Conjugations with Glutathione

DISTRIBUTION OF GLUTATHIONE S-ARYLTRANSFERASE IN VERTEBRATE SPECIES

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Booth, Boyland & Sims (1961) described and partially purified an enzyme from the soluble supernatant of rat liver which catalyses conjugations of glutathione with aromatic compounds to form thioethers as a first stage in mercapturic acid biosynthesis, and an apparently similar enzyme has been shown to be involved in processes leading to the biliary excretion of sulphobromophthalein (phenoltetrabromophthaleindisulphonate) conjugates (Combes & Stakelum, 1961). It is suggested that this enzyme, which has previously been called 'glutathio kinase' (Boyland, 1962; Al-Kassab, Boyland & Williams, 1963), should be named 'glutathione S-aryltransferase'. The conjugation of glutathione with aliphatic halogen compounds is catalysed by a liver enzyme that can be separated from that involved in conjugations of glutathione with aromatic compounds (Johnson, 1963). There must therefore be more than one glutathione transferase.

The present paper concerns the distribution of the enzyme described by Booth *et al.* (1961) in several vertebrate species and its apparent identity with the enzyme implicated in biliary sulphobromophthalein excretion.

MATERIALS AND METHODS

1,2-Dichloro-4-nitrobenzene was recrystallized from ethanol. Glutathione was obtained from Sigma Chemical Co., and sulphobromophthalein as the sodium salt from Samore Ltd.

Liver homogenates and soluble supernatant fractions were prepared by the methods described by Booth *et al.* (1961). Enzyme activities towards 1,2-dichloro-4-nitrobenzene were measured by the rate of increase in the extinction at 344 m μ (Booth *et al.* 1961) in a Unicam SP.500 spectrophotometer equipped with a Unicam SP.570 constant-temperature cell housing and coupled to a Gilford model 200 Optical Density Converter (Gilford Instrument Laboratories Ltd., Oberlin, Ohio, U.S.A.) and an Autograf model 3 Two-Axis Recorder (F. L. Moseley Co., Pasadena, Calif., U.S.A.) fitted with a time-base unit. These instruments were standardized against a K₂Cr₂O₇ standard solution (Ketelaar, Fahrenfort, Haas & Brinkman, 1955) of 0.345 g. of K₂Cr₂O₇ in 1 l. of 0.01 N-H₂SO₄, which had $E_{344\text{ m}\mu}^{1\text{ cm}}$ value of 3.55.

Proteins were determined by a modification of the biuret method (Fincham, 1954).

RESULTS

Inhibition of 1,2-dichloro-4-nitrobenzene conjugation by sulphobromophthalein. Sulphobromophthalein was found to be a competitive substrate in the reaction of 1,2-dichloro-4-nitrobenzene with glutathione. This inhibition was competitive (Fig. 1), with a K_i for sulphobromophthalein of 3×10^{-6} M. In reaction mixtures containing glutathione (10 mM), 1,2-dichloro-4-nitrobenzene (1 mM) and rat-liver supernatant in 0.1 M-pyrophosphate buffer, pH 8.0, 0.02 mM-sulphobromophthalein halved the rate of 1,2-dichloro-4-nitrobenzene conjugation.

A glutathione conjugate of sulphobromophthalein was identified, by paper chromatography, after

Fig. 1. Competitive-type Dixon (1953) plot obtained by regarding 1,2-dichloro-4-nitrobenzene (O, 0.75 mM; ●, 1.25 mM) as the substrate and sulphobromophthalein as the inhibitor. Reaction mixtures containing glutathione (10 mM) and rat-liver supernatant in 0.1 M-pyrophosphate buffer, pH 8.0, were incubated at 37°. The enzyme activity, v , was measured by the increase in the extinction at 344 m μ /min. (Booth *et al.* 1961).

