2-methylpropan-2-ol-4·25N-ammonia (4:1, v/v). Many solvent systems for these compounds have been proposed (see, for example, Kubota, 1961); the information is given because the system seems to be a very versatile one. It was used (Vining & Taber, 1957) for α -amino acids and α -hydroxy acids, and later (Russell, 1960) for *N*-methyl-amino acids. It is a stable one-phase system, and gives compact spots with DNP-derivatives, the R_f values lying between 0·1 and 0·8 approximately. The DNP-derivatives of leucine and isoleucine are particularly well separated.

SUMMARY

1. Solutions of DNP-amino acids and -peptides in dilute aqueous sodium hydrogen carbonate were examined photochemically.

2. The α -DNP-amino acids were all rapidly decomposed by light with the exception of DNP-tryptophan; β -, δ - and ϵ -DNP-amino acids were stable, and DNP-peptides were either stable or decomposed much more slowly than α -DNP-amino acids.

3. Formation of 4-nitro-2-nitrosoaniline on photolysis appeared to be a property exclusively of derivatives possessing the structure

but not all compounds possessing this structure yield 4-nitro-2-nitrosoaniline.

4. The solvent system 2-methylpropan-2-ol-4·25N-ammonia (4:1, v/v) was useful for the paper chromatography of DNP-amino acids.

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An Enzyme from Rat Liver Catalysing Conjugations with Glutathione

2. REPLACEMENT OF NITRO GROUPS*

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Booth, Boyland & Sims (1961) and Coombes & Stakelum (1961) showed that the soluble supernatant fraction of rat liver contained an enzyme catalysing the formation of glutathione *S*-conjugates with replacement of the active halogen of aromatic compounds. Booth *et al.* (1961) found this enzyme to be specific for glutathione although Coombes & Stakelum (1961) state that a small reaction occurred with cysteine and cysteinylglycine. Booth *et al.* (1961) also showed that several compounds that are known to be excreted as mercapturic acids by the body can act as the

acceptor (or second substrate) for the glutathione in the enzymic reaction. The enzyme has been called 'glutathio kinase' (Al-Kassab, Boyland & Williams, 1962; Boyland, 1962).

In the metabolism of some polychloronitrobenzenes the nitro group is displaced during metabolism, not the halide (Bray, Hybs, James & Thorpe, 1953; Betts, James & Thorpe, 1955; Betts, Bray, James & Thorpe, 1957). Evidence is now presented that the enzyme that catalyses the replacement of halogen will also displace the nitro group in these compounds. It also increases the rate of reaction of 4-nitropyridine *N*-oxide and of the carcinogen 4-nitroquinoline *N*-oxide with

* Part 1: Booth, Boyland & Sims (1961).

glutathione. The reaction of 4-nitroquinoline *N*-oxides with sulphhydryl groups may have an important bearing on their carcinogenic action (Nakahara & Fukuoka, 1959). Heppel & Hilmoe (1950) described an enzyme in pig and rat liver which catalysed the reaction between glycerol trinitrate and glutathione; this might be the same enzyme as that considered in the present paper.

MATERIALS AND METHODS

The chloronitrobenzenes were commercial samples recrystallized from ethanol. Some batches of commercial glutathione that were particularly prone to aerobic oxidation were not used. The carcinogenic 4-nitroquinoline *N*-oxide was prepared by British Drug Houses Ltd. 4-Nitropyridine *N*-oxide was a commercial sample (L. Light and Co. Ltd.).

