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The Biochemical Evaluation of Paper Chromatograms of Parathion, its Isomers and Analogues

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Parathion, its analogues and isomers were separated from mixtures by paper chromatography. The chromatograms were evaluated biochemically by dividing the chromatogram into sections, the cholinesterase inhibiting effects of which were measured against a system of acetylcholine and purified enzyme. The enzyme inhibiting power of several pure *p*-nitrophenyl esters of phosphoric and thiophosphoric acids was determined and the biochemical evaluation of the chromatograms was carried out with four samples (pure compound, heterogeneous technical grade compound, 96.5 % technical grade parathion, and a commercial formulation for insecticidal use). The various derivatives formed by heating parathion and methyl-parathion, can be demonstrated with the new technique.

In connection with the testing of various methods for the chemical assay of parathion, including the chromatographic separation on paper of the different constituents present in technical grade preparations [1], certain biochemical studies were made, and these results are the basis of the present paper.

Methods for paper chromatography of parathion (O,O-diethyl-O-*p*-nitrophenyl thiophosphate) and related compounds have been described in recent publications. A variety of solvents and stationary phases have been tested but the most convenient technique reported so far is that described by Metcalf and March [2]. This method gives the best results according to the experience of our laboratory and has therefore been used, partly modified throughout the present investigation. Other methods use ethanol-ammonia-water [3,4] as solvent or ethyl ether in water-saturated petroleum ether [5]. The detection of parathion and its degradation product (*p*-nitrophenol) in these methods is based either upon ultraviolet absorption [3,5] or upon spraying with Millon's reagent [4].

In the biochemical analysis of paper chromatograms such as that described below, the enzyme (cholinesterase) inhibition by various sections or spots of the chromatograms was measured. The technique used is suggested as a valuable complement for the evaluation of paper chromatograms of organophosphorus compounds.

METHODS AND MATERIAL

Paper chromatography. Filter paper (Munktell OB) was impregnated with silicone (MS 550) from a 5 % hexane solution and air dried. The phosphate esters (ethanol solutions) were applied to the paper and chromatographed using the ascending technique. The solvent used was the upper phase from a mixture containing water-ethanol-chloroform (6 : 10 : 10). The paper was afterwards dried in an air stream, sprayed with 5 % alcoholic potassium hydroxide solution, and finally kept for some minutes in steam over boiling water. The various components were visible as yellow spots (*p*-nitrophenolate).

Measurement of enzyme inhibition. Undeveloped chromatograms, *i. e.*, those not treated with alcoholic potassium hydroxide solution, were used to study the cholinesterase inhibition by separated phosphate and thiophosphate esters. The chromatogram was cut into sections of the same size, usually 20 sections from the starting point to the solvent front and extracts of each section were made in a bicarbonate buffer solution. Cholinesterase inhibition by these extracts was assayed, using a technique described previously [6], with acetylcholine chloride as substrate and a purified preparation from human blood serum as enzyme source. Before the addition of substrate the enzyme was incubated for 50 min with the extract. The location of the active constituents thus obtained, evaluated as per cent enzyme inhibition, was compared with a chromatogram developed with the technique described above.

Phosphate and thiophosphate esters. Most compounds (see Table 1) used in the present study were kindly supplied by Dr. Gerhard Schrader [7]. Gifts of pure parathion were made by Plant Protection (England) and the American Cyanamid Co. (USA).

RESULTS

Paper chromatograms. The results obtained on analysing the various phosphate and thiophosphate esters by the paper chromatographic technique are summarised in Table 1. It gives the R_F values of all the pure grade compounds tested and, in addition, the values for three unknown constituents which were traced in a parathion preparation heated for 40 h at 140° C. Some of these unknown compounds were also found in some technical preparations. The *o*- and *m*-nitrophenyl esters had the same R_F values as the corresponding *p*-nitrophenyl ester, as demonstrated with the S-phenyl isomers of parathion and methyl-parathion.

Cholinesterase inhibiting effect. The I_{50} values (*i. e.*, molar inhibitor concentration giving 50 % enzyme inhibition) are recorded in Table 1. These values were obtained by incubating a cholinesterase preparation for 50 min at 25° C with various concentrations of the inhibitor prior to the addition of substrate (acetylcholine). The most active inhibitor of cholinesterase was found to be the S-phenyl isomer of parathion, confirming a recent observation made by Hecht and Wirth [9] with horse serum as an enzyme source. The bis-(*p*-nitrophenyl) ethyl ester of phosphoric acid came next, and then paraoxon and methyl-paraoxon. The inhibiting power of parathion was relatively low. The least active inhibitors in this series were methyl-parathion and bis-(*p*-nitrophenyl) ethyl thiophosphate. Amongst the three unknown compounds traced on the chromatograms, the two slow running esters had no measurable inhibiting effect on cholinesterase activity. The compound with R_F 0.90, on the other hand, did inhibit the enzyme, but its nature could not be determined. Double spotting cannot be excluded, however, in these cases.

