3. Unlike other known active phenolases the enzyme released from a broad-bean leaf extract by Teepol is adsorbed strongly on diethylaminoethylcellulose.

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An Enzyme from Rat Liver Catalysing Conjugations with Glutathione

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Many compounds are excreted in urine and bile as mercapturic acids, which are thio ethers of acetylcysteine, and there has been considerable speculation on the source of the cysteine used for the biosynthesis of these compounds. Evidence that glutathione is involved was supplied by the work of Barnes, James & Wood (1959), who found that the levels of glutathione in the liver decreased when mercapturic acid-forming compounds were administered to rats. Furthermore, when *p*-chlorobenzyl chloride was given to a rabbit, investigations with paper chromatography provided evidence for the presence of *S*-(*p*-chlorobenzyl)-glutathione in the liver, and this compound could be converted into *S*-(*p*-chlorobenzyl)cysteine by liver slices and homogenates and by glutathionase (Bray, Franklin & James, 1959*a*). The *N*-acetylation of some of the *S*-substituted cysteines to the corresponding mercapturic acids was demonstrated *in vivo* and in tissue preparations *in vitro* (Bray, Franklin & James, 1959*b*).

It has been shown that rat-liver slices convert naphthalene into *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione (Booth, Boyland & Sims, 1960*b*) and 1:2-dihydronaphthalene into *S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)glutathione. This latter reaction could also be accomplished by rat-liver microsomes in the presence of reduced triphosphopyridine nucleotide, oxygen and glutathione, and the amount of glutathione derivative formed was increased by the addition of the soluble fraction of rat liver (Booth, Boyland, Sato & Sims, 1960*a*).

This paper describes the partial purification and some of the properties of the liver enzyme which catalysed the formation of glutathione derivatives from various compounds.

EXPERIMENTAL

Materials. Triphosphopyridine nucleotide (TPN) as the dihydrate of the sodium salt, reduced TPN ($TPNH_2$) as the sodium salt, diphosphopyridine nucleotide (DPN), reduced DPN ($DPNH_2$) as the disodium salt, glucose 6-phosphate as the disodium salt, flavin mononucleotide as the monosodium salt dihydrate, adenosine 5-triphosphate (ATP) as the disodium salt and glucose 6-phosphate dehydrogenase were obtained from Sigma Chemical Co. Pyrophosphate buffers were prepared from $K_4P_2O_7$ and $Na_2H_2P_2O_7$ and phosphate buffer from KH_2PO_4 and Na_2HPO_4. Concentrations of the constituents of the reaction mixtures refer to the final concentrations at the beginning of the incubations.

S-(2-Chloro-4-nitrophenyl)-L-cysteine. This was prepared from 2-chloro-4-nitroaniline by the method of Parke & Williams (1951), when it formed pale-yellow needles from aq. ethanol, m.p. 176–178° (decomp.) (Found: N, 10·15; Cl, 12·45. Calc. for $C_9H_9O_4N_2ClS$: N, 10·1; Cl, 12·8%). Bray *et al.* (1959*b*) report m.p. 182° for this compound prepared by the method of Suter (1895). Acetylation of the compound with acetic anhydride in 2N-NaOH yielded *N*-acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine in cream needles from ethanol, m.p. 193°. Bray, James & Thorpe (1957) report m.p. 192–194° for this derivative.

S-(2-*Chloro*-4-*nitrophenyl*)*glutathione*. This compound was prepared from glutathione (2 g.) and 2-chloro-4-nitroaniline (1·1 g.) by the method described for the preparation of 1-naphthylglutathione (Booth *et al.* 1960*b*). S-(2-*Chloro*-4-*nitrophenyl*)*glutathione* (0·95 g.) separated from aq. 90% ethanol–ether as a pale-yellow powder, m.p. 214° (decomp.) (Found: N, 12·6; Cl, 7·8; S, 6·8. $C_{16}H_{19}O_8N_4ClS$ requires N, 12·1; Cl, 7·7; S, 6·9%). Like the corresponding cysteine derivative, the compound turned brown on exposure to daylight. When the compound was heated with HBr (sp.gr. 1·7) for 4 hr. and the products were examined on paper chromatograms (with solvent 1, Table 2), compounds indistinguishable from *S*-(2-chloro-4-nitrophenyl)-cysteine, glycine and glutamic acid were detected.

N-Acetyl-L-cysteine was prepared by the method of Pirie & Hele (1933) and *trans*-1:2-dihydro-1:2-dihydroxynaphthalene and *trans*-9:10-dihydro-9:10-dihydroxyphenanthrene by reduction of the corresponding *o*-quinones with lithium aluminium hydride (Booth, Boyland & Turner, 1950). *trans*-1:2-Dihydro-1:2-dihydroxyphenanthrene was isolated from the urine of a rabbit dosed with phenanthrene (Boyland & Wolf, 1950).

A solution of oxidized glutathione (GSSG) was prepared by adding a small excess of 50 mM-potassium ferricyanide to a solution of 50 mM-GSH (10 ml.) and keeping the mixture at room temperature for 1 hr. The solution was adjusted to pH 4 with acetic acid and the GSSG absorbed on charcoal (400 mg.). The charcoal was filtered off and washed with water until the washings were free of potassium ferricyanide (no blue colour with $FeCl_2$). The GSSG was eluted with 10 ml. of methanol–benzene–aq. NH_3 (sp.gr. 0·88) (87:10:3, by vol.). The eluate was evaporated to dryness and the GSSG was dissolved in 0·1 M-pyrophosphate buffer, pH 8·0 (10 ml.).