Nitrite was determined by the method of Snell & Snell (1949). Acetic anhydride (0.1 ml./ml. of incubation mixture) was added 5 min. before diazotization to remove the excess of glutathione. Then 2 vol. of 1% (w/v) sulphanilamide in 20% (w/v) HCl followed by an equal amount of 0.02% *N*-(1-naphthyl)ethylenediamine hydrochloride were added to 1 vol. of incubation mixture. The tubes were read at 540 m μ after being left for 15 min. and centrifuged. An incubation mixture containing 0.1 mM-nitrite gave a solution of extinction 0.807. Beer's law was obeyed over the range 0.01–0.2 mM-nitrite. Reaction between nitrite and glutathione in the incubation mixture was negligible in 1 hr. at 37.5° at pH 8, but was appreciable at pH 7.4. This prevented the determination of pH-activity curves with substrates from which nitrite was liberated. Good recoveries (96–100%) were obtained of nitrite added to the system at pH 8.

Glutathione uptake was determined by titration with iodate, in the presence of iodide, in sulphosalicylic acid (Woodward & Fry, 1932). The protein of the incubation mixture was precipitated by the addition to an equal volume of 4% (w/v) sulphosalicylic acid; when nitrite was present it was removed by the addition of 0.1 ml. of 10% (w/v) urea/ml. The mixture was then left at room temperature for 45–60 min. before titration. This prevented iodine, which would have been liberated by the reaction of iodide with nitrite, from oxidizing some of the glutathione before the titration with iodate. This caused the artifact of two molecules of glutathione appearing to react for each NO₂⁻ ion liberated described by Al-Kassab *et al.* (1962), since each NO₂⁻ ion had liberated one I⁻ ion. Rapid titration under ice-cold conditions prevented the aerobic oxidation of nitric oxide from liberating further I⁻ ions.

Because of the low solubility of the polychloronitrobenzenes a sensitive method of determining chloride was required. The method used was a modification of that given by Dr J. F. Marten for the AutoAnalyzer (Technicon Instrument Co.). Tris-acetate (0.1M) buffer was employed instead of pyrophosphate buffer as the latter interfered with the determination. The reagent used was prepared in two parts: (a) ferric ammonium sulphate (60 g.) was dissolved in water (500 ml.), conc. nitric acid (sp.gr. 1.42) (384 ml.) was added slowly and the volume was adjusted to 1 l.; (b) mercuric thiocyanate (1 g.) was shaken with water (1 l.) for 2 hr. and filtered. Just before use 1 vol. of (a) was

mixed with 1 vol. of (b) and added to 2 vol. of incubation mixture. The final mixture was centrifuged and the extinction of the ferric thiocyanate complex read at 463 m μ . The colour was stable for at least 30 min. A reading of 0.485 was given by a 1 cm. light-path cell with 1 mM-sodium chloride in the incubation mixture; by using 4 cm. light-path cells concentrations down to 0.2 mM-chloride could be determined. In the presence of liver supernatant, however, high blanks were obtained.

To determine the uptake of glutathione incubations were carried out in Thunberg tubes to prevent aerobic oxidation. Oxidation was rapid at pH 8, but negligible in the acid solutions (approx. pH 5) obtained by dissolving glutathione in water. Glutathione solution and the enzyme were placed in the side arm of the tube, and the other substrate and buffer in the main part of the tube before evacuation. Rat-liver supernatant had a greater protective action than egg albumin or gelatin against aerobic oxidation, and in some cases aerobic oxidation caused glutathione removal to be more rapid in the absence of liver supernatant than in its presence. Aerobic oxidation was particularly troublesome in experiments with polychloronitrobenzenes as their solubility limited their concentration to 0.1 mM. Similar low concentrations (0.2 mM) of glutathione had to be employed and up to 50% was oxidized in a 30 min. incubation. In other cases concentrations of 1 or 0.5 mM of second substrate were employed; these were obtained by dissolving the substrate in the minimum amount of ethanol before dilution.

The enzyme preparations and the determination of reactions with 3,4-dichloronitrobenzene were those described by Booth *et al.* (1961). The incubation times used for 3,4-dichloronitrobenzene were 5–10 min. and for the polychloronitrobenzenes 1 hr., both at 37.5° and at pH 8.

