SUMMARY

1. Cell-free extracts, nearly free from bacteria, have been prepared from suspensions of the two genera of rumen holotrich Protozoa, *Dasytricha* and *Isotricha*.

2. The ability of these extracts to split a number of di-, tri- and poly-saccharides has been investigated. Hydrolytic activity of the extracts corresponded to the ability of the living Protozoa to ferment the substrates.

3. *Dasytricha ruminantium* extracts contained appreciable cellobiase and β-glucosidase activity, and moderate maltase activity. Extracts of mixed *Isotricha intestinalis* and *I. prostoma* contained hardly any maltase, a trace of cellobiase and a small amount of β-glucosidase. Neither genus gave extracts able to hydrolyse lactose, melibiose, trehalose, melezitose or xylobiose.

4. Considerable invertase activity was found in the protozoal extracts, especially those from *Isotricha*. Sucrose, raffinose, inulin and a bacterial levan were all hydrolysed, but not melezitose. The transferring activity of the invertase was examined, and some products of transfructosylation were isolated and identified. The protozoal invertase resembles most closely that of yeast.

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The Formation of Mercapturic Acids

1. FORMATION OF MERCAPTURIC ACID AND THE LEVELS OF GLUTATHIONE IN TISSUES

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Since Baumann & Preusse (1879) and Jaffe (1879) showed that the administration of monohalogenobenzenes to the dog led to the excretion of mercapturic acids [*N*-acetyl-*S*-(*p*-halogenophenyl)-L-cysteines] many other mercapturic acids have been isolated from the urines of several species. These compounds may be classified in four types as shown in Table 1 and are, in effect, formed by elimination between precursor and acetylcysteine of 2H (type I), halogen hydracid (type II), HNO_2 (type III) or $HO_3S \cdot CH_3$ (type IV). The suffix *a*, *b* or *c* to the type numbers indicates an aryl, alkyl or aralkyl compound.

Baumann & Preusse (1879) and Jaffe (1879) suggested that mercapturic acids were produced in urine from an acid-labile compound. Boyland, Sims & Solomon (1957) and Boyland & Sims (1958) have shown that naphthylmercapturic acid is an

artifact formed by decomposition of a compound, probably *N*-acetyl-*S*-(1:2-dihydro-2-hydroxynaphthyl)-L-cysteine, which is excreted in the urine of several animals dosed with naphthalene. A reduction of the aromatic ring during the formation of mercapturic acid had been postulated by Rhode (1923), who suggested that *S*-(4-bromo-2-hydro-1-hydroxyphenyl)cysteine was an intermediate in the conversion of bromobenzene into bromophenylmercapturic acid. Knight & Young (1958) have obtained similar evidence for premercapturic acids from precursors of type I but not for benzyl chloride. In this Laboratory we have not been able to detect a premercapturic acid from 3:4-dichloronitrobenzene (unpublished result). It would seem, therefore, that mercapturic acids may be formed from precursors of type I by a different process from that by which mercapturic acids are formed from precursors of type II. It may be relevant that type I precursors yield mercapturic acids by, in effect, deprotonation whereas in types II, III and IV the elimination of a molecule of an acid is involved.

Although the details of the formation of mercapturic acid have not been elucidated it is usually held that the precursor is converted into an *S*-cysteine derivative which is then acetylated. Indirect evidence of this was obtained by several workers (e.g. Stekol, 1938; Zbarsky & Young, 1943; West, Mathura & Black, 1951; West & Mathura, 1954) who administered *S*-substituted cysteines to animals and isolated the corresponding mercapturic acids from the urine. More direct evidence was obtained by Mills & Wood (1956), who detected *p*-iodophenylcysteine as an intermediate in the formation of the mercapturic acid from *p*-iodobenzene by rat-liver slices and in the liver after oral administration of iodobenzene to rat.

The immediate source of the cysteine required for conjugation with the mercapturic acid precursor has not been established. Possible sources are dietary cysteine, cysteine residues of tissue protein, and glutathione. Stekol (1937*a–d*, 1939*a*) concluded that the addition of sulphur-containing amino acids or glutathione to the food of animals maintained on a balanced diet had no effect on the formation of mercapturic acid but that the addition of these acids augmented the synthesis in rats and dogs maintained on a low-sulphur diet. Simultaneous measurements of the nitrogen and mercapturic acid excretion led Stekol to suggest that the augmentation observed was a result of replenishment of tissue sulphur rather than evidence of the direct union of the mercapturic acid precursor with dietary amino acids. Stekol's conclusion was based on the results of estimations carried out on 24 hr. urine samples. Barnes & James (1957) used detailed excretion curves, based on the analysis of frequent successive urine samples, and showed that the administration of cysteine, cystine or methionine did not increase the rate of synthesis of mercapturic acid in the rabbit. Gutmann & Wood (1950) showed that less than 10% of [^{35}S]cysteine administered simultaneously with bromobenzene to rats was excreted as *N*-acetyl-*S*-(*p*-bromophenyl)-L-cysteine. Marsden & Young (1958) also found that only a small proportion of ^{35}S given as L-[^{35}S]cystine was excreted as 1-naphthyl[^{35}S]mercapturic acid by rats dosed with naphthalene. There is therefore evidence that dietary sulphur-containing amino acids are not the immediate source of more than a small fraction of the cysteine used in mercapturic acid formation.

