The Separation of Multiple Forms of Housefly 1,1,1-Trichloro-2,2-bis-(*p*-chlorophenyl)ethane (DDT) Dehydrochlorinase from Glutathione *S*-Aryltransferase by Electrofocusing and Electrophoresis

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Electrophoresis in a sucrose gradient at pH values between 5 and 8 separated housefly DDT [1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane] dehydrochlorinase into two major fractions. GSH *S*-aryltransferase under similar conditions migrated as a single peak of activity. Separation of housefly homogenates or partially purified enzyme preparations by electrofocusing in a natural pH gradient also showed the presence of multiple forms of DDT dehydrochlorinase.

The detoxication of DDT* in houseflies is catalysed by the enzyme DDT dehydrochlorinase, which previous studies have shown to be inhibited by compounds that also inhibit GSH *S*-aryltransferase (Clark, Darby & Smith, 1967; Balabaskaran, Clark, Cundell & Smith, 1968; Balabaskaran & Smith, 1970). This is not unexpected, since both enzymes have a requirement for GSH, in the one case as a substrate and as a cofactor or activator in the other. Kinetic experiments with inhibition of GSH *S*-aryltransferase from both houseflies and grass grubs gave rise to simple patterns when plotted according to the double-reciprocal method of Lineweaver & Burk (1934) or by Dixon's (1953) method, which suggested that the inhibitors were competing with GSH for this enzyme (Clark *et al.* 1967; Balabaskaran & Smith, 1970). The inhibition of DDT dehydrochlorinase did not give simple plots under comparable conditions, and one reason for this that was considered was the possible presence of multiple forms of this enzyme in houseflies with different affinities for substrate or inhibitor.

Some justification for this view can be found in the report by Oppenoorth (1965) that the DDT dehydrochlorinase in a very DDT-resistant strain of houseflies differed in substrate-specificity from that in more susceptible strains.

We have therefore examined crude and partly purified enzyme extracts from houseflies, and it is shown below that, whereas GSH *S*-aryltransferase migrates as a single zone in electrophoretic experiments, DDT dehydrochlorinase can be separated into at least two fractions.

* Abbreviation: DDT, 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane.

METHODS

Houseflies. These (*Musca domestica*) were reared on a milk powder, yeast and agar medium and adults were fed on sucrose and water for 3 days before use. The fly strain (SP2AB) was obtained from Dr D. Spiller, Plant Diseases Division, Department of Scientific and Industrial Research, Auckland, New Zealand, and was the progeny of a single-pair mating possessing a low degree of resistance (LD₅₀ 1.7 µg of DDT/female) dependent on the presence of DDT dehydrochlorinase. The SP2AB strain was derived from a polymorphic strain originally obtained from Canberra and had been reared in the laboratory since before the use of DDT and had never been exposed to insecticide.

Enzyme preparations. Flies were homogenized in twice their weight of ice-water in a top-drive MSE homogenizer and the mixture was centrifuged at 10000g for 20 min at 0°C. The supernatant was poured into 50 vol. of dry acetone at -20°C, and the buff-coloured precipitate was filtered and washed with dry acetone at -20°C and dried *in vacuo*. In a number of experiments in which 25–100g of flies was used, the yield of dry powder was 5–6% of the weight of the flies. These powders had specific activities in the range 0.030–0.033 µmol/min per mg for GSH *S*-aryltransferase and in the range 0.045–0.050 nmol/min per mg for DDT dehydrochlorinase. For some experiments the crude acetone-dried powder was partially purified by passage through a large gel-filtration column (see below).

Before application to the sucrose-gradient electrophoresis or electrofocusing apparatus the crude acetone-dried powders were desalted by gel filtration.

Gel filtration. Sephadex G-25 and G-100 (Pharmacia Ltd., Uppsala, Sweden) was used. For desalting a column of Sephadex G-25 with 100 ml bed volume that had been equilibrated with water was used. Protein solutions (20 mg/ml) were passed through, and the eluates were freeze-dried and stored at room temperature in a desiccator.

An analytical column (100 cm × 1.5 cm) was used

containing Sephadex G-100 equilibrated with 0.02M-pyrophosphate buffer, pH 7.2 (0.02M- $\text{Na}_4\text{P}_2\text{O}_7$, adjusted to pH 7.2 with M-HCl). Buffer was circulated upwards through the bed and 1 ml of enzyme solutions (20 mg of protein/ml) injected. Fractions (1.5 ml) were collected and assayed for the two enzymes as described below. Molecular weights of the enzymes were deduced by comparison with the behaviour of proteins of known molecular weight on this column (Andrews, 1964).