For the heat-inactivation experiment rat-liver supernatant was heated with an equal volume of 0.2M-pyrophosphate buffer, pH 8, for various intervals at 50°, cooled in ice and centrifuged if necessary. This supernatant was then tested for activity towards 3,4-dichloronitrobenzene and 4-nitropyridine *N*-oxide.

In assays with 4-nitropyridine *N*-oxide as the substrate, solutions of 0.1M-pyrophosphate buffer, pH 8, 6mM-glutathione and 1mM-4-nitropyridine *N*-oxide were incubated for 15 min. at 37.5°. The rate of the spontaneous reaction between this substrate or 4-nitroquinoline *N*-oxide and glutathione had to be determined and taken into account during measurements of enzymic activity.

RESULTS

4-Nitropyridine *N*-oxide and 4-nitroquinoline *N*-oxide reacted spontaneously with glutathione but the reaction was appreciably accelerated by rat-liver supernatant. The reaction was carried out in 0.1M-pyrophosphate buffer, pH 8, the initial concentrations of the reactants being 6 mM for glutathione and 1 mM for the nitro compound. The nitro compounds were added as the ethanolic solution, the final concentration of ethanol in the reaction mixture being 5% (v/v), and the reaction was followed by the liberation of nitrite. In experiments with 4-nitropyridine *N*-oxide and 4-nitroquinoline *N*-oxide it was necessary to add

these compounds to the enzyme in the presence of glutathione, otherwise inactivation occurred by chemical reaction with the enzyme. Because of the rapid spontaneous reaction, incubation for 1–2 min. was used with the 4-nitroquinoline *N*-oxide. The apparent affinity of 4-nitropyridine *N*-oxide was dependent on the concentration of glutathione used: at 1 mM, K_m was 0.473 mM, but at the optimum concentration of glutathione (6 mM; Fig. 1) the affinity was so low that the reaction was pseudomonomolecular (Fig. 2). Fig. 2 also shows that the reaction was pseudomonomolecular for 2,3,4,6-tetrachloronitrobenzene, although in this case the maximum concentration that can be used is limited by the low solubility. The inhibition of the liberation of nitrite from 4-nitropyridine *N*-oxide or from 2,3,4,6-tetrachloronitrobenzene by 3,4-dichloronitrobenzene was non-competitive (Figs. 3 and 4). Booth *et al.* (1961) obtained a similar result with 3,4-dichloronitrobenzene as the substrate and benzyl chloride and 1,2-epoxy-1,2,3,4-tetrahydronaphthalene as inhibitors.

In all cases one NO_2^- ion was liberated for each glutathione molecule conjugated. With 2,3,5,6-tetrachloronitrobenzene, 2,3,4,6-tetrachloronitrobenzene and pentachloronitrobenzene, nitrite was released and no detectable chloride was liberated, although owing to the low solubility of these compounds and the high blanks given by the incubate

Fig. 1. Glutathione concentration-activity curves obtained with 0.5 mM 4-nitropyridine *N*-oxide (○), 0.25 mM 4-nitropyridine *N*-oxide (△) and 1 mM 3,4-dichloronitrobenzene (●) as the second substrate. Incubation was in 0.1M-pyrophosphate buffer, pH 8, for 15 min. at 37°. Almost identical graphs were obtained with 4-nitropyridine *N*-oxide (1 mM) and 2,3,4,6-tetrachloronitrobenzene (0.1 mM) in the presence of 1 mM 3,4-dichloronitrobenzene, and with 3,4-dichloronitrobenzene in the presence of either of the other two substrates.

some (perhaps, on a molecular basis, 10–20% of the nitrite liberated) could have been released without being detected. No nitrite was liberated from the 3,4- and 2,4-dichloronitrobenzene, but one Cl^- ion was liberated for each glutathione molecule that reacted. No reaction was observed with 2,4,5-trichloronitrobenzene.