Tissue preparations. The liver fraction soluble in phosphate buffer was prepared by homogenizing livers from male adult rats in 4 vol. of 0·1 M-phosphate buffer, pH 7·4, with a Potter & Elvehjem (1936) type of homogenizer with a Teflon pestle. The homogenate was centrifuged for 30 min. at 2000*g* and the supernatant centrifuged for 1 hr. at 95 000*g* (centre of tube) in a Spinco model L preparative ultracentrifuge with rotor no. 40. The supernatant from the high-speed centrifuge was dialysed against water and the precipitate which separated was removed by centrifuging for 30 min. at 2000*g*. The supernatant was adjusted to pH 7·4 with 0·1 M-$K_4P_2O_7$ and stored at −2°. This preparation is referred to as the soluble liver fraction. Kidneys from male adult rats were homogenized in 4 vol. of 0·154 M-NaCl immediately before use. All operations were carried out at between 3° and 8°.

Metabolism of 3:4-*dichloronitrobenzene*. This was investigated by incubating the following reaction mixtures at 37° for 1 hr. (1) Soluble liver fraction (0·5 ml.), GSH, cysteine or acetylcysteine (5 mM) and 3:4-dichloronitrobenzene (1 mM, added in 0·1 ml. of ethanol) in a total volume of 6 ml. of 0·1 M-pyrophosphate buffer, pH 8·0; (2) *S*-(2-chloro-4-nitrophenyl)glutathione (1 mM) and kidney homogenate (1 ml.) in a total volume of 6 ml. of 0·1 M-pyrophosphate buffer, pH 8·0; (3) *S*-(2-chloro-4-nitrophenyl)-cysteine (1 mM) and slices of liver (approx. 5 g. wet wt.) from adult rats in 50 ml. of Ringer phosphate solution, pH 7·4 (Umbreit, Burris & Stauffer, 1949). At the end of the incubation period the reaction mixtures were studied by paper chromatography.

Paper chromatography. The reaction mixtures were added to acetic acid (0·5 ml.) and centrifuged, and the supernatant was added to activated charcoal (50 mg.), shaken and again centrifuged. This supernatant was discarded and the charcoal was resuspended in water (6 ml.) and filtered. The products were eluted from the charcoal with 10 ml. of methanol–benzene–aq. NH_3 (sp.gr. 0·88) (87:10:3, by vol.) and the eluate was evaporated to a small volume and applied to Whatman no. 1 paper for chromatography. Descending chromatograms were developed for 18 hr. with one of the solvent systems listed in Table 2. The dried chromatograms were examined under u.v. light and then sprayed with (1) 20% Na_2CO_3 (Bray *et al.* 1957) or (2) 0·1 M-$K_2Cr_2O_7$–acetic acid (1:1), followed by 0·1 M-$AgNO_3$ (Knight & Young, 1958), or (3) diazotized *p*-nitroaniline (0·02% in 0·1 N-HCl), followed by aq. 10% (w/v) Na_2CO_3, or (4) diazotized *p*-nitroaniline (0·02% in 4 N-HCl), after which the papers were heated at 70° until colours appeared (about 5 min.) and then sprayed with aq. 10% (w/v) Na_2CO_3. Other papers were dipped in a solution of ninhydrin (0·4%) in acetone, heated in an oven at 70° for 5 min. and then dipped in a solution of $Cu(NO_3)_2$ (0·2%) in acetone.

Estimation of enzyme activity. The substrate chosen for the estimation of enzyme activity was 3:4-dichloronitrobenzene, because of the change in the ultraviolet-absorption spectrum which occurs when this compound is converted into *S*-(2-chloro-4-nitrophenyl)glutathione (Figs. 1 and 2). In obtaining the readings in Fig. 2, a cell containing all the constituents of the reaction mixture except 3:4-dichloronitrobenzene was used as the blank, so that the rate of increase of *E* at 344 mμ is a measure of the rate of formation of *S*-(2-chloro-4-nitrophenyl)glutathione. This increase was measured on a Unicam SP. 500 spectrophotometer with a Unicam SP. 570 constant-temperature-cell housing. The reaction mixtures contained GSH (5 mM), various concentrations of 3:4-dichloronitrobenzene (added in 0·1 ml. of ethanol) and enzyme in a total volume of 3·0 ml. of 0·1 M-pyrophosphate buffer, pH 8·0. The enzyme was diluted so that the rate of increase in *E* was less than 0·2/min. The reactions were carried out at 37°, the constituents being brought to this temperature before the determination and the reaction was started by the addition of the enzyme solution to the cell. After stirring, readings at 344 mμ were taken at 30 sec. intervals for 5·5 min., beginning 30 sec. after mixing. Under these conditions the initial reaction rates were constant for at least 10 min. and 1 unit of enzyme is defined as that amount which will form 1 μmole of *S*-(2-chloro-4-nitrophenyl)glutathione/min. Specific activity is expressed as units/mg. of protein. Protein was estimated by a modification of the biuret method (Fincham, 1954) or by the method of Warburg & Christian (1941).

Location of the enzyme. To determine the intracellular location of the enzyme, a 1 in 10 homogenate of adult male rat liver in 0·25 M-sucrose was prepared as described for the phosphate-buffer homogenate. The sucrose homogenate was fractionated by the method of Schneider & Hogeboom (1950), except that the microsome fraction was separated from the soluble fraction by centrifuging at 95 000*g* (centre of the tube) for 1 hr. in a Spinco model L preparative ultracentrifuge with rotor no. 40. The particulate fractions were suspended in 0·1 M-pyrophosphate buffer, pH 8·0, in volume equivalent to those of the original homogenate and the fractions were diluted with this buffer for measurements of enzyme activity.