Stekol (1937*a*; 1939*b*) and Smith, Spencer & Williams (1950) favoured the view that the first stage in the formation of a mercapturic acid consisted in the conjugation of the precursor with

Table 1. *Types of mercapturic acid precursors*

Type	Precursor	Examples	Group replaced by acetylcysteyl	Reference
I	Aromatic hydrocarbon or halogenobenzene	Benzene	H	Zbarsky & Young (1943)
		Chlorobenzene	H	Jaffe (1879)
		Naphthalene	H	Bourne & Young (1934)
II*a*	Halogenonitrobenzene	2:4-Dichloronitrobenzene	Cl	Bray, James & Thorpe (1957)
		p-Fluoronitrobenzene	F	Bray, James & Thorpe (1958*a*)
II*b*	Halogenoparaffin	Bromoethane	Br	Thomson, Maw & Young (1958)
		Bromobutane	Br	Bray & James (1958)
II*c*	ω-Halogenoalkylbenzene	Benzyl chloride	Cl	Stekol (1938)
		Phenethyl bromide	Br	Bray *et al.* (1958*b*)
III*a*	Halogenonitrobenzene	Pentachloronitrobenzene	NO_2	Betts, James & Thorpe (1955)
		2:3:5:6-Tetrachloronitrobenzene	NO_2	Bray, Hybs, James & Thorpe (1953)
III*b*	Nitroparaffin	Nitrobutane	NO_2	This paper
IV	Ester	Ethyl methanesulphonate	$HO_3S{\cdot}CH_3$	Roberts & Warwick (1957)

tissue protein. Stekol (1938, 1939*b*) cited as evidence the observation of Landsteiner & Jacobs (1936) that benzyl chloride produced sensitization in the guinea pig. The fact that this animal excretes very little mercapturic acid (see Bray, Franklin & James, 1959), however, detracts from the value of this argument, unless the reason for the small excretion is that any mercapturic acid formed is deacetylated or otherwise changed before excretion. Mills & Wood (1956) isolated a protein, containing radioactive material, from the liver of a rat which had been given [^{131}I]iodobenzene, but the possibility that the radioactivity was due to adsorbed material not removed by washing, rather than to the formation of a conjugate, was not excluded. These workers also prepared, by the action of diazotized *p*-iodoaniline on serum protein, a product which yielded *p*-iodophenylcysteine on hydrolysis. When the product was administered orally or intravenously to rats it was converted into *p*-iodophenylmercapturic acid, so it is possible that tissue protein may provide some of the cysteine required for mercapturic acid formation. However, the large amounts of mercapturic acid excreted after the administration of some mercapturic acid precursors to the rabbit, and the rapid rate at which they are formed, make it improbable that the tissue protein could furnish all the cysteine required for conjugation. Thus if liver protein is synthesized at the rate of 1·9 g./kg. of rabbit/day (Henriques, Henriques & Neuberger, 1955) about 2 mg. of protein S/2·5 kg. rabbit/hr. will be formed. Mercapturic acid can be formed from benzyl chloride at the rate of about 40 mg./2·5 kg. rabbit/hr. (Barnes & James, unpublished work) and this would require about 5 mg. of S/2·5 kg. rabbit/hr. Furthermore, Anson (1940, 1941), Anson & Stanley (1941) and Barron (1951) reported that not all the –SH groups of tissue protein are reactive and a proportion may not be available for combining with the foreign molecule.