The rate of inactivation of rat-liver supernatant by heating at 50° was the same for the enzymic reactions with 3,4-dichloronitrobenzene and 4-nitropyridine *N*-oxide (Fig. 5). There was also no difference in the activity ratios between the rat-liver supernatant and the purified ammonium

Fig. 2. Substrate concentration-activity curves for 4-nitropyridine *N*-oxide (○) and for 2,3,4,6-tetrachloronitrobenzene (●) in the presence of 6 mM-glutathione and 0.1M-pyrophosphate buffer, pH 8. Incubation was for 15 min. at 37°.

Fig. 3. Non-competitive-type Dixon (1953) plot obtained by regarding 4-nitropyridine *N*-oxide (○, 0.5 mM; ●, 0.25 mM) as the substrate and 3,4-dichloronitrobenzene as the inhibitor. Incubation was for 15 min. at 37° in the presence of 6 mM-glutathione and 0.1M-pyrophosphate buffer, pH 8. The enzymic activity was measured by the increase in nitrite release over that released spontaneously.

Fig. 4. Non-competitive-type Dixon (1953) plot obtained by regarding 2,3,4,6-tetrachloronitrobenzene (O, 0.1 mM; ●, 0.05 mM) as the substrate and 3,4-dichloronitrobenzene as the inhibitor. Incubation was for 15 min. at 37° in the presence of 6 mM-glutathione and 0.1M-pyrophosphate buffer, pH 8. The non-enzymic release of nitrite was negligible.

Fig. 5. Reduction in the enzymic activity towards 3,4-dichloronitrobenzene (O) and 4-nitropyridine *N*-oxide (●) by heating rat-liver supernatant at 50° with an equal volume of 0.2M-pyrophosphate buffer, pH 8.

Table 1. *Rate of formation of glutathione conjugates in the presence of rat-liver supernatant*

The incubation mixtures contained 0.1M-pyrophosphate buffer, pH 8, 6 mM-glutathione, 0.1–3% rat-liver supernatant and 0.1 mM-second substrate. The second substrates were added last as the ethanolic solutions, the final concentration of ethanol being 5% (v/v). The formation of conjugates was determined by the liberation of nitrite except with 3,4-dichloronitrobenzene where the absorption at 344 mμ was used to measure the enzymic activity (see Booth *et al.* 1961). Incubation was for 1 min. with 4-nitroquinoline *N*-oxide owing to the rate of the enzymic and chemical reactions and for 15 min. in other cases. The ratios of the activities at 37° and 25° were 4:3 with 4-nitropyridine *N*-oxide and 2:1 with 3,4-dichloronitrobenzene respectively. Slight opalescence was seen with pentachloronitrobenzene. The activity with 4-nitropyridine *N*-oxide is taken as a 'relative activity' of 1.0.

Second substrate	Relative activity		Approx. <i>k</i> for chemical reaction (M ⁻¹ min. ⁻¹)	
	37°	25°	37°	25°
4-Nitropyridine <i>N</i> -oxide	1.0	1.0	1.03	0.26
4-Nitroquinoline <i>N</i> -oxide	6.5	4.65	41	26
2,3,4,6-Tetrachloronitrobenzene	1.31	0.96	Too small to be measured over the incubation time and temperature	
2,3,5,6-Tetrachloronitrobenzene	1.39	0.76		
Pentachloronitrobenzene	0.54	0.51		
2,4,5-Trichloronitrobenzene	0	0		
3,4-Dichloronitrobenzene	0.42	0.29		

sulphate fraction as prepared by Booth *et al.* (1961). Table 1 compares the ratios of conjugate formation when different acceptors for the glutathione are used. Low concentrations of second substrates were employed, because of the limited solubility of the polychloronitro compounds.

In experiments where the uptake of glutathione was being compared with the liberation of nitrite or chloride it was necessary to add sufficient enzyme to remove a large proportion of the glutathione for accuracy in measurement. In other cases care was taken that not more than about 20% of the substrate was used up in the reactions, both enzymic and spontaneous (see Dixon & Webb, 1958*a*). Under these conditions the time-activity relationships were linear for the incubation times and

temperatures mentioned. Particular care had to be employed with the polychloronitrobenzenes because of their low solubility.