Table 1. R_F values and cholinesterase inhibiting power of various *p*-nitrophenyl esters of phosphoric and thiophosphoric acids. pI_{50} = negative logarithm of molar inhibitor concentration (during enzyme incubation) giving 50 % inhibition.

R_F at 22° C	Formula	Code name	pI_{50}
0.01	$(C_2H_5O)_2P(S)-O-C_6H_4-NO_2$	Bis-nitrophenyl thioester *	<2.3
0.03	$(C_2H_5O)_2P(S)-O-C_6H_4-NO_2$	Parathion, E 605	< 4
0.06	$(CH_3O)_2P(S)-O-C_6H_3-NO_2Cl(m)$	Chlorthion	— **
0.11	$(CH_3O)_2P(S)-O-C_6H_4-NO_2$	Methyl-parathion	< 2
0.12	Unknown	—	No inhibitor
0.17	Unknown	—	No inhibitor
0.22	$(C_2H_5O)_2P(O)-O-C_6H_4-NO_2$	Bis-nitrophenyl ester *	8.2
0.40	$C_2H_5O(C_2H_5S)P(O)-O-C_6H_4-NO_2$	S-ethyl isomer	~ 6
0.51	$(C_2H_5O)_2P-S-C_6H_4-NO_2$	S-phenyl isomer of parathion	8.55
0.60	$CH_3O(CH_3S)P(O)-O-C_6H_4-NO_2$	S-methyl isomer	— **
0.70	$(C_2H_5O)_2P(O)-O-C_6H_4-NO_2$	Paraoxon, E 600	8.18
0.72	$(CH_3O)_2P-S-C_6H_4-NO_2$	S-phenyl isomer of methyl-parathion	6.5
0.79	$HO-C_6H_4-NO_2$	pNP	No inhibitor
0.84	$(CH_3O)_2P(O)-O-C_6H_4-NO_2$	Methyl-paraoxon	6.0
0.90	Unknown	—	Inhibitor

* The corresponding methyl ester probably has an R_F close to this.

** Pure compound not available. DuBois *et al.*[8] give for chlorthion a pI_{50} of 4.4 (rat serum; compound "more than 95 % pure"), Hecht and Wirth [9] for the S-methyl isomer a pI_{50} of 5.55 (horse serum).

The I_{50} values, as shown in Table 1, do not always give the correct measure of the inhibitory effects of a compound. This is especially true for parathion and its analogues because they are mostly very difficult to obtain in a pure state and the presence of an impurity may not be detected in a simple determination of the 50 % inhibition concentration (I_{50}). A better technique was introduced by Aldridge and Davison [10], who used bimolecular rate constants (for reaction of inhibitor with cholinesterase) instead of I_{50}. Despite this criticism of a comparison of the inhibitory power based upon I_{50} values, we believe the values of Table 1 to be of value for the technique described in the present paper. In fact, the 50 % inhibition concentration is a useful property of a preparation, pure or not, in deciding the amount to be applied to the filter paper for chromatography with subsequent enzymic evaluation.

The effect of solvent and silicone treated filter paper on the inhibitory effect. Before measuring the cholinesterase inhibition by various sections of paper chromatograms, the effects on enzyme inhibition by the solvent and the silicone treated paper were studied. There is a possibility that a compound may

Table 2. Change of cholinesterase inhibiting power of parathion and methyl-parathion applied on filter paper. Samples kept at room temperature for various periods of time before testing.

Compound	Days	% Inhibition		
		Untreated paper	Paper treated with silicone	Paper treated with silicone + solvent
Parathion	0	29	14.5	15
	1	64.5	32	39.5
	10	87	81	91.5
Methyl-parathion	0	13.5	3.5	9
	1	25.5	8.5	9.5
	8	28	13.5	17.5
	15	32.5	16	17.5

undergo isomerisation or oxidation during chromatography or spontaneous reactions may take place on a chromatogram kept for some time before biochemical evaluation.

The following experiments were made. Ethanol solutions of parathion and methyl-parathion respectively were placed on chromatographic paper

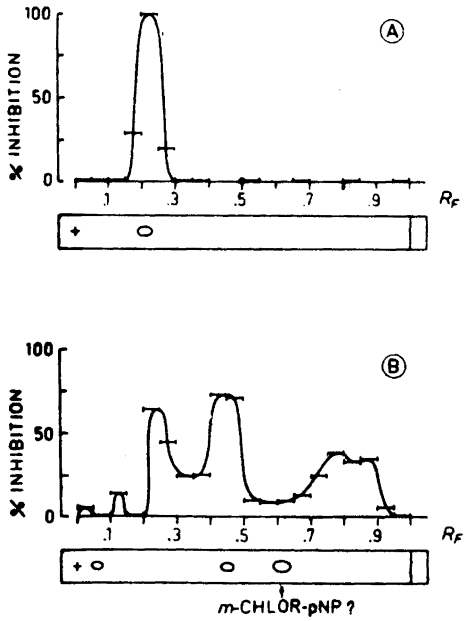

Fig. 1. Inhibition of cholinesterase by various sections of the paper chromatograms of bis- (*p*-nitrophenyl) ethyl phosphate (A), and a chlorthion preparation ("Chlorthion-Forte, Bayer") (B).