The possible role of glutathione in furnishing the three amino acids utilized for conjugation with foreign molecules was considered by Waelsch (1930) and, although later evidence (Waelsch & Rittenberg, 1941) excluded the possibility that the sole source of glycine for hippuric acid formation was glutathione, the possibility that the cysteine residue was used in the formation of mercapturic acids has not been similarly excluded (Waelsch, 1952). Stekol (1940, 1941) gave benzyl- and *p*-bromobenzyl-glutathione to rats and isolated benzyl- and *p*-bromobenzyl-mercapturic acids from the urine. This is not unequivocal evidence that the mercapturic acid precursor combines with glutathione in the body since the substituted glutathione may have been hydrolysed in the gut (see Bray *et al.* 1959) and there is no proof that the glutathione conjugate itself was absorbed. Nakashima (1934) showed that the glutathione content of the eye lens and of the liver was depleted 24 hr. after the administration of naphthalene to the rabbit. Yamamoto (1940) related the fall in the level of liver glutathione in the rabbit dosed with bromobenzene to the excretion of *p*-bromophenylmercapturic acid. Binet & Wellers (1951) observed a decrease in the level of glutathione in the livers and kidneys of rats given bromobenzene. Snyder & Cornatzer (1958) showed that the amounts of acid-soluble –SH compounds, but not the protein sulphur-containing amino acids, of rat liver decreased after administration of bromobenzene.

Evidence for the participation of glutathione in the formation of mercapturic acids has been given in preliminary communications by Barnes & James (1957) and Bray & Franklin (1957) and some details of this work are now reported. This paper describes the effect of the administration of typical mercapturic acid precursors upon the level of glutathione in tissues of the rat and rabbit.

MATERIALS

All melting points are uncorrected.

Chlorobenzene, benzyl chloride, *p*-chlorobenzyl chloride, 1-bromobutane, 3:4-dichloronitrobenzene and butyl chloral hydrate were purchased. 2:4:6- and 3:4:5-Trichloronitrobenzenes were prepared by the method of Holleman & Haeften (1921) and 2:3:4:6-tetrachloronitrobenzene as described by Betts, James & Thorpe (1955). 1-Nitrobutane, b.p. 151°, was prepared by the interaction of 1-bromobutane and silver nitrite. It contained 0·8% of Br (i.e. 98% pure). Glutathione and oxidized glutathione were purchased from Boehringer und Söhne G.m.b.H., Mannheim.

METHODS

Animals and diet. Female rats (approx. 200 g.) were used for the determination of tissue glutathione. For excretion experiments they were housed in metabolism cages set over a funnel which was arranged over a fraction-cutter (Bray, Thorpe & White, 1951). The animals were maintained on a diet of rat cubes and water. The rabbits used were does (about 2·5 kg.) maintained on rabbit pellets and water. All doses were administered by stomach tube as suspensions in water.

Determination of mercapturic acid. For mercapturic acids formed from chlorobenzene, 2:4:6-trichloronitrobenzene, 2:3:4:6-tetrachloronitrobenzene and butyl chloral hydrate the modified Stekol (1936) method (Betts *et al.* 1955) was used; for those from benzyl chloride, *p*-chlorobenzyl chloride, 1-bromobutane and 1-nitrobutane the nitroprusside method (Bray, James & Thorpe, 1958*b*) was employed. For the last two the final concentration of NaOH used for the hydrolysis was increased to 4N and the time was 1·5 hr. Mercapturic acids formed from 3:4-dichloronitrobenzene and 3:4:5-trichloronitrobenzene were determined by the colorimetric method of Bray, James & Thorpe (1956).

The maximum rates of excretion of the mercapturic acid were measured by determining the concentration of mercapturic acid in the urine produced during the period from 2 to 6 hr. after dosing. During this period the excretion curves are nearly linear (see Fig. 1) and the rate of excretion is approximately constant. Urine was expelled from the bladder by gentle pressure 2hr. after the dose had been given. This urine was discarded. The rats were then given water (3 ml.) by stomach tube and from this time the urine was collected. A further 3 ml. of water was given after 5 hr. After 6 hr. the urine was expelled from the bladder as before and the combined samples were analysed. Usually six rats were used for each compound studied and a correction was made for base-line excretion of material reacting with the reagents by deducting one-sixth of the average normal 24 hr. values for undosed rats from the amount excreted by experimental rates during the 4 hr. In some experiments complete excretion curves of the mercapturic acid were obtained by analysis of successive urine samples for longer periods.

Determination of glutathione. The glutathione (GSH) and oxidized glutathione (GSSG) content of blood was determined by the iodometric method of Woodward & Fry (1932). In early experiments the GSH content of the tissues was determined by the enzymic method of Woodward (1935) as modified by Bhattacharya, Robson & Stewart (1955). In later experiments the method of Martin & McIlwain (1959) was used for GSH and GSSG. In this method GSSG is reduced by preincubation with the glutathione reductase of yeast.