DISCUSSION

Mercapturic acids are often excreted in the urine and bile as metabolic products of foreign compounds. Booth *et al.* (1961) showed (as suggested by Bray, Franklin & James, 1959) that the formation of these acids can take place in three stages. The soluble supernatant fraction of rat liver contains an enzyme that catalyses the conjugation of glutathione and 3,4-chloronitrobenzene with replacement of the 4-chloro group. This was the first stage of mercapturic acid formation; in the

following steps, rat-kidney homogenate removed the glycine and glutamic acid residues of the glutathione, the cysteine derivative then being acetylated to the mercapturic acid by rat-liver slices. Booth *et al.* (1961) also showed by paper chromatography that the liver supernatant would also catalyse the formation of glutathione conjugates with several other compounds that give rise to mercapturic acids.

With some polychloronitrobenzenes, however, the nitro group is replaced in the body (Bray *et al.* 1953; Betts *et al.* 1957). Soluble rat-liver supernatant has been shown to catalyse the conjugation of 2,3,5,6- and 2,3,4,6-tetrachloronitrobenzene and of pentachloronitrobenzene, one NO_2^- ion being released for every glutathione molecule taken up. No chloride release was detected, and is unlikely in view of the equivalence between the nitrite release and glutathione uptake. Conversely, no nitrite was released during the enzymic reaction of 3,4- and 2,4-dichloronitrobenzene, but glutathione uptake and chloride release were exactly equivalent. In each case the most chemically reactive group on the ring was displaced in the reaction. No reaction took place with 2,4,5-trichloronitrobenzene, which is chemically inert, being stable to boiling ethanolic sodium hydroxide (Betts *et al.* 1955). None of the chloronitrobenzenes tested reacted with glutathione at pH 8 in 1 hr. at 37.5° in the absence of the enzyme.

Nakahara, Fukuoka & Sugimura (1957) showed that some 4-nitroquinoline *N*-oxide derivatives are carcinogenic, and Nakahara & Fukuoka (1959) suggested that, as both the 4-nitro and the *N*-oxide groups were necessary for activity, the carcinogenic action was due to the reaction of the nitro group with sulphhydryl compounds. The spontaneous reaction between 4-nitroquinoline *N*-oxide and glutathione is rapid (k is approx. $41 \text{ M}^{-1} \text{ min}^{-1}$ at 37.5°) at pH 8, but by using short incubation times (1–2 min.) the increase in the rate in the presence of rat-liver supernatant was demonstrated. On the basis of Nakahara & Fukuoka's (1959) suggestions, it is, however difficult to predict what effect the enzyme would have on carcinogenicity, for although the rate of the chemically rapid reaction is increased, there may be some alteration in the energy changes during the reaction.

4-Nitropyridine *N*-oxide is probably non-carcinogenic (Nakahara, 1961); this might be due to the lower activity of the nitro group (k is $1.03 \text{ M}^{-1} \text{ min}^{-1}$) under the same conditions. This compound was used in preference to the polychloronitrobenzenes for the determination of the rate of enzymic displacement of nitro groups by glutathione, since its greater solubility (1 mM compared with 0.1 mM) enabled sufficient nitrite to be liberated for

accurate determination before an appreciable amount of the substrate had reacted. It did, however, suffer from the disadvantage that the spontaneous chemical reaction had to be taken into account in the determination of the enzymic activity. That the nitro group was displaced by the same enzyme that removes chloride from 3,4-dichloronitrobenzene was indicated by the fact that the rates of inactivation of the soluble rat-liver fraction for the two processes were the same. The ratios of activities for the two processes in a crude rat-liver supernatant and a partially purified sample prepared according to Booth *et al.* (1961) were also the same.