To determine the distribution of the enzyme in the soluble fraction of various rat tissues, the tissues were homogenized in 0·1 M-phosphate buffer, pH 7·4, and the phosphate buffer-soluble fractions prepared as described for liver. Blood was added to an equal volume of water and after 10 min. an equal volume of 0·1 M-phosphate buffer was added. The supernatant after centrifuging for 1 hr. at 95 000*g* was then used for measurements of enzyme activity.

Partial purification of the enzyme. Steps 1 and 2 are the homogenization of rat liver and the preparation of the soluble fraction as described above. *Step* 3. The undialysed soluble liver fraction was adjusted to pH 5·2 with acetic acid (approx. 0·55 ml./100 ml.), centrifuged for 15 min. at 2000*g* and the ppt. discarded. The supernatant was dialysed against water for 18 hr., centrifuged as before and the ppt. again discarded. The supernatant was adjusted to pH 8·0 with 0·1 M-$K_4P_2O_7$ and the centrifuging repeated. *Step* 4. To the supernatant from step 3, $(NH_4)_2SO_4$ (47·2 g./100 ml.) was added and, after 30 min., the ppt. was removed by centrifuging and discarded. A small portion of the supernatant was dialysed against water and used for activity measurements. *Step* 5. The supernatant from step 4 was adjusted to pH 4·5 with 5% acetic acid and centrifuged for 30 min. at 2000*g*. The ppt., which contained the activity, was dissolved in 0·1 vol. of 0·1 M-pyrophosphate buffer, pH 7·4, and stored in 0·5 ml. portions at −15°. Immediately before use a portion was allowed to thaw at room temperature and was diluted 40 times with 0·1 M-pyrophosphate buffer, pH 8·0, so that when 0·25 ml. of this solution was present in 3 ml. of reaction mixture the total dilution factor was 480 and the protein concentration was 0·0117 mg./ml. This gave a change in E at 344 mμ of 0·15 to 0·20/min. under the optimum conditions. All operations were carried out at between 3° and 8°. The partial purification of the enzyme by these steps is summarized in Table 1.

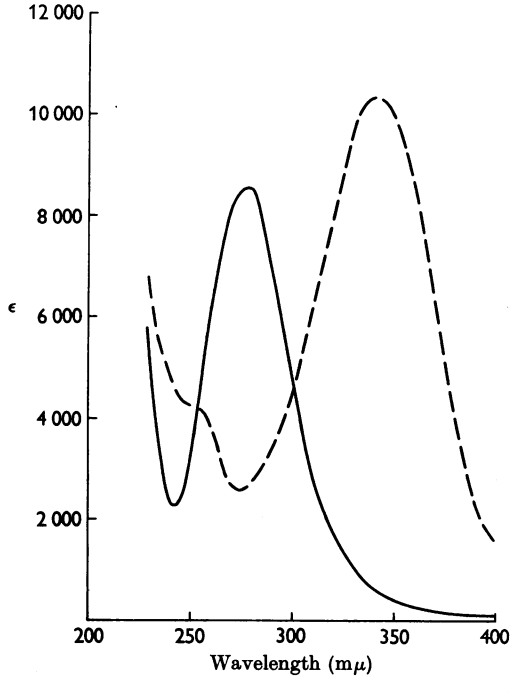

Fig. 1. Absorption spectra in 0·1 M-pyrophosphate buffer, pH 8·0: —, 3:4-dichloronitrobenzene; - - -, *S*-(2-chloro-4-nitrophenyl)glutathione.

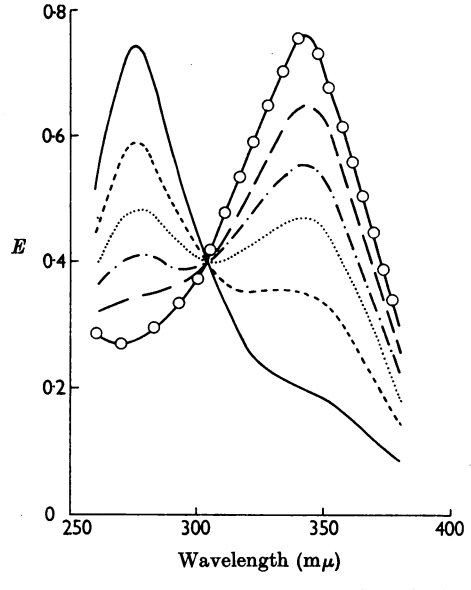

Fig. 2. Effect on ultraviolet absorption of incubating 3:4-dichloronitrobenzene with GSH and enzyme. Reaction mixtures contain 3:4-dichloronitrobenzene (0·1 mM), GSH (5 mM) and 0·25 ml. of enzyme solution in 3 ml. of 0·1 M-pyrophosphate buffer, pH 8·0. The readings for each curve occupied approx. 1 min. and were started at the following times (min.) after adding the enzyme solution —, 1; - -, 6; · · ·, 11; —·—, 16; — —, 21; O—O, 26.