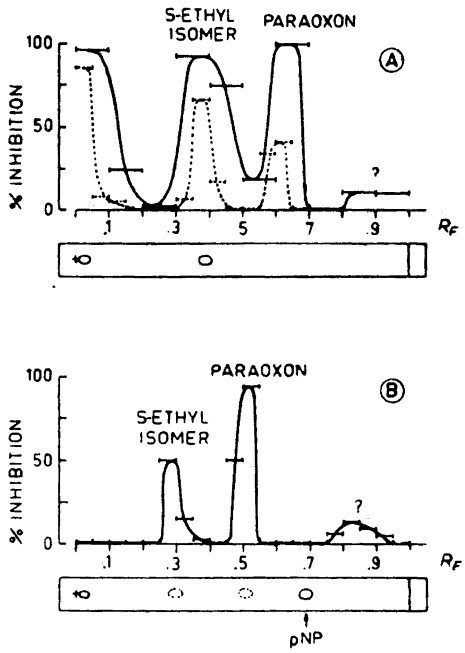

Fig. 2. Inhibition of cholinesterase by various sections of the paper chromatograms of a 96.5 % parathion preparation (American Cyanamid Co.) (A) (two separate experiments with various amounts applied on the paper), and a commercial formulation ("Ara-Parathion 35") for insecticidal use containing 35 % parathion (B).

(untreated, treated with silicone, and with silicone + solvent). After varying times, extracts of the spots were made in bicarbonate buffer and the cholinesterase inhibitory effect tested in the usual way. Table 2, recording the results, shows that both compounds studied underwent transformations to more active cholinesterase inhibitors, parathion more rapidly than its methyl analogue. It will be noted that the inhibitory effect of the samples on the silicone treated paper was about 50 % lower than the effect shown by the samples on untreated paper, except for very high inhibition values. The derivatives produced in these reactions are most probably the oxygen analogues (paraoxon and methyl-paraoxon) and the S-ethyl and S-methyl isomers which are known to be formed rather easily under a variety of experimental conditions. It is therefore to be expected that the section of a chromatogram containing parathion will show enzyme inhibition if it is not tested immediately after chromatographic separation, although parathion itself should not show this property (*cf.* Table 1). This is in accord with the experimental results (see Fig. 2 A).

The biochemical evaluation of chromatograms of pure and "pseudo pure" compounds. The results obtained in the biochemical evaluation of paper chromatograms are exemplified by four analyses. Fig. 1 A illustrates the

results obtained with bis-(*p*-nitrophenyl) ethyl phosphate. This highly active cholinesterase inhibitor gave only a single spot on the developed chromatogram and the biochemical results confirmed the purity of the preparation used. Fig. 1 B illustrates the chromatographic and biochemical results obtained with a technical grade preparation of chlorthion. Pure chlorthion is probably a weak enzyme inhibitor and it would therefore be expected that biochemical means would not be able to detect it on the chromatogram. The heterogeneity of the preparation used in this case was clear from the developed chromatogram but was still more pronounced when the various sections of the chromatogram were tested against a cholinesterase preparation. No efforts were made in the present investigation to identify the various constituents shown in the diagram, because pure preparations of chlorthion, its isomers and analogues were not available.

The chromatogram (Fig. 2 A) of a 96.5 % parathion preparation showed, in addition to the parathion area, an area of R_F 0.40 represented by the S-ethyl isomer. The biochemical results demonstrated the presence of a third constituent identified as the oxygen analogue of parathion (paraoxon). The results shown are from two separate experiments in which various amounts of the preparation were applied on the filter paper. The weak inhibitory effect of the area with R_F 0.9 was due to an unknown constituent demonstrated in several other parathion preparations analysed. It will be noted that the parathion section inhibited cholinesterase, an effect which would not be expected from the known weak inhibiting effect of this compound. The chromatograms in this case were kept for several days at room temperature before being analysed biochemically and during this period more active enzyme inhibitors (S-ethyl isomer and paraoxon) had been produced by spontaneous reactions (see above for proof of this explanation).

