydrolysis of acetylcholine ($\log C_0/C$) with the concentration of rat brain, the period of hydrolysis being constant at 15 min.

The variation in the cholinesterase activity of different rat-brain homogenates is not large; the mean percentage hydrolysis in 15 min. from thirteen tests is 59 with a coefficient of variation of 6.4%.

Measurement of inhibition

Solutions of the inhibitors to be examined were prepared containing 100 mg./100 ml. ethanol and a series of dilutions in phosphate saline were made. To 2 ml. of the brain homogenate was added 1 ml. of the inhibitor solution and the mixture incubated at 37° for 30 min. Then 2 ml. of substrate

were added and incubation continued for a further 15 min., after which the residual acetylcholine concentration C_I was estimated. If E_I is the activity of the enzyme in the presence of inhibitor, the fractional residual activity E_I/E_0 is equal to $(\log C_0/C_I)/(\log C_0/C)$.

(a)

(b)

(c)

Fig. 3a, b, c. Variation in the term $\log E_0/E_I$ with the concentration of inhibitor; the key to the numbers referring to the various inhibitors is given in Table 1.

RESULTS

Aldridge's (1950) investigation into the kinetics of red-cell cholinesterase inhibition by parathion has indicated that it has the characteristics of a

Table 1. *Inhibition of brain cholinesterase by various preparations of parathion and related compounds*

(The inhibitory activity is the reciprocal of the concentration needed for 50% inhibition of the enzyme.)

Ref. no.	Description	Concn. for 50% inhibition (mg./ml.)	Inhibitory activity	S-ethyl isomer (%)
1	Parathion purified by chromatography	—	—	<0.4
2	'Pure' parathion	23	0.43	0.5
3	'Pure' parathion	0.06	17.5	9.6
3a	Sample 3 extracted with N-NaOH	0.96	1.04	4.0
4	Technical parathion (sample X)	0.28	3.6	2.0
5	Technical parathion (sample Y)	0.35	2.9	0.5
6	Technical parathion (sample Z)	1.2	0.83	0.8
6b	Sample 6 heated 3 hr. at 150°	0.023	43.5	16.0
7	'OS-Diethyl O-p-nitrophenyl thiophosphate'	0.009	111	62.0
8	'OO-Diethyl S-p-nitrophenyl thiophosphate'	0.007	143	0.6
9	'OO-Diethyl O-p-nitrophenyl phosphate' (E 600)	0.002	500	1.3

bimolecular reaction with one component in large excess. If Aldridge's equation is assumed, the inhibitor concentration should be proportional to $\log E_0/E_1$, where E_0 is the original activity of the enzyme and E_1 the residual activity after inhibition. Figs. 3a, b and c show the results obtained when various samples of parathion and analogous compounds are investigated and the inhibitor concentration plotted against $\log E_0/E_1$. A key to the numbering in these figures is given in Table 1. The points fall tolerably well on straight lines, which differ very widely in slope. The points at which the ordinate $\log 2$ intersects these lines corresponds with the concentrations required for 50% inhibition of the enzyme; these values are given in Table 1. The reciprocals of the 50% inhibition concentrations are taken as a measure of the inhibitory activity of the samples.

DISCUSSION

All the samples examined for *in vitro* cholinesterase inhibition were subjected to the analytical procedure which has been described elsewhere (Gage, 1951). It became evident that, with a few exceptions, the very wide range in inhibitory activity, which is disclosed in Fig. 3 and Table 1, was related to the variation in the percentage of OS-diethyl O-p-nitrophenyl thiophosphate, the S-ethyl isomer of parathion (II). A sample of parathion which had been subjected to a repeated chromatographic purification procedure and which contained only a trace of the isomer (sample 1) had a very low *in vitro* activity; 50% inhibition could not be achieved with a saturated aqueous solution. Fig. 4 shows that the points obtained by plotting the inhibitory activity of the parathion samples against their percentage of the S-ethyl isomer fall approximately on a straight line passing through the origin. Samples stated to be the S-phenyl isomer of parathion (III) and the oxygen analogue of parathion, E 600 (IV), do not conform; these have an *in vitro* inhibition which is not attributable to their content of the S-ethyl isomer.