A large preparative column (80 cm long) was also used containing 3 litres of Sephadex G-100 equilibrated with 0.02M-pyrophosphate buffer, pH 7.2. Fly enzyme acetone-dried powder (5–7 g) was dissolved in 20 ml of buffer and passed up the column. Enzymes were located in the 50 ml fractions collected by assays as described above, and the pooled fractions were freeze-dried.

Enzyme assays. GSH *S*-aryltransferase was measured at 37°C by using 0.1 mM-1-chloro-2,4-dinitrobenzene and 2 mM-GSH at pH 8.3 in a Perkin-Elmer model 137 u.v. recording spectrophotometer (Clark *et al.* 1967). In some experiments the method of Booth, Boyland & Sims (1961) was used, with 0.5 mM-3,4-dichloro-1-nitrobenzene as substrate. In these experiments the extinction was measured after 0.5 h in a Hilger Uvispek spectrophotometer.

DDT dehydrochlorinase was assayed by the g.l.c. method described by Balabaskaran & Smith (1970).

Electrophoresis. The apparatus described by Svensson (1960) was used. The 30 cm sucrose gradient (10–50% w/v) contained a low-conductivity buffer composed of a saturated solution of benzoic acid adjusted to the pH required with 0.01 M-triethanolamine. Some initial runs were also made with 0.01 M-triethanolamine–0.01 M-acetic acid buffer. The side limbs and electrode compartments of the apparatus were filled with 0.1 M-KCl into which acid (5 M- H_2SO_4) and alkali (5 M- NaOH) locks with 60% sucrose were interposed as described by Svensson (1960).

Cooling water at 0°C was circulated through the jacket round the sucrose-gradient section of the apparatus. Power applied to the apparatus was limited to about 9 W, overloading being readily shown by the appearance of convection cells in the gradient. With these buffers and conditions about 200 V could be applied to the electrodes, and direct measurement from insulated probes inserted at the top and bottom of the gradient section showed that the voltage fall across this was about 120 V or approx. 4 V/cm.

A 5 ml portion of the gradient near the middle was removed through the central capillary tube to form a density shelf and the desalted enzyme powder (approx. 10 mg) dissolved in 2 ml of the lighter part of the removed gradient. The enzyme solution was then slowly injected back on to the density shelf, where it formed a stable turbid zone about 0.5 cm deep. The capillary tube was then lowered and about 20 drops were bled from it to remove any residual enzyme.

After equilibration for 0.5 h to make sure no flotation or droplet formation was developing (Svensson, 1960), power was applied to the apparatus from a constant-voltage power supply.

After 12 h the current was turned off and the gradient run out downwards. Fractions (3.2 ml) were collected and suitable portions assayed for the two enzymes after

the pH of the tubes had been checked. These were within ± 0.2 pH unit of the nominal buffer value.

Electrofocusing. An apparatus similar to that described by Vesterberg & Svensson (1966) and Haglund (1967) was used with a working volume of approx. 100 ml. Ampholine buffers (LKB Produkter AB, Stockholm, Sweden) were made up as described by Haglund (1967) and used to form the pH gradient. In all experiments the anode was at the lower end of the apparatus, since in experiments with housefly preparations insoluble proteins accumulated at this end. A 50–10% (w/v) sucrose gradient was used to stabilize the system against convection and the maximum power applied to the apparatus was limited to about 1.0 W. A constant-current power supply was used, the voltage being adjusted periodically to keep the power used below the critical level. After the stabilization of the pH gradient, which occurred after about 12 h, the voltage could be increased to about 700 V.

Separations were normally carried out for 36 h after the gradient had been formed, with water at 5°C circulating through the cooling jacket. After completion of the separation the column was allowed to run out and the pH and enzyme content of 2 ml fractions were measured as described above.

RESULTS

Stability of the enzymes. The two enzymes were assayed after being kept at 5°C in 40% (w/v) sucrose and 0.01 M-triethanolamine–benzoate buffer for up to 24 h at pH values between 4 and 8.7. No loss of DDT dehydrochlorinase activity was observed in 12 h between pH 5.1 and 7.8, but at pH 4 and 8.7 half the activity was lost after 12 h at 0°C. At pH 6 no DDT dehydrochlorinase activity was lost in 50 h at 0°C, and no inhibition was observed in 1% (w/v) Ampholine in 20% (w/v) sucrose solution or in fractions of such a solution that had been focused for 12 h. DDT dehydrochlorinase was very sensitive to frothing, even in crude preparations. Air bubbled through a solution containing 1 mg of protein/ml caused the loss of 80% of the activity in 20 s. Shaking gently for 20 s caused a similar loss.