Plan of experiments and collection of tissues

Rats. The dose, or in control experiments water alone, was administered to rats which had been starved for 19 hr. The animals were killed after the required time by a blow on the back of the neck. Blood was collected from the nostrils into potassium oxalate (37·5 mM), deproteinized and analysed for glutathione. The liver, kidneys and small intestine were quickly removed. A sample of the liver (about 1–2 g.) was separated, chopped up and placed in ice-cold 3% (w/v) sulphosalicylic acid (10 ml.) in a tared centrifuge tube. Whole kidneys were treated in the same way. The small intestine was slit longitudinally and the contents were washed out with water and discarded. The intestine was finely chopped and immersed in sulphosalicylic acid as with the liver and kidney. The tissues were homogenized with a Teflon pestle, diluted with 3% sulphosalicylic acid (10 ml.) and centrifuged. The supernatant was separated off. The residue was mixed with a further 10 ml. of 3% sulphosalicylic acid and centrifuged. Samples of the combined supernatants were used for determination of GSH and GSSG.

For the continuous injection experiments (see Fig. 2) the rat was kept in a cylinder of wire mesh, which prevented undesirable movement of the animal. The cylinder was held in a polythene funnel cut to fit it. The appropriate isotonic fluid was injected continuously into the caudal vein of the rat through fine polythene tubing which had been inserted while the tail was anaesthetized with procaine (cf. Kellogg, Burack & Isselbacher, 1954). The fluid was kept in a 50 ml. glass syringe held rigidly in a horizontal position. The plunger was fitted with a cap and wires so arranged that the plunger could be gradually drawn in by winding the wire on a slowly rotating spindle operated by an electric motor with a 10^6:1 reduction gear. The spindle was 'stepped' in several diameters so that the rate of movement of the plunger could be varied. When the wire was wound on a step of 1 cm. diameter the plunger was drawn in at the rate of 6 mm./hr. This apparatus is a modification of a device shown to us by Dr R. Keeler. Under the continuous injection of fluid the rat passed urine at frequent and fairly uniform intervals (see Fig. 2). After each sample the rat and funnel were washed with a small amount of water which was combined with the urine sample.

Rabbits. Blood samples (about 3 g.) were collected from an ear vein directly into 25 ml. of 37·5 mM-potassium oxalate.

RESULTS

Isolation of mercapturic acids from rat urine

N-Acetyl-S-(2-chloro-4-nitrophenyl)-L-cysteine. The urine of three rats which had each received 150 mg. of 3:4-dichloronitrobenzene was acidified and extracted with ether. *N*-Acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine crystallized from the extract. After recrystallization from aqueous ethanol it had m.p. 192°, alone and in admixture with an authentic specimen. Yield 100 mg. The spectrum was in agreement with that recorded for the synthetic material (Bray, James & Thorpe, 1957).

N-Acetyl-S-(2:3:4:6-tetrachlorophenyl)-L-cysteine. Evaporation of the solution obtained by continuous ether extraction of the urine of six rats which had each received 75 mg. of 2:3:4:6-tetrachloronitrobenzene gave crystals of *N*-acetyl-*S*-(2:3:4:6-tetrachlorophenyl)-L-cysteine. After recrystallization from aqueous ethanol the crystals (90 mg.) had m.p. 194°, alone and in admixture with a specimen isolated from rabbit urine (Betts *et al.* 1955); $[\alpha]_D^{24}$ was $+21 \pm 2°$ in ethanol (*c*, 0·66).

Extent of formation of mercapturic acid in the rat

The extent of the formation of mercapturic acid after administration of examples of precursors of types I, II and III to the rat is given in Table 2, from which it can be seen that the rat closely resembles the rabbit in its ability to form mercapturic acid from typical precursors. The maximum rates of excretion of some of the mercapturic acids during the period from 2 to 6 hr. after administration of the dose, the nearly linear part of the excretion curve, are given in Table 3. Fig. 1 shows typical excretion curves over longer periods obtained by the analysis of consecutive urine samples from rats given benzyl chloride, 3:4-dichloronitrobenzene and 3:4:5-trichloronitrobenzene.

Glutathione levels of tissues

Liver. The amounts of GSH and GSSG found in the liver of rats are given in Table 4. The administration of water by stomach tube to a rat had a

Table 2. *Excretion of mercapturic acid by the rat*

Values are means with ranges in parentheses; the superior figures indicate the number of experiments. Doses for the rabbit were of the same order as for the rat. Samples of urine were collected until excretion of mercapturic acid could no longer be detected (usually 24 hr.).