The conclusion to be drawn from this investigation is that pure parathion has no appreciable *in vitro* inhibition of cholinesterase when examined by the method described. The substrate concentration used in this investigation was $1.2 \times 10^{-4} M$; this is considerably lower than that used by previous investigators in this field, but the very low activity of the pure sample has been confirmed with $10^{-3} M$ -acetylcholine by an electrometric method. It may be

Fig. 4. Graph showing the linearity between the percentage of S-ethyl isomer and the inhibitory activity of the various samples. The samples are numbered as in Table 1.

concluded that the high cholinesterase inhibition ascribed by the earlier workers to parathion was due to active impurities in the samples used.

SUMMARY

1. The *in vitro* cholinesterase inhibition of a series of samples of parathion (OO-diethyl O-p-nitrophenyl thiophosphate) and of similar compounds has been studied using a rat-brain homogenate as a source of specific cholinesterase.

2. It has been shown that the *S*-ethyl and *S*-phenyl isomers of parathion and the oxygen analogue of parathion have a powerful inhibitory action.

3. The variable inhibition produced by samples of parathion can be entirely ascribed to contamination

with the *S*-ethyl isomer of parathion, and a highly purified sample, containing only a trace of this isomer, has a very low inhibitory action.

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Iron Metabolism and Haemoglobin Formation in the Embryonated Hen Egg

2. SOME OBSERVATIONS ON THE EMBRYONIC BLOOD SUPPLY

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In a recent paper (Ramsay, 1950) some evidence was presented suggesting that the membranes of the developing egg contain a substantial proportion of the total quantity of haem iron in the egg, and hence of the haemoglobin and blood. This paper discusses in greater detail the changes which take place in the amount of haem iron and its distribution between the body of the embryo and its membranes during incubation.

EXPERIMENTAL

General

Eggs from Brown Leghorn hens weighing 50–60 g. were incubated for various periods and frozen as soon as possible after removal from the incubator in thoroughly prechilled acetone- CO_2 . The shell was removed, and the embryo body dissected from the remainder of the egg contents. In this way the separation was easily made without loss of blood, and it is believed that the freezing was sufficiently rapid to prevent any serious redistribution of blood taking place.

Homogenates prepared with the addition of suitable quantities of ice-cold water from the embryo and the 'rest' (the 'rest' comprised yolk, white, allantoic and amniotic fluids, and the membranes, but not the shell) were separately analysed for non-haem and total Fe by the methods described below. In the interpretation of the results it has been assumed that the haem Fe in the 'rest' was derived entirely from the haemoglobin of the blood circulating in the membranes. These constitute the only living tissue outside the body of the embryo, and they are such highly vascular structures that the cytochrome and other haem compounds of the membrane cells themselves must surely be even less

important quantitatively than in less vascular systems. Where necessary, the rather less justifiable assumption has been made that in the embryo body also the amount of non-haemoglobin haem Fe might be neglected. This is based on the work of Drabkin (1948), who has shown that in the bodies of several species cytochrome and myoglobin do not account for more than about 10% of the total haem.

Non-haem iron

Reagents. Dipyriddy reagent: a solution containing 2.5 M-acetate, pH 4.6, 2,2'-dipyridyl, 0.2%, NH_4OH , HCl, 0.5%. Ethanol, redistilled.

Procedure. Homogenate (1 ml.) was treated with 4 ml. dipyriddy reagent and the mixture heated for 5 min. in a boiling-water bath. After cooling, the contents of the tube were made up to 12 ml. with ethanol and filtered through a no. 42 paper when the precipitate had coagulated. The Unicam D.G. Spectrophotometer at a wavelength setting of 520 m μ . was found very suitable for measuring the density of the pink colour. 'Blanks' were done in the same way, but with acetate buffer alone instead of the complete reagent.

Total iron

The technique for estimation of total Fe was based on the belief that the procedure just described does in fact determine all non-haem Fe, and on the knowledge (Peters, 1947) that haem pigments can be decomposed by H_2O_2 . It has been found that the decomposition is not always effective at low temperatures, perhaps because catalytic destruction of H_2O_2 interferes with the desired reaction. Moreover, H_2O_2 interferes with the subsequent dipyriddy reaction, but excess can be easily destroyed with Na_2SO_3 .

Reagents. H_2O_2 , 3% (w/v) in 2 M-acetate, pH 4.6. $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$, 40% (w/v). Neither of these reagents should be more than a week old.