The Inhibition of 1,1,1-Trichloro-2,2-bis-(*p*-chlorophenyl)ethane (DDT) Dehydrochlorinase and Glutathione *S*-Aryltransferase in Grass-Grub and Housefly Preparations

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The inhibition of DDT [1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane] dehydrochlorinase and glutathione *S*-aryltransferase by diphenylmethane and triphenylmethane derivatives was examined. Bis-(3,5-dibromo-4-hydroxyphenyl)methane and similar compounds were excellent inhibitors of both enzymes, but only DDT dehydrochlorinase was inhibited by compounds similar to bis-(*N*-dimethylaminophenyl)methane. Colour salts of the basic triphenylmethyl dyes were excellent inhibitors of both enzymes. All the inhibitors examined appeared to act by competition with glutathione for its binding site on the two enzymes.

It has been shown that vertebrate tissues (Boyland & Chasseaud, 1969) and insects (Clark, Murphy & Smith, 1969; Clark, Darby & Smith, 1967; Ishida & Dahm, 1965) contain a group of enzymes that catalyse the formation of *S*-substituted derivatives of GSH. One of these, GSH *S*-aryltransferase, which is present in high concentration in a number of insect species, can be inhibited by some dicarboxylic acids as well as by low concentrations of phthaleins and similar structures (Cohen, Smith & Turbert, 1964; Clark *et al.* 1967). Some of these inhibitors are also effective on the GSH-dependent enzyme systems in insects that detoxify gammexane (γ -1,2,3,4,5,6-hexachlorocyclohexane) and pentachlorocyclohexenes (Clark *et al.* 1969).

A similar insect enzyme detoxifies DDT* by removal of hydrogen chloride from the molecule and, although there is no evidence for the formation of any *S*-substituted GSH as an intermediate in this reaction, DDT dehydrochlorinase has a specific requirement for GSH as a cofactor (Lipke & Kearns, 1960). The common requirement for GSH by the GSH *S*-aryltransferases and DDT dehydrochlorinase suggested that the latter enzyme might also be inhibited by molecules that act as competitive inhibitors of the GSH-binding site in GSH *S*-aryltransferase, and that a study of the inhibition of DDT dehydrochlorinase and GSH and *S*-aryltransferase would be valuable. A secondary consideration was that information about structures required for inhibition of DDT dehydrochlorinase might permit the selection or design of compounds acting as synergists for DDT in DDT-resistant insects.

MATERIALS AND METHODS

Inhibitors. Compounds prepared by standard methods were leucobenaurin (m.p. 156°C), 3,3',5,5'-tetrabromo-leucobenaurin (m.p. 158°C), 3,3',5,5'-tetrabromophenolphthalin (m.p. 138°C), 3,5-dibromo-4-hydroxybenzoylbenzoic acid (m.p. 246°C), bis-(3,5-dibromo-4-hydroxyphenyl)methane (m.p. 225°C), bis-(3,5-dibromo-4-hydroxyphenyl)methanol (m.p. 173°C), 3,3',5,5'-tetrabromo-4,4-dihydroxybenzophenone (m.p. 218°C), 3-(3',5'-dibromo-4'-hydroxyphenyl)phthalide (m.p. 223°C), dimethyl undecanedioate (b.p. 145°C/2 mmHg) and methyl hydrogen undecanedioate (b.p. 165°C/2 mmHg). Other compounds, indicators and dyes were commercial samples used without further purification.

Preparation of enzymes. Grass-grub (*Costelytra zealandica*) acetone-dried powder was prepared as described by Clark *et al.* (1967). Houseflies (*Musca domestica*) from a DDT-resistant strain (SP2AB, LD₅₀ 1.7 μ g of DDT/female insect, obtained from Dr D. Spiller, Plant Diseases Division, Department of Scientific and Industrial Research, Auckland, New Zealand) were homogenized at 5°C in an equal volume of water and centrifuged at 10000*g* for 30 min at 5°C. The supernatant was poured with stirring into a large volume of acetone at -15°C, and the precipitate was filtered, washed with dry acetone at -15°C and dried *in vacuo*. Both powders were freely soluble in water, and all kinetic experiments with DDT dehydrochlorinase were carried out with 1 mg of the fly preparation dissolved in 2 ml of incubation mixture.