Table 1. *Summary of the partial purification of the enzyme from a* 1 *in* 5 *homogenate of rat liver in* 0·1 M-*phosphate buffer*, pH 7·4

Step	Volume (ml.)	Activity (units/ml.)	Total units	Protein (mg./ml.)	10^3 × Specific activity (units/mg. of protein)	Recovery (%)
(1) Whole homogenate	360	21	7560	45·1	47	100
(2) Soluble liver fraction	245	27	6615	26·2	104	87
(3) Precipitation at pH 5·2 and dialysis	270	19	5130	12·1	158	68
(4) $(NH_4)_2SO_4$ fractionation at pH 8·0	330	9	2970	1·4	643	39
(5) $(NH_4)_2SO_4$ fractionation at pH 4·5	33	90	2970	5·6	1607	39

Table 2. *Paper chromatography of 3:4-dichloronitrobenzene metabolites*

Solvent systems: (1) butanol–acetic acid–water (12:3:5); (2) butanol–acetic acid–water (2:1:1). All metabolites showed dark absorption in u.v. light and gave a positive reaction with the $K_2Cr_2O_7$–$AgNO_3$ reagent.

Reaction mixture or reference compound	R_F Solvent (1)	R_F Solvent (2)	Colour with ninhydrin and $Cu(NO_3)_2$	Colour with Na_2CO_3
3:4-Dichloronitrobenzene, dialysed soluble liver fraction and GSH	0·45	0·62	Red	Yellow
S-(2-Chloro-4-nitrophenyl)glutathione	0·44	0·61	Red	Yellow
S-(2-Chloro-4-nitrophenyl)glutathione and kidney homogenate	0·57	0·67	Red	Yellow
S-(2-Chloro-4-nitrophenyl)-L-cysteine	0·59	0·67	Red	Yellow
S-(2-Chloro-4-nitrophenyl)-L-cysteine and liver slices	0·89	0·86	Orange	Pale yellow
N-Acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine	0·89	0·86	Orange	Pale yellow

Identification of glutathione conjugates with various substrates. Reaction mixtures contained soluble liver fraction (2 ml.), GSH (5 mM) and 0·1 M-pyrophosphate buffer, pH 8·0, in a final volume of 6 ml. The substrates were added in ethanol (0·2 ml.) to give a final concn. of 1 mM; some of the compounds were partially precipitated and with these the excess of substrate was allowed to remain in suspension. With the substrates iodobenzene, naphthalene, 1-chloronaphthalene and phenanthrene the following cofactors were also added: $DPNH_2$ (0·2 mM), nicotinamide (2 mM), TPN (0·2 mM), glucose 6-phosphate (2 mM), glucose 6-phosphate dehydrogenase (0·01%) and 1 ml. of microsome suspension prepared as described by Booth *et al.* (1960*a*). Two incubation flasks were used for each substrate, one containing 2 ml. of soluble liver fraction and the other either 2 ml. of soluble liver fraction which had been heated at 100° for 5 min. before the incubation or 2 ml. of buffer solution.

The mixtures were incubated at 37° for times which varied between 2 min. and 1 hr., according to the substrate, the same time being used for each pair of flasks containing the same substrate. The products were adsorbed on charcoal and eluted as described above, and the eluates were evaporated to dryness and methanol (0·25 ml.) containing aq. 1% NH_3 (sp.gr. 0·88) was added to the residues. For each substrate the same volume of the methanol extract from the two experiments (boiled and fresh soluble liver fraction) was applied with micropipettes to chromatography paper for comparison. With some substrates only a small fraction of the methanol was used, to avoid overloading. The dried chromatograms were treated with the ninhydrin reagent followed by the $Cu(NO_3)_2$ solution. The spots were cut out, the coloured compounds eluted with 50% methanol and the extinctions measured at 510 mμ on the Unicam SP. 500 spectrophotometer. A reaction mixture which contained soluble liver fraction and GSH but no substrate was treated in the same way for use as a blank.

In the experiments with naphthalene and phenanthrene, other paper chromatograms were treated with the diazotized *p*-nitroaniline reagents (3) and (4).

RESULTS

Metabolism of 3:4-dichloronitrobenzene. The conversion of 3:4-dichloronitrobenzene into *N*-acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine (Bray *et al.* 1957) in three stages is demonstrated by the results in Table 2. The first reaction, which is catalysed by the soluble liver fraction, is the replacement of the *para* chlorine atom by GSH to form *S*-(2-chloro-4-nitrophenyl)glutathione. If GSH is replaced by cysteine or acetylcysteine in the reaction mixture no corresponding derivatives can be detected on the chromatograms. Kidney homogenate removes glycine and glutamic acid from *S*-(2-chloro-4-nitrophenyl)glutathione to form *S*-(2-chloro-4-nitrophenyl)-L-cysteine, which is then acetylated by liver slices to the mercapturic acid, *N*-acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine.

Table 3. *Intracellular location of the enzyme*

Samples of the fractions of 1 in 10 rat-liver homogenate in 0·25 M-sucrose were incubated with GSH (5 mM) and 3:4-dichloronitrobenzene (1 mM) in 0·1 M-pyrophosphate buffer, pH 8·0 (total vol. 3 ml.).

Intracellular fraction	Activity (units/ml.)	Activity (as % of whole homogenate)
Whole homogenate	15·8	100
Nuclei	0·8	5
Mitochondria	0·8	5
Microsomes	0·7	4
Soluble fraction	13·0	82

Table 4. *Distribution of the enzyme in the soluble fractions of rat tissues*

Reaction mixtures contained GSH (5 mM), 3:4-dichloronitrobenzene (1 mM) and soluble liver fractions in 0·1 M-pyrophosphate buffer, pH 8·0 (total vol. 3 ml.).