Precursor	Type	Dose for rat (μmoles/100 g.)	Mercapturic acid excreted (% of dose) Rat	Rabbit
Chlorobenzene	I	187	18 (15–23)[3]	20*
Benzyl chloride	II*c*	158	27 (20–32)[4]	49†
p-Chlorobenzyl chloride	II*c*	124	37 (21–47)[3]	23†
1-Bromobutane	II*b*	175	44 (21–64)[5]	45‡
1-Nitrobutane	III*b*	165	63 (62–64)[3]	23‡
3:4-Dichloronitrobenzene	II*a*	182	19 (4–29)[12]	45§
3:4:5-Trichloronitrobenzene	II*a*	110	24 (21–29)[3]	24§
2:4:6-Trichloronitrobenzene	III*a*	110	2 (0–4)[3]	<1§
2:3:4:6-Tetrachloronitrobenzene	III*a*	144	32 (29–38)[3]	37‖
2:3:5:6-Tetrachloronitrobenzene	III*a*	144	11 (8–14)[3]	14¶
Benzyl alcohol	—	171	0[3]	2†
Butyl chloral hydrate	—	65	0[3]	—

* Spencer & Williams (1950).
† Bray *et al.* (1958*b*). The value for benzyl alcohol is probably not significant.
‡ Unpublished results. § Bray *et al.* (1957). ‖ Betts *et al.* (1955).
¶ Only about 34 % of this compound is absorbed by the rabbit and 76 % by the rat.

Table 3. *Maximum rates of excretion of mercapturic acid by the rat*

Successive samples of urine were collected during the period from 2 to 6 hr. after dosage as described under Methods. Rates for this period are given as means with ranges in parentheses; superior figures indicate the number of experiments.

Precursor	Dose (μmoles/100 g.)	Rate (μmoles/100 g./hr.)
Chlorobenzene	169	3·5 (1·2–5·9)[6]
Benzyl chloride	158	5·8 (3·5–7·8)[6]
p-Chlorobenzyl chloride	124	2·9 (1·8–3·8)[6]
1-Bromobutane	157	4·9 (2·3–7·4)[6]
3:4-Dichloronitrobenzene	130	1·0 (0·4–1·9)[6]
3:4-Dichloronitrobenzene	182	2·1 (1·1–2·9)[6]
3:4:5-Trichloronitrobenzene	110	2·0 (0·9–2·5)[6]
2:3:4:6-Tetrachloronitrobenzene	144	3·2 (2·6–4·3)[5]

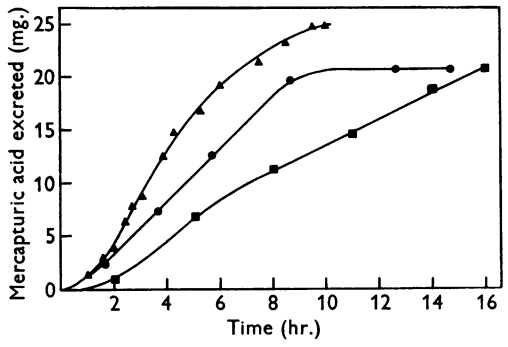

Fig. 1. Excretion of mercapturic acid by the rat. The dose was given at 0 hr. and water (3 ml.) was given 2-hourly. Doses were: ▲, benzyl chloride, 158 μmoles/100 g.; ●, 3:4:5-trichloronitrobenzene, 110 μmoles/100 g.; ■, 3:4-dichloronitrobenzene, 130 μmoles/100 g.

pronounced effect on the relative proportions of GSH and GSSG. If the rats were killed immediately after water had been given, the GSH level was about half the amount found when rats which had not been given water were killed; there was a corresponding rise in the GSSG level and the total glutathione was approximately constant.

When mercapturic acid precursors were administered to rats there was usually a significant fall in the glutathione levels which reached a minimum value with most compounds about 4 hr. after the administration of the dose. This is most clearly seen where the dose of precursor exceeded 130 μmoles/100 g. The results with doses below 130 μmoles/100 g. are less striking, although significant for at least one time period. The effect of dose level is seen with 3:4-dichloronitrobenzene, with which for a dose of 130 μmoles/100 g. there is a marked fall in GSH level only at 2 hr. after dosage; at 180 μmoles/100 g. the fall in GSH level is pronounced even 6 hr. after dosage. The fall in glutathione levels is particularly great with benzyl chloride and chlorobenzene. In most experiments the glutathione content after 6 hr. suggests the beginning of a return towards the normal value. After about 10 hr. the excretion of mercapturic acid is usually approaching completion (see Fig. 1),

Table 4. *Effect of mercapturic acid precursors on levels of glutathione in rat liver*