Enzyme assays. The assay method for DDT dehydrochlorinase was similar in principle to that of Oppennoorth & Voerman (1965) and based on the g.l.c. measurement of DDE. Incubations were for 30 min at 37°C in air in a total

* Abbreviations: DDT, 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane; DDE, 1,1-dichloro-2,2-bis-(*p*-chlorophenyl)ethylene.

volume of 2ml of pyrophosphate buffer (0.02M- $\text{Na}_4\text{P}_2\text{O}_7$, adjusted to pH 7.4 with 10M-HCl) containing 1mg of acetone-dried powder. GSH was added as a 20mM solution in pyrophosphate buffer, pH 7.4, containing 1mM-EDTA, to give a final concentration of 1.0mM-GSH for routine assays or in the range 0.2–1.0mM for kinetic studies. DDT in 0.02ml of dimethyl sulphoxide was added to start the reaction and to give a concentration of $10\mu\text{M}$ for routine assays or in the range 1–5 μM in the kinetic experiments. Inhibitors were added in 0.025–0.1ml of water or dimethyl sulphoxide, depending on their solubility, to give concentrations in the range 1–50 μM . After 30min 1ml of 3% (w/v) HClO_4 was added and the solution shaken with 1ml of propan-2-ol and 2ml of sulphur-free xylene (May and Baker Ltd., Dagenham, Essex, U.K.). A further 20ml of water was added and, after shaking and centrifuging to separate the layers, DDE was assayed in the xylene layer by g.l.c. Control incubations that lacked enzyme or DDT or GSH produced no DDE.

GSH *S*-aryltransferase from flies or grass grubs was assayed with 1-chloro-2,4-dinitrobenzene as described by Clark *et al.* (1967) by using a Perkin-Elmer model 137 u.v. recording spectrophotometer.

G.l.c. A Perkin-Elmer model 811 gas chromatograph fitted with an electron-capture detector was used. The glass column measured 40 cm $\times$ 0.3 cm and was packed with 0.5% silicone oil (Embaphase; May and Baker Ltd.) on 80-mesh Celite. The oven temperature was 170°C, and the flow rate was adjusted to about 80ml/min to give retention times of about 1 min for DDE and 2 min for DDT. Quantitative measurements were made by comparison of the experimental peak heights with similar ones from standard solutions of DDE, and recoveries of 0.1–1 μg of DDE added to the standard enzyme assay mixture were in the range 95–105%.

Plotting of results. The symbols used are those defined by Dixon & Webb (1964, p. 786). Graphical methods described by Webb (1963, p. 149) were used to interpret the type of inhibition, and in experiments to determine K_i values five concentrations of substrate were used at each of four inhibitor concentrations chosen to give a suitable spread of experimental values for plotting. The results were plotted on axes of $1/v$ against $1/s$ (type I), $1/v$ against i (type II) and $i\alpha/(1-\alpha)$ against s (type III). Apparent values of K_i were taken from the intersection of lines on the plot of $1/v$ against i , or from the value of $i\alpha/(1-\alpha)$ at $s = 0$.

Considerable variation of estimated K_i values can arise from plotting errors in these methods, but values obtained from the type II and type III plots were in agreement and replicate determinations of values quoted in Table 1 were within $\pm 50\%$ of the quoted values.