The next example is given in Fig. 2 B which illustrates the results obtained with a commercial parathion formulation. The lower R_F values obtained for the four visible constituents on the chromatogram (parathion, S-ethyl isomer, paraoxon, and *p*-nitrophenol) were most probably due to the disturbing effects of other unknown constituents (65 %) in this mixture. The biochemical analysis confirmed the presence of the S-ethyl isomer and paraoxon; in addition, the typical (for many technical grade parathion preparations) but unknown derivative with R_F 0.9 was present.

Isomerisation of parathion and methyl-parathion at high temperature. It is known from previous investigations [7,11,12] that parathion isomerises on heating, one of the products being the S-ethyl isomer. The strong cholinesterase inhibitor derived from parathion *in vivo*, on the other hand, is paraoxon [13]. After heating pure parathion for 40 h in a sealed tube at 140° C, chromatographic analysis of the mixture revealed the presence of eight constituents (Fig. 3 B). Four of these could be seen without difficulty on the developed chromatogram, *i. e.*, the bis-nitrophenyl ester, the S-ethyl isomer, paraoxon and *p*-nitrophenol. The other four (parathion and the three unknown constituents with R_F 0.12, 0.17 and 0.90) were visible when a greater amount of the heated mixture was chromatographed. Amongst these eight constituents, four were known to be highly active cholinesterase inhibitors and they were demonstrated clearly in the biochemical analysis (Fig. 3 B).

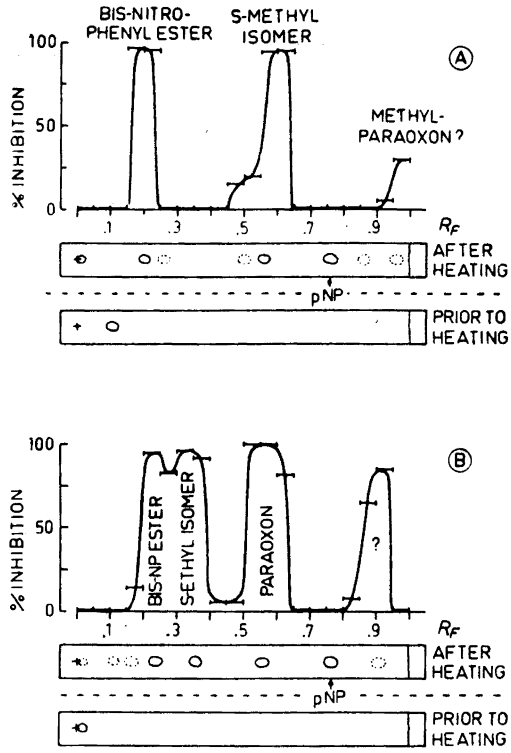

Fig. 3. Inhibition of cholinesterase by various sections of the paper chromatograms of methyl-parathion (A) and parathion (B); both preparations heated at 140° C for 40 hours.

The various constituents present in a methyl-parathion preparation heated in the same way were not as easily identified (Fig. 3 A). Eight constituents were present in this mixture also, some of which were traced from known R_F values. The slow running compound (R_F 0.02) was probably bis-(*p*-nitrophenyl) methyl thiophosphate. Two constituents were found to be strong cholinesterase inhibitors and they were almost certainly identical with bis-(*p*-nitrophenyl) methyl phosphate (R_F 0.20) and the S-methyl isomer (R_F 0.60), respectively. Except for *p*-nitrophenol the remaining constituents were unknown; one of the two fast running constituents was presumably identical with methyl-paraoxon.

DISCUSSION

Paper chromatography has been a valuable tool in analysing organophosphorus insecticides, especially technical grade parathion preparations [1]. It is proposed that this technique should be combined with a biochemical evaluation of the chromatograms as described in the present paper. Many parathion

preparations are more or less heterogeneous. The toxicity of these preparations is greatly dependent on the amounts of S-ethyl isomer, bis-(*p*-nitrophenyl) ethyl phosphate, and paraoxon present. These derivatives, especially in small amounts, are difficult to assay by available chemical methods. Paper chromatography alone is not sufficient in many cases, as its sensitivity is not high enough. A knowledge of the presence of these highly active constituents is important since otherwise misleading values may be obtained for toxicity, cholinesterase inhibitory power, hydrolysis rates, and insecticidal usefulness. These highly toxic isomers and analogues of parathion are formed spontaneously under various conditions and are especially important in the case of preparations which have been stored under uncontrolled conditions.

The technique described should be valuable in studying the metabolism of parathion, methyl-parathion, *etc.* in animals and plants as well as the mode of action of such compounds in mammals and insects and its usefulness may be extended to other organophosphorus compounds, many of which are highly active cholinesterase inhibitors. For parathion, and several related compounds, the toxicity and other properties are dependent on the presence of trace amounts of isomers or analogues which may give misleading results in pharmacological, biochemical and chemical studies. The homogeneity of preparations under examination can be tested easily by the technique as described in the present paper for a few compounds, the biochemical properties of which are characteristic of a larger group of toxic agents.

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