Doses suspended in 3 ml. of water were administered at 0 hr. to rats which had fasted for 19 hr. Results are expressed as means with ranges in parentheses; the superior figure indicates the number of experiments. The total glutathione values from dosed animals were all significantly lower by *t* test than the corresponding control values, except those for benzyl alcohol (*P* 0·2 and 0·3 for 2 and 6 hr. respectively) and butyl chloral hydrate (*P* 0·2 for 0·5 hr.). The water control values for 0·5, 2, 4 and 6 hr. against 0 hr. gave *P* 0·2, 0·2, 0·5 and 0·1 respectively. The 6 hr. values for bromobutane and 3:4-dichloronitrobenzene gave *P* 0·05 and 0·02.

Compound administered	Dose (μmoles/100 g. of rat)		Glutathione (mg./100 g. of liver) after 0 hr.	0·5 hr.	2 hr.	4 hr.	6 hr.
None	—	GSH	122 (114–134)[4]	—	—	—	—
		GSSG	48 (39–61)[4]	—	—	—	—
Water only	—	GSH	79 (59–112)[4]	120 (113–139)[6]	163 (139–181)[6]	140 (117–149)[6]	133 (113–169)[12]
		GSSG	110 (91–143)[4]	53 (47–57)[6]	14 (0–39)[6]	35 (23–49)[6]	34 (25–39)[4]
Chlorobenzene	169	GSH	—	—	52 (37–75)[6]	23 (13–28)[4]	21 (16–32)[4]
		GSSG	—	—	14 (0–23)[5]	8 (6–13)[4]	5 (2–6)[4]
Benzyl chloride*	158	GSH	—	38 (11–63)[11]	16 (0–35)[11]	20 (0–49)[11]	27 (10–57)[5]
		GSSG	—	21 (4–35)[5]	4 (0–10)[6]	15 (0–28)[5]	—
p-Chlorobenzyl chloride	124	GSH	—	—	105 (63–196)[7]	70 (30–136)[8]	100 (60–151)[5]
1-Bromobutane	157	GSH	—	—	67 (52–86)[4]	52 (32–74)[4]	84 (55–127)[6]
		GSSG	—	—	18 (7–32)[4]	20 (8–29)[4]	27 (13–38)[6]
1-Nitrobutane	165	GSH	—	—	—	63 (36–81)[4]	—
		GSSG	—	—	—	38 (26–54)[4]	—
3:4-Dichloronitrobenzene	130	GSH	—	183 (165–195)[8]	95 (60–151)[8]	127 (60–148)[8]	137 (102–152)[5]
	182	GSH	—	—	80 (36–114)[6]	67 (34–99)[6]	89 (66–110)[4]
		GSSG	—	—	31 (17–50)[6]	22 (12–42)[5]	44 (35–53)[4]
3:4:5-Trichloronitrobenzene†	110	GSH	—	157 (114–175)[6]	118 (70–162)[6]	95 (70–115)[6]	85 (44–121)[6]
2:4:6-Trichloronitrobenzene	110	GSH	—	—	161 (130–199)[5]	140 (127–154)[6]	—
2:3:4:6-Tetrachloronitrobenzene	144	GSH	—	—	75 (58–89)[4]	40 (20–58)[4]	46 (20–56)[4]
		GSSG	—	—	22 (10–34)[4]	16 (10–18)[4]	17 (10–20)[4]
Benzyl alcohol	171	GSH	—	—	114 (108–121)[4]	102 (87–111)[6]	96 (80–110)[6]
		GSSG	—	—	44 (37–49)[4]	33 (23–42)[4]	53 (31–65)[6]
Butyl chloral hydrate	65	GSH	—	124 (102–139)[6]	200‡ (148–274)[6]	—	—
		GSSG	—	35 (29–46)[6]	—	—	—

* The GSH values after 10 hr. were 70 (40–98)[6].

† The values after 18 hr. were 186 (180–190)[3].

‡ After 1 hr. This value is significantly higher than the control 0·5 hr. value for GSH (*P* 0·01) but not significantly different from the 2 hr. control for GSH (*P* 0·1) or the 0·5 hr. control for GSH + GSSG (*P* 0·2).

so that the period of decrease in the level of glutathione in liver is approximately coincident with the period of mercapturic acid excretion.