RESULTS

Factors affecting the DDT dehydrochlorinase assay. The rate of formation of DDE in incubations containing 1mg of housefly acetone-dried powder was constant for at least 1h under the assay conditions described above when DDT concentrations in the range 2–50 μM were used. Reaction

Fig. 1. Effect of amount of enzyme acetone-dried powder on rate of formation of DDE by DDT dehydrochlorinase from houseflies. Assay mixtures contained 10 μM -DDT and 1.0mM-GSH in 2ml of 0.02M-pyrophosphate buffer, pH 7.4, and DDE was assayed by g.l.c. as described in the text. Two separate enzyme preparations were used for the two curves.

rates were also constant for at least 1h with GSH concentrations in the range 0.1–2mM.

Values for the K_m for DDT (with 1.0mM-GSH) derived from a large number of experiments made on different occasions were in the range 2–3 μM . Values for the K_m for GSH (with 10 μM -DDT) were also obtained and were in the range 0.1–0.3mM. The optimum pH for the reaction in 0.02M-pyrophosphate buffer was at pH 7.4, but some assays carried out in 0.02M-tris-HCl or 0.02M-sodium barbital buffers showed maximum activity at pH 8.4.

The rate of formation of DDE was not proportional to the amount of enzyme when increasing amounts of acetone-dried powder were used if the concentrations of GSH and DDT were 1mM and 10 μM respectively (Fig. 1). This effect was more pronounced with some preparations of powder, but it was not observed when less than 1mg of powder/tube was used. With 50 μM -DDT the rate was proportional to the amount of enzyme used up to 5mg/tube, but the large amounts of DDT were a hindrance to g.l.c. analyses of DDE.

Addition of bovine serum albumin (Sigma Chemical Co., St Louis, Mo., U.S.A.) to an assay system with 10 μM -DDT containing 0.5mg of acetone-dried powder/ml also progressively diminished the yield of DDE, 3mg/ml decreasing the apparent activity of the system to half. Recovery of DDE from control preparations was not affected

by increasing the amount of acetone-dried powder in the assay mixture.

Determination of substrate constants for DDT dehydrochlorinase. The procedure of Florini &

Vestling (1957) was employed with three separate preparations of acetone-dried powder enzyme from houseflies and values of the four substrate constants for DDT dehydrochlorinase were estimated from the graphs. Mean values of K_{GSH} and K'_{GSH} obtained from these experiments were 0.4 (0.3–0.5) mM and 0.3 (0.2–0.4) mM. Values for K_{DDT} and K'_{DDT} were 4 (3–5) μM and 4 (2.5–5) μM .

Measurement of apparent inhibition constants. Assays of activity for GSH *S*-aryltransferase in houseflies and grass grubs and DDT dehydrochlorinase in houseflies were made in a series of inhibited incubation mixtures set up as described above with a range of GSH and inhibitor concentrations, and the results were plotted as described.

Aliphatic dicarboxylic acids were relatively poor inhibitors of GSH *S*-aryltransferase (Fig. 2). Traumatic acid (*trans*-dodec-2-ene-1,12-dioic acid) (K_i 60 μM) and 3,3'-dicarboxydiphenylmethane (K_i 45 μM) were the most effective inhibitors; all the acids gave type I and type II plots indicative of competition with GSH.

All other inhibitors of GSH *S*-aryltransferase studied also gave rise to simple plots, suggesting competition with GSH, and the K_i values quoted in Table 1 were derived from the values of i at the intersection of the five lines on type II plots.

No significant inhibition (<25%) of grass-grub

Fig. 2. Inhibition of grass-grub GSH *S*-aryltransferase by dicarboxylic acids. Inhibition constants were measured with 0.1 mM-1-chloro-2,4-dinitrobenzene with a range of GSH concentrations as described in the text. ○, α,ω -Dicarboxylic acids from C_7 to C_{16} ; △, traumatic acid; □, 3,3'-dicarboxydiphenylmethane.

Table 1. *Inhibition kinetics of housefly and grass-grub GSH S-aryltransferases*

Assays were made as described in the text in solutions containing 0.1 mM-1-chloro-2,4-dinitrobenzene at a range of GSH concentrations. Apparent K_i values were taken from the value of i at the intersection of lines on plots of $1/v$ against i . All compounds gave competitive-type plots on axes of $1/v$ against $1/[\text{GSH}]$. N.T., Not tested.