Tissue	$10^2 \times$ Specific activity (units/mg. of protein)
Liver	96·2
Heart	2·9
Kidney	2·4
Lung	1·6
Spleen	0·5
Blood	0·1

Location of the enzyme. The estimations of enzyme activity in the intracellular fractions of a rat-liver homogenate in 0·25 M-sucrose shows that 82% of the activity is present in the soluble fraction (Table 3). The activities of the soluble fractions of various rat tissues are listed in Table 4, which shows that liver has between 30 and 200 times the activity of the other tissues investigated.

Some properties of the partially purified enzyme. The activity is completely destroyed by heating at 100° for 5 min. and is not affected by dialysis. The initial reaction rate under the conditions described is constant for at least 10 min. and is directly proportional to enzyme concentration when this is adjusted to give a change in E at 344 mμ of 0·2/min. or less. The pH–activity curve (Fig. 3) shows a broad region of optimum activity between 7·5 and 9·0, but a rapid decrease on either side of these values. Replacement of pyrophosphate buffer by Na_2CO_3–$NaHCO_3$ buffer, of the same molarity and pH, at the pH of optimum activity had no effect on the reaction rate.

GSH was used throughout at a concentration of 5 mM, and increasing this value produced no increase in enzyme activity. Although it could be demonstrated that a decrease in GSH concentration caused a decrease in activity, a quantitative study of this factor was not undertaken as it was uncertain what proportion of GSH remained in the reduced form during the experiments. No activity was observed when GSH was replaced by the same concentration of GSSG, cysteine or acetylcysteine. The absorption spectra of the GSH, cysteine and acetylcysteine derivatives of 3:4-dichloronitrobenzene are all very similar (cf. Bray *et al.* 1959*b*), so that if any reaction had taken place a change in E at 344 mμ would have been apparent. This confirms the results obtained with paper chromatography, in which GSH was the only one of these three SH compounds which was converted into a derivative of 3:4-dichloronitrobenzene by the soluble liver fraction.

Investigations on the effect of 3:4-dichloronitrobenzene concentration were restricted by the solubility (1 mM) of this substrate, and it was not possible to saturate the enzyme. However, the effect of various substrate concentrations below this value are shown in Fig. 4 in the form of a Lineweaver & Burk (1934) plot, and the K_m calculated from these values was $2·5 \times 10^{-3}$ M (2·5 mM). The inhibition of *S*-(2-chloro-4-nitrophenyl)glutathione formation by other compounds which were

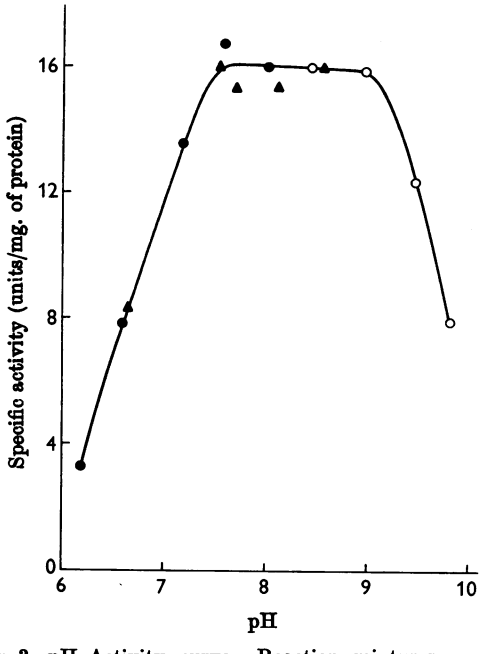

Fig. 3. pH–Activity curve. Reaction mixtures contain 3:4-dichloronitrobenzene (1 mM), GSH (5 mM) and 0·25 ml. of enzyme solution in 3 ml. of 0·1 M-buffer solution, the final pH values being shown. ●, Expt. 1 in pyrophosphate buffer; ▲, Expt. 2 in pyrophosphate buffer; ○, Expt. 3 in Na_2CO_3–$NaHCO_3$ buffer.

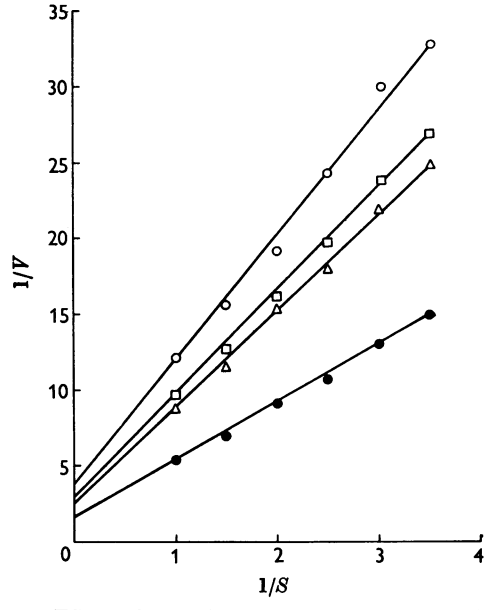

Fig. 4. Effect of benzyl chloride and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene on the rate of *S*-(2-chloro-4-nitrophenyl)glutathione formation. *S*, 3:4-Dichloronitrobenzene concn. (mM); *V*, initial rate of formation of *S*-(2-chloro-4-nitrophenyl)glutathione (μmoles/ml./min.). Reaction mixtures contain 3:4-dichloronitrobenzene, GSH (5 mM) and 0·25 ml. of enzyme solution in 3 ml. of 0·1 M-pyrophosphate buffer, pH 8·0, with the following additions: ●, none; △, 0·3 mM- and ○ 0·6 mM-1:2-epoxy-1:2:3:4-tetrahydronaphthalene; □, 0·6 mM-benzyl chloride.