incubation (at 37° for 15 min.) of a reaction mixture containing glutathione (3.75 mM), sulphobromophthalein (1 mM) and partially purified enzyme from rat-liver soluble supernatant fraction (Booth *et al.* 1961) in 0.1 M-pyrophosphate buffer, pH 8.0 (4.0 ml.). Ethanol (5 ml.) was added to the mixture and, after standing (for 20 min.) and centrifuging (for 10 min. at 2000g), portions of the supernatant were chromatographed on Whatman no. 1 paper with propan-1-ol-acetic acid-water (10:1:5, by vol.) (Combes, 1959). The glutathione derivative had R_F 0.50, was ninhydrin-positive and gave a purple colour with ammonia vapour. Some conjugate was formed chemically in reaction mixtures containing boiled enzyme preparation.

Distribution of the 1,2-dichloro-4-nitrobenzene-conjugating enzyme in vertebrate species. Liver homogenates and soluble supernatant fractions were prepared from freshly killed adult animals or from samples that had been cooled with solid carbon dioxide immediately after death. The enzyme acti-

vities of these preparations towards 1,2-dichloro-4-nitrobenzene were measured and the values are shown as specific activities (units/mg. of protein) in Table 1. Dilution of most of the liver supernatant preparations was necessary before enzyme-activity measurements; dilution experiments with rat-liver preparations failed to show the presence of any endogenous reversible inhibitors of enzymic 1,2-dichloro-4-nitrobenzene conjugation with glutathione. Fresh or freshly frozen liver samples were used for activity measurements, although experiments had shown that no appreciable fall in the 1,2-dichloro-4-nitrobenzene-conjugating activity of rat-liver supernatant occurred when intact rats were kept at room temperature for up to 16 hr. *post mortem*.

The enzyme activity towards 1,2-dichloro-4-nitrobenzene was not enhanced but was decreased by pretreatment of rats with aromatic hydrocarbons (3-methylcholanthrene, 3,4-benzo[*a*]pyrene) or narcotics (phenobarbitone, chlorotone) (Fig. 2), com-

Table 1. *Distribution of liver glutathione S-aryltransferase in different animals*

Enzyme activities towards 1,2-dichloro-4-nitrobenzene were measured by recording the increase in extinction at 344 m μ (Booth *et al.* 1961) of reaction mixtures containing glutathione (3.75 mM), 1,2-dichloro-4-nitrobenzene (1.25 mM) and liver preparations in 0.1 M-pyrophosphate buffer, pH 8.0 (4 ml.), at 37°. One unit of enzyme is defined as the amount which will form 1 μ mole of *S*-(2-chloro-4-nitrophenyl)glutathione/min. The values in parentheses refer to the numbers of livers homogenized and pooled for enzyme-activity measurements.

Species	10 ² × Specific activity (units/mg. of protein)	
	Soluble supernatant fraction	Liver homogenate
Mouse (<i>Mus musculus</i>) CBA/H strain (6), ♂	4.55	2.32
Mouse (<i>M. musculus</i>) CBA/H strain (6), ♀	9.60	4.71
Rat (<i>Rattus norvegicus</i>) August strain (2), ♂	8.80	4.54
Rat (<i>R. norvegicus</i>) Slonaker strain (2), ♂	8.78	3.93
Rat (<i>R. norvegicus</i>) Chester Beatty strain (2), ♂	6.39	3.64
Rat (<i>R. norvegicus</i>) Chester Beatty strain (2), ♀	4.05	3.08
Dog (<i>Canis familiaris</i>)	5.30	2.25
Guinea pig (<i>Cavia porcellus</i>), ♂	3.46	2.66
Guinea pig (<i>C. porcellus</i>), ♀	2.90	1.82
Cat (<i>Felis catus</i>)	2.77	1.49
Golden hamster (<i>Mesocricetus auratus</i>) (4), ♂	1.94	1.33
Sheep (<i>Ovis aries</i>)	1.10	0.55
Horse (<i>Equus caballus</i>)	1.03	0.52
Rabbit (<i>Oryctolagus cuniculus</i>), ♂	0.99	0.53
Monkey (<i>Macaca mulatta</i>)	0.87	0.73
Lizard (<i>Lacerta viridis</i>), ♂	0.68	0.09
Lizard (<i>L. viridis</i>), ♀	0.66	0.15
Pig (<i>Sus scrofa</i>)	0.39	0.24
Man		
Biopsy specimen, ♂	0.34	0.20
Post-mortem specimen, ♀	0.28	0.18
Foetus (20-week)	0.25	0.18
Chicken (<i>Gallus domesticus</i>)	0.20	—
Goldfish (<i>Carassius auratus</i>), ♂	0.13	0.12
Goldfish (<i>C. auratus</i>), ♀	0.16	0.09
Duck (<i>Anas platyrhynchos</i>), ♂	0.11	0.03
Duck (<i>A. platyrhynchos</i>), ♀	0.11	0.05
Frog (<i>Rana temporaria</i>), ♂	0.05	0.02
Frog (<i>R. temporaria</i>), ♀	0.11	0.10
Ox (<i>Bos</i> sp.)	0.11	0.08
Duckling (1-day-old) (12)	0.02	0.006