The amounts of glutathione found in the livers of rats given 2:4:6-trichloronitrobenzene were not significantly lower than those from undosed control animals. This compound forms only very small amounts of mercapturic acid (Table 2). Two compounds which are not mercapturic acid precursors are included in Table 4. So far as is known the metabolism of benzyl alcohol does not involve any reaction with a cysteine derivative. Of the results with this compound for total glutathione in Table 4 only those for 4 hr. are significantly lower (P 0·01) than those for the controls. Butyl chloral hydrate was chosen because, although it does not form mercapturic acid (Table 2), it is a compound which readily loses chloride and probably combines with –SH compounds in rabbit-liver extracts to an extent comparable with that for benzyl chloride (Bray, Thorpe & Vallance, 1952). It is doubtful whether this compound decreases the level of glutathione, but because of its anaesthetic properties the dose given was much smaller than that for the other compounds.

Kidney and small intestine. The glutathione content of these tissues after rats had been dosed with 3:4:5-trichloronitrobenzene is shown in Table 5. There was no significant change in the GSH content of kidney and the decrease in small intestine was barely significant. When rats were given butyl chloral hydrate (65 μmoles/100 g.) the GSH values for these tissues 1 hr. after dosage were not significantly lower than those for undosed rats. Expressed as in Table 5 they were 49 (20–91)[6] for kidney and 44 (36–49)[6] mg./100 g. for intestine.

Blood. The levels of glutathione observed in rat blood are recorded in Table 6. A significant and rapid decrease in glutathione content was found in rats dosed with butyl chloral hydrate, but the decrease in those dosed with 3:4:5-trichloronitrobenzene was not significant.

The GSH levels in the blood of three rabbits dosed with 3:4:5-trichloronitrobenzene are given in Table 7. There was a slight decrease only in rabbit 2. In the other two and in a rabbit given water only, the level is approximately constant over 5 hr. The administration of a large dose of benzyl chloride to rabbit 6 caused no significant decrease in the total glutathione of the blood (Table 7).

When glutathione was added to the blood of a rat by continuous injection no significant difference in the rate of excretion of mercapturic acid was observed in several experiments. The results of a typical experiment are summarized in Fig. 2. For these experiments 3:4-dichloronitrobenzene was used because the excretion of mercapturic acid from this compound is relatively slow (see Table 3 and Fig. 1) and there was adequate time for establishing the rate of excretion before the injection of glutathione was started.

Table 5. *Effect of* 3:4:5-*trichloronitrobenzene on levels of glutathione in rat kidney and intestine*

3:4:5-Trichloronitrobenzene (110 μmoles/100 g. of rat) was administered to rats which had fasted for 19 hr. Results are expressed as means with ranges in parentheses; the superior figures indicate the number of experiments. Values for 0 hr. are for undosed rats.

Time after dosage (hr.)	GSH (mg./100 g. of tissue)		
	Kidney	Small intestine	P for intestine
0	46 (23–65)[6]	46 (39–54)[6]*	—
0·5	40 (31–52)[6]	38 (31–46)[6]	0·05
1·0	—	38 (29–46)[8]	0·02
2·0	54 (45–78)[6]	42 (38–47)[6]	0·2
4·0	34 (25–56)[6]	37 (25–44)[6]	0·05
6·0	39 (27–55)[6]	40 (33–50)[6]	0·1
18·0	47 (39–55)[3]	52 (45–58)[3]	0·2

* Values for undosed animals which had fasted for 22 hr. and 25 hr. were 46 (40–55)[8] and 43 (38–47)[8] respectively.

Table 6. *Effect of* 3:4:5-*trichloronitrobenzene and butyl chloral hydrate on levels of glutathione in rat blood*

Rats were killed at times indicated. Results are expressed as means with ranges in parentheses; superior figures indicate the number of rats used. Values for 0 hr. are for undosed control rats.

Compound administered ...	3:4:5-Trichloronitrobenzene		Butyl chloral hydrate	
Dose (μmoles/100 g.) ...	110		65	
Time after dosage (hr.)	GSH (mg./100 ml.)	P	GSH (mg./100 ml.)	P
0	49 (38–65)[12]	—	49 (38–65)[12]	—
0·5	43 (42–46)[3]	0·2	34 (17–47)[5]	0·01
1·0	—	—	27 (21–35)[6]	<0·01
2·0	43 (41–45)[6]	0·1	25 (8–33)[4]	<0·02
4·0	43 (40–44)[3]	0·2	—	—
6·0	48 (40–60)[3]	0·8	—	—

Table 7. *Effect of mercapturic acid precursors on levels of glutathione in the blood of rabbits*

Doses were given to rabbits from which food had been withheld for 18 hr. Blood samples were withdrawn at the times indicated after dosage. Values for 0 hr. are for blood taken immediately before administration of the dose.