GSH *S*-aryltransferase activity in triethanolamine–benzoate buffers and 20% (w/v) sucrose was completely lost in 1 h at pH 4.1, but recovery of activity between pH 5.1 and 7.8 was greater than 85% after 12 h at 0°C.

Gel filtration of enzymes. Separation of the acetone-dried powder (20 mg, 1 ml) on the analytical column gave coincident elution bands of the two enzymes, both activities 'peaking' at fraction 64. Calibration of this column with standard proteins (Andrews, 1964) suggested that the molecular weights of the two enzymes were about 40 000. Some samples of acetone-dried powder were fractionated by passage through the preparative Sephadex column, from which 5 g of acetone-dried powder yielded 1 g of desalted freeze-dried product

Fig. 1. Sucrose-density-gradient electrophoresis of housefly enzymes at pH 8.0 (a), pH 6.8 (b), pH 6.1 (c) and pH 5.0 (d). Desalted partially purified enzymes prepared as described in the text were separated at 0°C in triethanolamine-benzoate buffers for 12 h. The voltage gradient was 5 V/cm at pH 5 and 4 V/cm at other pH values. Eluted fractions were assayed with 1-chloro-2,4-dinitrobenzene for GSH *S*-aryltransferase (●) or by the g.l.c. method for DDT dehydrochlorinase (○) as described in the text, and migration distances were calculated from the column dimensions and fraction numbers.

containing 80% of the DDT dehydrochlorinase activity of the original powder.

Electrophoretic separation. Desalted acetone-dried powders and desalted powder purified through the preparative gel-filtration column were used for electrophoretic experiments, but no difference in behaviour was observed. All runs were made at least twice and concordant results were obtained with peak values in agreement to within one eluted fraction. Results obtained at four pH values with a sample of powder that had been partially purified by passage through a Sephadex G-100 column are shown in Fig. 1. Plots of mobility against pH showed that the left-hand peak of DDT dehydrochlorinase was isoelectric at pH 5.6 and the right-hand one isoelectric at pH 6.7 (Fig. 2).

Additional runs were made at pH 6.1, where the

Fig. 2. Electrophoretic mobility of housefly enzymes. Partially purified enzyme preparations made as described in the text were used and assayed for GSH *S*-aryltransferase (●) or DDT dehydrochlorinase (○, left-hand peak of Fig. 1; □, right-hand peak of Fig. 1). Conditions for the electrophoresis were as given in Fig. 1.

two DDT dehydrochlorinase bands were migrating in opposite directions, and were continued for 24 h, by which time the two peaks of activity had moved 2 cm towards each electrode.

Separations of enzyme from male and female houseflies were also carried out. Enzyme was prepared from the males and females sorted from a group of 2500 3-day-old flies and the desalted acetone-dried powder used for separation at pH 7.9 (Fig. 3).

Electrofocusing separation. Electrofocusing experiments carried out with the buffer range pH 3–10 showed that DDT dehydrochlorinase activity was concentrated near pH 6. Runs made with the narrow-range buffers at pH 5–7 or 6–8 gave major peaks of DDT dehydrochlorinase activity corresponding to enzymes isoelectric in the regions pH 6.4–6.7 and 7.4–7.8 with minor-activity peaks at pH 5 and 6. In eight experiments, however, with the sample of enzyme powder used in the experiments of Fig. 1 the amount of enzyme associated with these two zones was not reproducible after focusing for 48 h at 700 V. Similar results were obtained in five experiments where a housefly water homogenate, prepared and desalted by gel filtration as described above, was used directly without acetone precipitation.

Experiments with the sample of male housefly enzyme used in the experiment shown in Fig. 3 showed at least three zones of DDT dehydrochlorinase activity with apparent isoelectric points of 6.3, 6.9 and 7.4 when focused for 3 days at 600 V, but when focusing was carried on for 9 days after establishment of the pH gradient only one of these peaks was observed (Fig. 4).

Fig. 3. Sucrose-density-gradient electrophoresis at pH 7.9 of male (a) and female (b) housefly DDT dehydrochlorinase. Conditions were as given in Fig. 1 except that enzyme from desalted acetone-dried powder from sexed groups of houseflies was used.

Fig. 4. Electrofocusing of housefly DDT dehydrochlorinase. Desalted freeze-dried samples of enzyme (25 mg) were focused and 3 ml fractions were assayed for enzyme by the g.l.c. method described in the text. The sample used to obtain the curve for the male housefly enzyme in Fig. 3 was used and assays were made 3 days (●) or 9 days (○) after the pH gradient had stabilized. The smooth curves show the pH values of the eluates of the 3-day (A) or the 9-day (B) experiments.