Fig. 2. Effect of administration of aromatic hydrocarbons and narcotics on the activity of the rat-liver enzyme conjugating 1,2-dichloro-4-nitrobenzene with glutathione. The enzyme activity of the supernatant fractions prepared from pairs of male Chester Beatty rat livers was estimated by the increase in the extinction at 344 $m\mu$ (Booth *et al.* 1961) of reaction mixtures containing glutathione (3.75 mM), 1,2-dichloro-4-nitrobenzene (1.25 mM) and rat-liver supernatant in 0.1 M-pyrophosphate buffer, pH 8.0 (4 ml.). Rats were given a single intraperitoneal injection, in arachis oil, of: Δ , 3-methylcholanthrene (10 mg.); $\blacksquare$, 3,4-benzo[a]pyrene (20 mg.); $\circ$, chloretone (40 mg.); $\bullet$, phenobarbitone sodium (20 mg.).

pounds that increase the activities of several liver-mitochondrial enzyme systems (Conney & Burns, 1962), one liver-soluble-supernatant enzyme, namely uridine diphosphate glucose dehydrogenase (Touster & Hollman, 1961), and ascorbic acid excretion (Boyland & Grover, 1961).

DISCUSSION

Enzymic conjugations of a variety of compounds with glutathione through a thio ether linkage have been reported by several authors. Heppel & Hilmoe (1950) described a reaction between glycerol trinitrate and glutathione that was catalysed by liver preparations with the release of inorganic nitrite. Booth *et al.* (1961) showed that rat-liver soluble supernatant fraction catalysed glutathione conjugations with both aromatic and aliphatic compounds, and Combes & Stakelum (1961) reported similar conjugations between glutathione and sulphobromophthalein. Johnson (1963) showed that rat-liver supernatant contained an enzyme that catalysed glutathione conjugations with aliphatic halogen compounds, and that could be separated from that involved in glutathione conjugation with aromatic compounds.

The Dixon (1953) plot (Fig. 1) obtained by the addition of various concentrations of sulphobromophthalein to reaction mixtures containing glutathione, 1,2-dichloro-4-nitrobenzene and rat-liver soluble supernatant showed that sulphobromophthalein is a strong competitive substrate or inhibitor of 1,2-dichloro-4-nitrobenzene conjugation ($K_i 3 \times 10^{-6}$ M), and probably indicates that the same enzyme is responsible for glutathione conjugations with both 1,2-dichloro-4-nitrobenzene (Booth *et al.* 1961) and sulphobromophthalein (Combes & Stakelum, 1961).