shown by paper chromatography to form GSH derivatives is also shown in Fig. 4. At all the 3:4-dichloronitrobenzene concentrations studied, i.e. 1, 0·67, 0·5, 0·4, 0·33 and 0·285 mM, the addition of 0·6 and 0·3 mM-1:2-epoxy-1:2:3:4-tetrahydronaphthalene caused 55 and 40 % inhibition respectively, whereas at the same concentrations of 3:4-dichloronitrobenzene, 0·6 mM-benzyl chloride caused 44 % inhibition. Furthermore, when the concentration of 3:4-dichloronitrobenzene was kept constant at 1 mM, the addition of 0·2, 0·4, 0·6, 0·8 and 1 mM-benzyl chloride produced 17, 33, 43, 58 and 68 % inhibition respectively. Therefore, regarding 3:4-dichloronitrobenzene as the substrate and the other two compounds as inhibitors, the inhibition is of a non-competitive nature since it is independent of substrate concentration but dependent on inhibitor concentration.

The addition of the following compounds at a final concentration of 1 mM had no effect on the initial reaction rate: $TPNH_2$, $DPNH_2$, TPN, DPN, flavin mononucleotide, ATP and $MgSO_4$, ascorbic acid, cysteine, acetylcysteine, ethylenediaminetetra-acetate, KCN, NaF, $ZnSO_4$ and $MgCl_2$.

Formation of glutathione conjugates with various substrates. The formation of the GSH conjugates was demonstrated by the appearance of ninhydrin-positive spots when the substrates, GSH and soluble liver fraction were incubated in 0·1 M-pyrophosphate buffer, pH 8·0. With some substrates a GSH conjugate could be detected only when fresh soluble liver fraction was present in the incubation mixture, indicating that these reactions had been brought about by an enzyme in the liver fraction. Other substrates formed a GSH conjugate in the presence of boiled liver fraction, but a greater amount of the same derivative, as determined by the difference in extinction at 510 mμ after reaction with ninhydrin and $Cu(NO_3)_2$, when the liver fraction was fresh. It was assumed that in these cases both chemical and enzymic reactions had occurred. In no case, however, was the new ninhydrin-positive compound formed if either the substrate or GSH was omitted. The difference in extinctions observed between the two experiments with the same substrate ranged from a three- to a ten-fold increase. However, they could serve only to indicate that an enzymic as well as a chemical reaction had taken place, since a kinetic study with each substrate and the synthesis or isolation of the corresponding GSH derivative would be needed to put them on a quantitative basis. Benzyl chloride, which reacts chemically and enzymically, was used to confirm the specificity of the system for GSH. Paper chromatograms of reaction mixtures in which GSH had been replaced by GSSG showed that no reaction had taken place, and when cysteine or *N*-acetylcysteine replaced the GSH the chemical reaction only was observed. With the substrates iodobenzene, naphthalene, 1-chloronaphthalene and phenanthrene there was no formation of GSH conjugates unless microsomes and $TPNH_2$ were also added. The properties on paper chromatograms of the GSH conjugates detected in these reactions are listed in Table 5.

Naphthalene also yielded a compound which gave a blue colour with reagent 4 and was indistinguishable from *trans*-1:2-dihydro-1:2-dihydr-

Table 5. *Paper chromatography of glutathione conjugates*

Solvent systems are as described in Table 1. All conjugates gave pink colours with the ninhydrin–$Cu(NO_3)_2$ reagent. Type of reaction is indicated by A, chemical and B, enzymic.

Substrate	R_F of GSH derivative: Solvent (1)	R_F of GSH derivative: Solvent (2)	Type of reaction observed	Reference to mercapturic acid excretion in urine
3:4-Dichloronitrobenzene	0·45	0·62	B	Bray, James & Thorpe (1957)
2:4-Dichloronitrobenzene	0·42	0·60	B	Bray *et al.* (1957)
2:3:5:6-Tetrachloronitrobenzene	0·62	0·72	B	Bray, Hybs, James & Thorpe (1953)
3:4:5:6-Tetrachloro-1:2-epoxy*cyclo*hexane	0·42	0·64	B	—
Benzyl chloride	0·50	0·67	A, B	Bray, James & Thorpe (1958)
Bromoethane	0·33	0·52	A, B	Thomson, Maw & Young (1958)
1-Bromopropane	0·44	0·62	A, B	Grenby & Young (1959, 1960)
Ethyl methanesulphonate	0·33	0·52	A, B	Roberts & Warwick (1958)
Sulphobromophthalein	0·19	0·35	A, B	—
1:2-Epoxy-1:2:3:4-tetrahydronaphthalene	0·49	0·63	A, B	Boyland & Sims (1960)
Iodobenzene*	0·40	0·56	B	Hele (1924); Mills & Wood (1953)
Naphthalene*	0·37†	0·47†	A, B	Bourne & Young (1934)
1-Chloronaphthalene*	0·49	0·65	B	Cornish & Block (1958)
Phenanthrene*	0·43† 0·38‡	0·61† 0·57‡	B	—

* Required microsomes and $TPNH_2$.
† Gave blue colours with diazotized *p*-nitroaniline and Na_2CO_3 (reagent 3).
‡ Gave orange colours with reagent (4).

oxynaphthalene on paper chromatograms. Phenanthrene yielded two compounds indistinguishable from *trans*-1:2-dihydro-1:2-dihydroxy- and *trans*-9:10-dihydro-9:10-dihydroxy-phenanthrene, which gave blue and orange colours respectively on paper chromatograms treated with reagent 4.