Inhibitor	Apparent K_i (μM)	
	Housefly enzyme	Grass-grub enzyme
Tetrabromophenolphthalein	20	0.5
Tetrabromophenolphthalein ethyl ester	N.T.	1
Tetrabromophenolphthalein isopropyl ester	N.T.	1
Tetrabromophenolphthalin	20	1
Bromophenol Blue	15	0.5
Tetrabromophenol Blue	N.T.	0.5
Bromothymol Blue	30	10
Bromocresol Green	20	0.5
Bromocresol Purple	50	2
Eosin	30	3
Fluorescein	200	N.T.
Phenol Red	60	N.T.
Phenolphthalein	N.T.	20
Leucobenzaurin	N.T.	40
Phenoltetrabromophthalein	N.T.	30
Phenoltetrabromophthaleindisulphonate	20	N.T.
Bis-(3,5-dibromo-4-hydroxyphenyl)methane	N.T.	1
Bis-(3,5-dibromo-4-hydroxyphenyl)methanol	N.T.	1
3,3',5,5'-Tetrabromo-4,4'-dihydroxybenzophenone	N.T.	2
3,5-Dibromo-4-hydroxyphenylphthalide	N.T.	6
Rhodamine B	N.T.	80

GSH *S*-aryltransferase was observed when bis-(4-aminophenyl)methane, bis-(*N*-dimethylaminophenyl)methane, leucoMalachite Green, Methylene Blue, Michler's ketone, 1,1-bis-(*p*-chlorophenyl)-

ethanol, 4,4'-dichlorobenzophenone or 3,5-dibromo-4-hydroxybenzoylbenzoic acid were tested as inhibitors at 0.1 mM.

In contrast with the GSH *S*-aryltransferase work, plots obtained from experiments with inhibited DDT dehydrochlorinase did not produce patterns characteristic of simple competitive or non-competitive inhibition, most inhibitors giving results suggesting 'mixed' or 'partial' inhibition on one or more of the axes used. Graphs obtained from experiments with inhibited DDT dehydrochlorinase on type III plots were linear, though in some cases a set of lines rather than a single line was obtained (Fig. 3). For these inhibitors values of $i\alpha/(1-\alpha)$ at $s = 0$ have been quoted in Table 2 as a range of apparent K_i values from the two extreme intersections. Since these cases also gave rise to non-linear type II plots, it was not possible to deduce values of K_i from this procedure.

Reliable results from which the nature of the inhibition could be deduced could not be obtained with aurintricarboxylic acid (Aluminon) or with the basic dyes such as Malachite Green and pyronin. Except for Rhodamine B, which was a competitive inhibitor of aryltransferase in higher concentrations, these compounds were immediately but incompletely reduced by GSH, the extent of reduction being dependent on the concentration of dye and GSH. Nevertheless, this group of dyes were effective

Fig. 3. Inhibition of DDT dehydrochlorinase by 3,3',5,5'-tetrabromophenolphthalein. Results from duplicate experiments were plotted by the method of Hunter & Downs (1945). Experiments were carried out with two batches of enzyme with inhibitor concentrations of: ●, 50 μ M; ○, 25 μ M; △, 12.5 μ M.

Table 2. Inhibition kinetics of housefly DDT dehydrochlorinase

Incubation mixtures were prepared as described in the text with a range of DDT, GSH and inhibitor concentrations and assayed after 30 min for DDE by g.l.c. Results were plotted on axes of $1/v$ against $1/s$ (I), $1/v$ against i (II) and $i\alpha/(1-\alpha)$ against s (III). The form of the plots is indicated as follows: N, a non-competitive pattern on I and III; C, competitive pattern on I and III; L, linear intersecting sets of lines on II; M, 'mixed-inhibition' pattern on I; A, anomalous patterns with curved lines on I and II or a set of lines on III. Sets of lines with a positive slope on III have been designated as competitive as well as anomalous and the apparent K value shown as a range taken from the extreme values of $i\alpha/(1-\alpha)$ at $s = 0$ on the type III plot. 'WARF anti-resistant' is *NN*-di-*n*-butyl-*p*-chlorobenzenesulphonamide.