GSH *S*-aryltransferase assays were also made, but this enzyme was never detected after electrofocusing, and since the electrophoretic work

suggests that the isoelectric point would be at a lower pH than those of the DDT dehydrochlorinases it was presumed to be in the region below pH 5 where the enzyme was not stable for 36 h.

DISCUSSION

The electrophoretic results obtained are qualitatively similar to those of Ishida (1968), who used enzyme from a more DDT-resistant strain of houseflies than that used in the present work. His use of gel-block ionophoresis, however, gave poor resolution of the enzymes, though it was shown that both GSH *S*-aryltransferase and DDT dehydrochlorinase moved towards the anode at pH 7.4, as we have found.

Our results demonstrate that these two enzymes are separate and suggest that the housefly GSH *S*-aryltransferase could be a single protein, since it migrated as a single band in electrophoretic experiments. DDT dehydrochlorinase, on the other hand, in the different samples of enzyme and in the two types of experiment always behaved as a mixture of enzymes.

In electrophoretic experiments different preparations of enzyme did not always appear to have the same proportions of activity in the two peaks detectable by this method, and it is possible that one of these may be an artifact formed by a minor change in the naturally occurring molecule. The electrophoretic heterogeneity of cytochrome *c* (Flatmark, 1966) and lactoperoxidase (Carlstrom & Vesterberg, 1967) has similarly been attributed to the hydrolysis of amide side chains in these molecules. If these changes did take place in the housefly enzyme they had little effect on the enzyme activity, since no significant loss was found under the conditions of the electrophoretic experiments at most of the pH values used.

The variability of the multiple forms of DDT dehydrochlorinase was more pronounced in electrofocusing experiments, which took longer to complete than the electrophoresis but were capable of greater resolution of the different activity zones. In the longest electrofocusing experiment (Fig. 4), which was carried on for 9 days at 5°C, only one major peak of activity remained and this was isoelectric at pH 7.4. At this time the total activity recorded was about one-third of that applied to the apparatus, and this suggested that the enzyme isoelectric at pH 7.4 was more stable than those isoelectric at more acid pH values. It is not possible, however, to make valid quantitative conclusions from this type of experiment, since no information is available as yet about the turnover numbers of the various forms of DDT dehydrochlorinase.

If the multiple forms of the enzyme are derived by partial degradation of the naturally occurring

molecule this could have occurred during the working-up of the enzyme. In the hope of being able to examine an enzyme that had received the minimum exposure to possible degradation procedures, experiments were therefore carried out with water homogenates of houseflies with the omission of any acetone treatment or freeze-drying. In these experiments, again, only the peaks corresponding to enzymes isoelectric at pH 6.5 and 7.8 were found.

It seems probable therefore that the shorter times used in the electrophoretic experiments allowed the detection of a form of DDT dehydrochlorinase that is isoelectric at pH 5.6, but that this changed to forms isoelectric at higher pH values during the much longer periods used for electrofocusing.

All the present experiments, electrophoretic as well as electrofocusing, were carried out with housefly enzyme that had been kept in solution at 0–5°C for at least 12h and that had usually been freeze-dried or treated with acetone. They do not necessarily show that housefly tissues contain two forms of the DDT dehydrochlorinase. The kinetic experiments carried out by Balabaskaran & Smith (1970), which could also be interpreted as evidence for the presence of more than one enzyme, were also carried out with acetone-dried powder or freeze-dried samples.

If the two enzymes found are not artifacts the presence of two enzymes that detoxify DDT could be of considerable interest in understanding the way in which DDT-resistance develops, since most cases of housefly resistance to DDT can be correlated with the presence of the detoxication enzyme. Lipke & Kearns (1959) isolated their enzyme with isoelectric point 6.5 from a highly resistant strain of houseflies and the product attacked 1,1-dichloro-2,2-bis-(*p*-chlorophenyl)ethane (DDD) more readily than DDT. This strain (L) Oppenoorth (1965) has also shown to differ from a low-resistance strain (Fc) and a susceptible strain (rca), which attack DDT more rapidly than 1,1-dichloro-2,2-bis-(*p*-chlorophenyl)ethane.

The housefly strain used in the present work was

selected without insecticide pressure to have a normal low resistance to DDT.

Oppenoorth (1965) has also suggested that the enzymes in the low-resistance strains Fc and rca were 'wild-types', and the enzyme in our strain may be similar since it has not been exposed to DDT. The L-type enzymes, he suggests, arose by mutation of the wild-type. It also seems possible that the different DDT dehydrochlorinases are representative of a wider group of GSH-dependent enzymes, including GSH *S*-aryltransferase, all with molecular weights near 40 000 (cf. Ishida, 1968) and that may have evolved from a common precursor.

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