The enzyme that conjugates glutathione with aliphatic halogen compounds is inactivated by dialysis against distilled water (Johnson, 1963), but the reaction of glutathione with glycerol trinitrate is catalysed by dialysed rat-liver supernatant preparations (K. Williams, unpublished work). Although enzymic reactions between glutathione and glycerol trinitrate are inhibited by 1 mM-copper sulphate (Heppel & Hilmoe, 1950), enzymic catalysis of the conjugation of glutathione with 1,2-dichloro-4-nitrobenzene is not affected by this concentration of Cu^{2+} ions.

These results indicate that there are two distinct enzymes concerned in the conjugation of organic compounds with glutathione. First, that described by Booth *et al.* (1961) and by Combes & Stakelum (1961), and since studied by Al-Kassab *et al.* (1963), which conjugates glutathione with aromatic halogen and nitro compounds; secondly, the enzyme described by Johnson (1963) which is responsible for the conjugation of glutathione with aliphatic halogen compounds.

The activities, in several vertebrate species, of the liver enzyme that catalyses glutathione conjugation with aromatic compounds are shown in Table 1. In general the livers of the rodents and carnivores examined possessed the highest specific activities whereas those of amphibia, birds and fish were low. There did not appear to be a consistent sex difference in the activity of the enzyme, for, though liver from male rats showed a higher specific activity than did the sample from females, the opposite was found for mouse livers.

In addition to the adult livers studied, samples of foetal human (20-week foetus) liver and of duckling (1-day-old) liver were examined. The specific activity of the foetal human liver sample was only slightly lower than the value for adult liver; this agrees with the work of Vest (1962), who, after examining the conjugation and excretion of sulphobromophthalein in new born infants, concluded that the delayed serum clearance was due to a low biliary secretion rather than to a deficiency in the hepatic conjugating mechanism. In other species there appears to be a maturation period for this enzyme which Combes & Stakelum (1962) have found to be

about 9 weeks in rats, the activity in new-born rats being only some 25 % of that in adults.

The distribution of this glutathione *S*-aryltransferase in different species is similar to that of other enzymes that are concerned with the metabolism and renal excretion of foreign compounds, as described by Brodie (1962). The enzyme is therefore probably concerned with the defence of the terrestrial animals against toxic foreign compounds. The activities were low in goldfish and frogs but higher in lizards and mammals. A remarkable difference between this enzyme and other drug-metabolizing systems is the low activities in chickens and ducks and the very low activity in ducklings. Bird livers in general have about the same activity for the oxidative metabolism of aromatic compounds such as acetanilide as does mammalian liver. The low activities of the enzyme in chicken and duck liver may indicate that these birds do not excrete large amounts of mercapturic acids. If birds do not possess some other mechanism for the metabolism and excretion of compounds usually excreted as mercapturic acids, then these compounds would probably be very toxic to avian species.

The urinary excretion of the mercapturic acids of foreign compounds by guinea pigs has been shown to be small in comparison with that of other rodents (Corner & Young, 1954; Bray, Franklin & James, 1959*a*). Since an appreciable activity of the enzyme catalysing glutathione conjugation with 1,2-dichloro-4-nitrobenzene was found in guinea-pig-liver supernatant (Table 1) guinea pigs can carry out the first stage of mercapturic acid synthesis. This agrees with the work of Bray, Franklin & James (1959*b*), who conclude that the lack of mercapturic acid excretion by the guinea pig is due to the inability of this species to acetylate arylcysteine derivatives, the final stage in mercapturic acid synthesis.

SUMMARY

1. The specific activity of the liver enzyme that catalyses the reaction of 1,2-dichloro-4-nitrobenzene with glutathione is high (1–10) in rats, mice, dogs, cats, hamsters, guinea pigs, horses and sheep, lower (0.1–1) in rabbits, monkeys, pigs, oxen, lizards, frogs, men, chickens, goldfish and ducks, and very low (0.02) in 1-day-old ducklings.

2. This enzyme is different from those catalysing the reaction of glutathione with methyl iodide or with glycerol trinitrate, but appears to be identical with that involved in the conjugation of sulphobromophthalein.

3. The enzyme in rat liver was decreased by treatment with aromatic hydrocarbons or narcotics.

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