	DDT substrate at 1 mM-GSH				GSH substrate at 10 μ M-DDT			
	Plot type on			K_i (μ M)	Plot type on			K_i (μ M)
	I	II	III		I	II	III	
Bromocresol Green	N	A	C, A	10-40	M	L	C	30
Phenol Red	N	L	N	55	M	L	C	60
Bromocresol Purple		—	—	—	M	L	C	120
Chlorophenol Red		—	—	—	M	L	C	25
Bromothymol Blue		—	—	—	M	L	C	35
Bromophenol Blue		—	—	—	A	L	C	25
Tetrabromophenolphthalein	A	A	C, A	15-40	M	A	C, A	20-40
Tetrabromophenolphthalein ethyl ester	M	L	C	20	A	A	C, A	5-15
Phenoltetrabromophthalein		—	—	—	C	A	C	1
Bis-(3,5-dibromo-4-hydroxyphenyl)methane		—	—	—	A	A	C, A	40-60
Bis-(<i>N</i> -dimethylaminophenyl)methane	N	L		22	M	A	C, A	5
'WARF anti-resistant'	C	L	C	10	N	L	N	15

Table 3. *Inhibitors of housefly DDT dehydrochlorinase*

Assays were carried out as described in the text except that 20 μ M-DDT was used as substrate. Inhibitor concentration was 50 μ M.

	Inhibition (%)	
	1.0 mM-GSH	0.25 mM-GSH
Sulphonphthaleins		
Tetrabromophenol Blue	80	90
Phthaleins		
Tetrabromophenolphthalein ethyl ester	52	67
Tetrabromophenolphthalein isopropyl ester	71	79
Phenoltetrabromophthalein	100	100
Phenoltetrabromophthaleindisulphonate	80	90
Pyronines		
Pyronin G	90	90
Fluoresceins		
Phloxin (tetrabromotetrachlorofluorescein)	88	90
Eosin (tetrabromofluorescein)	78	82
Ethyleosin	60	71
Di-iodo(R)fluorescein	64	68
Di-iodo(R)dimethyl(R)fluorescein	71	84
Dichlorofluorescein	78	84
Erythrosin A (tri-iodofluorescein)	51	61
Erythrosin B (tetraiodo(R)fluorescein)	50	60
Tetrachloro(P)tetraiodo(R)fluorescein	86	90
Triphenylmethane derivatives		
LeucoMalachite Green	70	65
Methyl Violet	90	80
Methyl Green	80	90
Fuschin basic	89	90
Corallin	80	75
Diphenylmethane derivatives		
Bis-(3,5-dibromo-4-hydroxyphenyl)methane	45	65
Bis-(3,5-dibromo-4-hydroxyphenyl)methanol	47	54

inhibitors of DDT dehydrochlorinase (Table 3) and of GSH *S*-aryltransferase, a 25 μ M solution of Malachite Green inhibiting the activity of the latter enzyme by 50% in 2 mM-GSH.

Since the initial kinetic studies with DDT dehydrochlorinase were not indicative of a simple situation, most measurements of the inhibition of this enzyme were made at a single concentration of inhibitor. Compounds were selected that contained dialkylamino or hydroxyl groups in similar orientation to the hydroxyl groups in the phthaleins, and those giving more than 50% inhibition at 50 μ M are listed in Table 3. Some compounds tested under the conditions of Table 3 produced less than 50% inhibition. These were Bromocresol Purple, Bromophenol Blue, Bromocresol Green, Bromopyrogallol Red, Glycine Cresol Red, Glycine Thymol Blue, phenolphthalein, tetrabromophenolphthalein, tetrabromophenolphthalin, gallein, Rhodamine B, Rhodamine 6G, Rhodamine 3GO, fluorescein, tetrachlorofluorescein, auramine, tetramethyldiamino-

benzophenone, diaminodiphenylmethane, safranin, phenosafranin, Nile Blue, Cresyl Fast Violet, 3,5-dibromo-4-hydroxybenzoylbenzoic acid, leucobenzaurin, 3,5-dibromo-4-hydroxyphenylphthalide, 2,7-diaminofluorene, Methylene Blue and proflavine.