DISCUSSION

The detoxication of foreign compounds, such as the monohalogenobenzenes (Baumann & Preusse, 1879; Jaffé, 1879), as mercapturic acids has been known for many years, and several GSH compounds have been suggested as the source of the cysteine residues. The possibility that GSH was involved in the detoxication of avertin was suggested by Waelsch (1930). Administration of naphthalene to rabbits caused a decrease in the GSH content of the eye lens and liver (Nakashima, 1934) and Yamamoto (1940) found that when bromobenzene was given to rabbits there was a decrease in the GSH level in several organs, particularly liver, which was paralleled by the amount of *p*-bromophenylmercapturic acid excreted. Bromobenzene, when given to rats, caused an increase in GSH levels in the liver and kidney 30 min. after administration, followed by a decrease in the levels lasting for 2 days, and it was suggested that this decrease was due to the formation of sulphuric acid esters and a mercapturic acid (Binet & Wellers, 1951). Barnes *et al.* (1959) gave a variety of mercapturic acid precursors to rats and found falls in the liver GSH level after the administration of the compounds; these falls could be related to the amounts of mercapturic acids formed. One of these compounds was 3:4-dichloronitrobenzene, and it had been shown that rabbits dosed with this substance excreted the mercapturic acid, *N*-acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine (Bray *et al.* 1957). The present work has shown that the biosynthesis of this metabolite can be carried out by tissue preparations in the three stages suggested by Bray *et al.* (1959*b*). The first reaction, which is catalysed by the soluble fraction of rat liver, is the replacement of the *para* chlorine atom by GSH. Since the activity of the fraction is destroyed by heat and is not affected by dialysis, it appears to be due to an enzyme which has no absolute cofactor requirements. The second stage is the conversion of *S*-(2-chloro-4-nitrophenyl)glutathione into *S*-(2-chloro-4-nitrophenyl)-L-cysteine by kidney homogenate, a reaction which is probably due to two enzymes previously found in pig kidney and which catalyse the removal of the glutamyl group from glutathione itself and the hydrolysis of cysteinylglycine (Olson & Binkley, 1950). That glutathione derivatives can also behave in this way has been shown by the conversion of *S*-(*p*-chlorobenzyl)-glutathione into *S*-(*p*-chlorobenzyl)-L-cysteine by liver slices of various species (Bray *et al.* 1959*a*). In the third stage, rat-liver slices were used to demonstrate the *N*-acetylation of *S*-(2-chloro-4-nitrophenyl)-L-cysteine to the mercapturic acid, and again other similar reactions have been reported, such as the *N*-acetylation of *S*-benzyl-DL-homocysteine by rat-liver and -kidney slices (Gutmann & Wood, 1951) and of *S*-(*p*-chlorobenzyl)-L-cysteine by liver slices (Bray *et al.* 1959*b*). The conversion of GSH derivatives into mercapturic acids *in vivo* has been demonstrated by Roberts & Warwick (1958), who found that when *S*-ethylglutathione was administered to rats it was excreted as *S*-ethylmercapturic acid, and Stekol (1940, 1941) showed that administration of *S*-benzylglutathione and *S*-(*p*-bromobenzyl)glutathione to rats resulted in the excretion of *N*-acetyl-*S*-benzyl-L-cysteine and *N*-acetyl-*S*-(*p*-bromobenzyl)-L-cysteine respectively.

The enzyme catalysing the reaction in which the *para* chlorine atom of 3:4-dichloronitrobenzene is replaced by GSH appears to be specific for GSH, but not for the other substrate, since it was found that GSH could not be replaced by GSSG, cysteine or *N*-acetylcysteine, whereas other compounds could replace 3:4-dichloronitrobenzene. Many of these are known to be converted into mercapturic acids in the body (see Table 5). With 2:4- and 3:4-dichloronitrobenzene (which yield mercapturic acids in which chlorine atoms are replaced by *N*-acetylcysteine), and 2:3:5:6-tetrachloronitrobenzene (which yields a mercapturic acid by the replacement of the nitro group by acetylcysteine), no GSH derivative was obtained if the soluble liver fraction was boiled before the incubation, indicating that these reactions were enzymic. 3:4:5:6-Tetrachloro-1:2-epoxy*cyclo*hexane also reacted with GSH only in the presence of the soluble liver fraction.

With the substrates bromoethane, 1-bromopropane, benzyl chloride, ethyl methanesulphonate and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene, all of which are alkylating agents, reactions with GSH both in the presence of fresh and of boiled soluble liver fraction was observed although the first reaction was much faster. These reactions were carried out at pH 8, which is the optimum pH of the enzyme for 3:4-dichloronitrobenzene, so that the observed chemical reaction, presumably requiring ionized SH groups of GSH, is not unexpected. Sulphobromophthalein, which also reacted with GSH both in the presence and the absence of the enzyme, is used as a test substance for liver function because it is removed from the blood by the liver and it is excreted in the bile partly in the form of a GSH conjugate (Javitt, Wheeler, Baker & Ramos, 1959; Combes & Stakelum, 1960). It is possible that this liver-

function test is actually a measure of the activity of the enzyme.