To obtain kinetic evidence for the identity of the enzyme replacing aromatic chloride and nitro groups a Dixon (1953) plot was made by assuming 3,4-dichloronitrobenzene to be the inhibitor and 4-nitropyridine *N*-oxide and substrate (Fig. 3). From this graph the inhibition appeared to be non-competitive. The substrate 4-nitropyridine *N*-oxide, however, might have some chemical effect on the enzyme as it reacts quickly with sulphhydryl groups. However, a similar result was obtained with 2,3,4,6-tetrachloronitrobenzene where the non-enzymic reaction is negligible (Fig. 4).

Since it is unlikely that the second substrate would be activated by different active sites on the enzyme, an alternative explanation is possible. The reaction between glutathione and the nitro compound may be regarded as taking place without definite combination of the second substrate with the enzyme. In such a case the rate of the reaction rises to a definite limit on increase of the first substrate (glutathione; Fig. 1) but should increase linearly on increasing the second substrate (Dixon & Webb, 1958*b*) (4-nitropyridine *N*-oxide or 2,3,4,6-tetrachloronitrobenzene; Fig. 2). This was observed up to the limits imposed by the solubility of the second substrate.

Dixon (1953), in considering competitive inhibition, showed that, in addition to intersection occurring in the first quadrant when $1/v$ is plotted against the inhibitor concentration, i , at different substrate concentrations, s , each straight line obtained cuts the base-line at $-K_i(s/K_m + 1)$, where v is the velocity of the reaction and K_i and K_m are the inhibitor and Michaelis constants respectively. With non-competitive inhibitors the intersection occurs on the base-line at $-K_i$.

The affinity of the enzyme-glutathione complex for 4-nitropyridine *N*-oxide or 2,3,4,6-tetrachloronitrobenzene, however, has been shown by the results in Fig. 2 to be low: K_m is large and s/K_m small. Therefore $-K_i(s/K_m + 1)$ is approximately equal to $-K_i$ and the results obtained experimentally will be indistinguishable from those that would be expected if the inhibition were non-competitive.

To simplify the kinetics all these experiments were carried out with the enzyme saturated with glutathione. The presence of 3,4-dichloronitrobenzene did not alter the optimum concentration curve for glutathione when 4-nitropyridine *N*-oxide or 2,3,4,6-tetrachloronitrobenzene was used as the substrate (Fig. 1).

The low affinity of the second substrate indicates that the main requirement of the second substrate should be that it has an electrophilic site to accept the nucleophilic sulphur atom of the glutathione and that, in effect, the action of the enzyme is to give an 'activated' glutathione; in the cases described here the group displaced is different, but the actual conjugation takes place on similar carbon atoms. The exact nature of the specificity of the second substrate is not yet known.

SUMMARY

1. Rat-liver supernatant contains an enzyme that catalyses the replacement of labile aromatic nitro groups with glutathione.

2. Evidence is presented for its identity with the enzyme that conjugates 3,4-dichloronitrobenzene and glutathione with the liberation of chloride.

3. An explanation of the non-competitive inhibition of one second substrate by another is suggested.

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Improvements to the Ultraviolet-Absorption Optical System of an Ultracentrifuge

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In the original ultracentrifuges the course of the sedimentation was followed by means of light-absorption, but the general accuracy and ease of use of refractometric methods led to the disuse of this type of measurement until quite recently, when the renewed interest in nucleic acids and nucleoproteins revived it. The manufacturers of the Spinco model E ultracentrifuge (Beckman, Spinco Division, Palo Alto, Calif., U.S.A.) now provide ultraviolet-absorption optics for this machine, and, though they are usable, their design is by no means without defect. However, it is possible to

improve their performance by a few simple modifications, and the object of this paper is to indicate what can be done without a complete re-design of the instrument.

The modifications that have been made to our own instrument, and the reasons why they have been made, are as follows.

Lamphouse

This consists of a brass block enclosing the 85w medium-high-pressure mercury lamp; the block is cooled by being in metallic contact with the rest of