Dicarboxylic acids did not inhibit DDT dehydrochlorinase at the concentrations used in Table 3, but at 0.3 mM inhibition was observed in 1.0 mM-GSH with traumatic acid (20%) and 3,3'-dicarboxydiphenylmethane (20%). At this concentration undecanedioic (20%) and brassylic acid (28%) were relatively poor inhibitors, though their dimethyl esters gave 35 and 75% inhibition respectively.

DISCUSSION

The results described above clarify some of the structural requirements for an inhibitor of GSH *S*-aryltransferase (Table 1). Substitution of halogens *ortho*- to the oxygen atoms decreased the K_i

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values about 20-fold. Eosin (I), tetrabromophenolphthalein, its ethyl ester and the sulphonphthalein, Bromophenol Blue (II), all had K_i values near $1 \mu\text{M}$ with the grass-grub enzyme.

Reduction of the quinonoid form to give tetrabromophenolphthalin (III) had little effect on the K_i value and the loss of phthalein structure in the dihydroxyphenylmethane derivatives also changed the K_i value insignificantly.

Loss of the four halogen atoms (e.g. as in Phenol Red or phenolphthalein) gave much less effective inhibition, as did the monoarylphthalide (IV). The results support the suggestion of Clark *et al.* (1967) that the two oxygen atoms in structures similar to 4,4'-dihydroxydiphenylmethane that are isosteric with oxygen atoms in the carboxyl groups of GSH are concerned in the binding of the inhibitors to the enzyme. All these compounds gave rise to plots in kinetic experiments characteristic of competition with GSH for the enzyme.

Analogous basic triphenylmethane and diphenylmethane derivatives were also tested, and with Rhodamine B, the only example tested from which reliable assays were obtained, inhibition of grass-grub GSH *S*-aryltransferase was found to be competitive. Other quinonoid forms of basic dyes tested against DDT dehydrochlorinase (Table 2) were also found to inhibit GSH *S*-aryltransferase, but since the dyes were partly reduced by GSH quantitative results were not obtained. Nevertheless this type of molecule, for instance Malachite Green, was a more effective inhibitor of GSH *S*-aryltransferase than the best of the brominated

phthaleins. In contrast with the phthaleins, the reduced forms of the basic compounds, i.e. leuco-Malachite Green and bis-(*N*-dimethylaminophenyl)-methane, did not inhibit GSH *S*-aryltransferase from houseflies or grass grubs at the concentrations used in Table 1.

In the phthalein group of inhibitors the behaviour towards GSH *S*-aryltransferase was a useful qualitative guide to their action on DDT dehydrochlorinase, both enzymes being affected by these compounds. The similarity also extended to the much less inhibitory dicarboxylic acids, where the best GSH *S*-aryltransferase inhibitors were also effective as DDT dehydrochlorinase inhibitors.

Inhibition of the two enzymes by the basic compounds was different, since both the oxidized forms, e.g. Malachite Green (Table 2), and reduced forms, e.g. leucoMalachite Green, were effective inhibitors of DDT dehydrochlorinase whereas only the oxidized forms inhibited GSH *S*-aryltransferase.

Quantitative comparisons of the inhibition of two housefly enzymes were not possible, since straightforward plots from which K_i values could be deduced were not obtained with most DDT dehydrochlorinase experiments (Table 2). Unusual kinetics have been observed previously with this enzyme by Lipke & Kearns (1960), though their use of DDT in a lipoprotein complex makes interpretation of their system difficult. The use of much lower concentrations of DDT made possible by the g.l.c. assay makes it more probable in the present study that DDT molecules were equilibrating with the enzyme, though here also the solutions of DDT used (approx.