The substrates iodobenzene, naphthalene, 1-chloronaphthalene and phenanthrene did not react with GSH in the presence of soluble liver fraction alone, but only if microsomes and $TPNH_2$ were also present. With naphthalene, one of the products thus obtained was indistinguishable on paper chromatograms from *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione, which has been previously identified as a metabolite of naphthalene formed by liver slices (Booth *et al.* 1960*b*) and in the bile of rats dosed with naphthalene (Boyland, Ramsey & Sims, 1961). The second product was *trans*-1:2-dihydro-1:2-dihydroxynaphthalene, which has been reported as a product of the action of microsomes and $TPNH_2$ on naphthalene (Mitoma, Posner, Reitz & Udenfriend, 1956; Booth & Boyland, 1957). Phenanthrene gave two GSH conjugates, and also yielded *trans*-1:2-dihydro-1:2-dihydroxy- and *trans*-9:10-dihydro-9:10-dihydroxy-phenanthrene, which are known metabolites in urine (Boyland & Wolf, 1950).

When 1:2-dihydronaphthalene is incubated with rat-liver microsomes and $TPNH_2$, perhydroxylation occurs with the formation of *trans*-1:2:3:4-tetrahydro-1:2-dihydroxynaphthalene, whereas if GSH is added *S*-(1:2:3:4-tetrahydro-1-hydroxy-1-naphthyl)glutathione is also formed. If rat soluble liver fraction is also present the amounts of the glutathione conjugate formed are much increased (Booth *et al.* 1960*a*). It was suggested that in these reactions the action of microsomes and $TPNH_2$ was to convert 1:2-dihydronaphthalene into 1:2-epoxy-1:2:3:4-tetrahydronaphthalene, which then reacted with GSH, either with or without the enzyme present in the soluble liver fraction, to yield the GSH conjugate. It seems probable that, in the present work, the four compounds requiring microsomes and $TPNH_2$ in addition to the enzyme are first converted into similar intermediates. With these compounds, however, the presence of the soluble liver fraction is usually necessary for the formation of the GSH conjugates, although occasionally small amounts of the GSH conjugate derived from naphthalene, presumably formed by a chemical reaction with the intermediate, have been detected in experiments in which boiled soluble liver fraction has been used.

Fig. 4 shows in effect that compounds which are apparently alternative substrates for the enzyme inhibit the reaction of GSH with 3:4-dichloronitrobenzene non-competitively. This indicates that these compounds are combining with the enzyme at a point other than the active centre for 3:4-dichloronitrobenzene and would seem to imply that the enzymic reactions involved are different so far as active centres are concerned. It seems likely that the enzyme involved in these reactions can produce an activated form of GSH which then reacts more readily with certain compounds. However, since the dialysed soluble liver fraction was used to catalyse the enzymic reactions shown in Table 5, the possibility that all these GSH conjugates are not formed by the same enzyme is not completely eliminated.

SUMMARY

1. An enzyme which catalyses the formation of *S*-(2-chloro-4-nitrophenyl)glutathione from 3:4-dichloronitrobenzene has been partially purified from the soluble fraction of rat liver.

2. *S*-(2-Chloro-4-nitrophenyl)glutathione is converted into *S*-(2-chloro-4-nitrophenyl)-L-cysteine by rat-kidney homogenate, and this compound can be acetylated to the mercapturic acid, *N*-acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine by rat-liver slices.

3. The soluble liver fraction can catalyse the formation of glutathione conjugates from a number of other mercapturic acid precursors.

4. With bromobenzene, naphthalene, 1-chloronaphthalene and phenanthrene, rat-liver microsomes and reduced triphosphopyridine nucleotide as well as the soluble liver fraction are necessary in the formation of the glutathione conjugates.

5. Glutathione cannot be replaced by oxidized glutathione, cysteine or *N*-acetylcysteine in these systems.

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The Intrinsic Viscosity of Myosin and the Interpretation of its Hydrodynamic Properties

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Early work suggested a particle weight of about 800 000 for the myosin molecule, with an axial ratio (J) of 100:1 (Portzehl, 1950), but more recently it has appeared that some of the data employed were in error. For the sedimentation coefficient, a detailed account has been given of the probable cause of this error in terms of molecular-transformation reactions occurring during the experiments (Johnson & Rowe, 1960). There may well have been similar errors in much recent as well as early diffusion work; also in viscosity, light-scattering and osmotic-pressure experiments. There has as yet, however, been no general agreement upon revised estimates of the size and shape of the myosin molecule.

For the complete definition of an ellipsoid of revolution hydrodynamically equivalent to the myosin molecule, three independent (usually kinetic) parameters must be known, together with the partial specific volume ($\bar{v}$). The method of Scheraga & Mandelkern (1953) then provides a convenient means for the calculation of the axial ratio and the effective volume/g. (V) of this ellipsoid. Let us take the extrapolated sedimentation and diffusion coefficients, $[S^0_{20}]$ and $[D^0_{20}]$, and the intrinsic viscosity, $[\eta]$, as the three kinetic parameters. $[S^0_{20}]$ has been accurately determined under conditions where myosin is known to be stable (Holtzer & Lowey, 1956, 1959; Johnson & Rowe, 1960), as also has $[D^0_{20}]$ (Parrish & Mommaerts, 1954), although the value given by Parrish & Mommaerts should be confirmed by an independent and equally thorough study. But the values given in the literature for $[\eta]$ show a surprising degree of scatter, the following being among those reported (dl./g.): 2·2 (Mommaerts, 1945, and Weber, 1950*a*, *b*); 1·8–2·0 (Weber & Portzehl, 1951; Portzehl, Schramm & Weber, 1951); 2·3, 2·2 and 2·4 (Holtzer & Lowey, 1956). Since the completion of this work Holtzer & Lowey (1959) have reported a value of 2·17 at 25°, and a rather higher value (2·24) at 0·7°. In view of the occur-