10 μM) were in excess of the water-solubility of this compound.

Three other possible explanations for the anomalous DDT dehydrochlorinase kinetics may be suggested. It is possible that DDT or GSH may modify the binding of the second substrate if already attached to the enzyme. Attempts were made to assess this effect by using the procedure of Florini & Vestling (1957), and although plotting errors in this method may be considerable, the results obtained above do not suggest that the substrate-substrate interaction with housefly DDT dehydrochlorinase is any more significant than is the case with grass-grub GSH *S*-aryltransferase (Clark *et al.* 1967).

The analogous effect of DDT on the binding of a GSH-competitive inhibitor is less easy to assess, since the kinetics of inhibited two-substrate enzymes are complex, and little theoretical attention has been given to these systems. Some mathematical investigations of these systems were therefore carried out to calculate the effect on the usual plotting patterns of substrate-substrate-inhibitor interactions (Clark, 1968, 1970).

A more likely possibility that could have given rise to anomalous DDT dehydrochlorinase kinetics is the competition of separate inhibitor molecules at both sites of the enzyme simultaneously. This could be expected, since many of the GSH-competitive inhibitors used can be regarded as structural analogues of DDT and could be competitive at the DDT site in the enzyme as well as at the GSH-binding site. Calculations based on this model show that the form of the plots is highly characteristic, those on type II axes being parabolic and those on type I axes having no common point of intersection except in certain limiting cases (Clark, 1968, 1970).

These types of plot were not encountered among the anomalous situations listed in Table 2, and it is presumed that at the inhibitor concentrations used in our experiments dual competition was not a significant factor.

Non-linear plots on type II axes could also be attributed to the presence of two DDT dehydrochlorinases having different affinities for the various inhibitors. This could produce plots similar to cases of partial inhibition. This possibility has been examined by electrofocusing and electrophoretic techniques (Goodchild & Smith, 1970), though the history of the housefly strain used in this work suggests that only a single resistance factor is concerned (D. Spiller, personal communication). Multiple forms of DDT dehydrochlorinase have, however, been reported, and highly resistant houseflies may contain an enzyme different in its substrate and inhibitor affinities from that found in susceptible houseflies (Oppenoorth, 1965).

In spite of the complexity of the kinetics the action

of most of the inhibitors examined was competitive with GSH for DDT dehydrochlorinase in that the inhibition was diminished by increasing the GSH concentration. These compounds represent new types of structure, which may serve as synergists for the insecticide in resistant insects where the resistance is due to increased detoxication.

Previous attempts to find synergists for DDT have concentrated on compounds having a structural similarity to DDT (March, Metcalf & Lewallen, 1952; Speroni, 1952; Neeman, Mer, Cwilich, Modiano & Zacks, 1957; Metcalf, 1965). These compounds have been assumed to compete with DDT for the detoxication enzyme and to decrease the amount of detoxication product formed (Moorefield & Kearns, 1955). With the sensitivity of the g.l.c. method it has been possible to demonstrate this in a formal kinetic experiment with 'WARF anti-resistant' (Table 2), where the type of plot given by this compound is of the typical competitive type with respect to DDT.

A possible disadvantage of the use of DDT analogues as synergists for DDT in resistant insects is the selection of synergist-insensitive forms of DDT dehydrochlorinase. This has been observed by Oppenoorth (1965), who showed that the form of the enzyme present in houseflies that were resistant to a mixture of DDT and 1,1-bis-*p*-chlorophenylethanol (DMC) was less readily inhibited by 1,1-bis-*p*-chlorophenylethanol than enzyme from houseflies resistant to DDT only. The differences between these two enzymes are not yet clear, but both required GSH as cofactor. The availability of a different series of enzyme inhibitors that compete for GSH rather than for DDT may be of value in countering this type of resistance.

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