	Water	3:4:5-Trichloronitrobenzene (88 μmoles/100 g.)	Water		Benzyl chloride (250 μmoles/100 g.)	
	GSH	GSH	GSH	GSSG	GSH	GSSG
Rabbit ...	1	2, 3, 4	5	5	6	6
Time after dosage (hr.)						
0	60	49, 42, 42	35	8	16	14
0·25	61	54, 51, 44	—	—	—	—
0·5	58	45, 47, 42	26	11	23	10
1·0	—	42, 44, 42	—	—	—	—
1·5	54	40, 46, 42	—	—	—	—
1·75	—	— — —	25	19	17	13
2·0	—	39, 46, 42	—	—	—	—
3·0	59	38, 42, 42	—	—	—	—
3·75	—	— — —	30	9	24	18
4·0	54	37, 40, 42	—	—	—	—
5·0	58	36, 40, 43	—	—	—	—
5·5	—	— — —	34	6	24	10
6·75	—	— — —	32	11	25	9

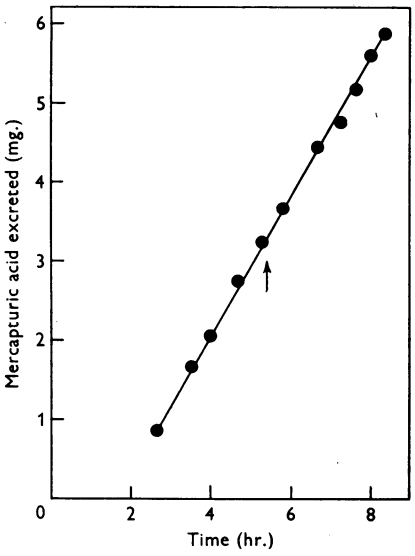

Fig. 2. Effect of injection of glutathione upon the rate of excretion of mercapturic acid by the rat. The rat was given 75 mg. of 3:4-dichloronitrobenzene in 3 ml. of water by stomach tube. Isotonic sodium chloride solution was then injected continuously into the caudal vein. At the time indicated by the arrow the injection fluid was changed to isotonic sodium chloride solution containing 1 mg. of GSH/ml. The injection rate was 3·3 ml./hr. over the period shown. The GSH equivalent to the mercapturic acid formed is 0·87 mg./hr.

DISCUSSION

It is clear from Table 2 that the rat can form mercapturic acids in effect by acetylcysteyldehalogenation and acetylcysteyldenitration from either aromatic precursors (types II*a* and III*a*) or aliphatic ones (types II*b* and III*b*) in amounts similar to those formed by the rabbit. The most conspicuous difference is that the rat excretes much less mercapturic acid from 3:4-dichloronitrobenzene than does the rabbit. For some reason rats seem to excrete this mercapturic acid relatively slowly (see Fig. 1).

No significant decrease in the GSH levels in blood was detected after the administration of 3:4:5-trichloronitrobenzene to rats or rabbits, nor was there any significant change in either GSH or GSSG levels in blood after the administration of benzyl chloride to rabbits. Furthermore, when glutathione was injected into the blood stream of the rat there was no detectable increase in the rate of excretion of mercapturic acid from 3:4-dichloronitrobenzene. The GSH content of rat blood was, however, considerably decreased after the administration of butyl chloral hydrate, which is not a mercapturic acid precursor. No change in the GSH level was detected in the kidneys of rats dosed with 3:4:5-trichloronitrobenzene, and in the intestine there was a slight decrease of doubtful significance. The intestine was examined because Anderson & Mosher (1951) found that a rapid synthesis of glutathione occurred in the intestinal mucosa of rats and it seemed possible that the mercapturic acid precursor would encounter this glutathione and react with it. The results in Table 5 suggest that if such a reaction occurs it can do so to only a very small extent.

Table 4 shows that when mercapturic acid precursors of types I, II and III are administered there is a fall in the level of liver glutathione which is most marked with benzyl chloride. As in general

there was no significant increase in GSSG the observed fall seems to be a true reduction in the concentration of GSH caused by the administered compound. This fall may be the result either of a direct combination of glutathione with the mercapturic acid precursor or of a secondary effect following the depletion of the cysteine in the animal as the mercapturic acid is formed. Binet & Wellers (1951) gave bromobenzene to rats and observed a slight rise in the level of total glutathione in the liver during the first hour after dosage and then a fall to about 40% of the initial level which persisted for about 48 hr. There followed on the third day a recovery to a level about 160% of the initial one and this gradually declined over nine days towards the initial level. They obtained similar results with kidney. The dose given by Binet & Wellers was 1900 μmoles/100 g. subcutaneously to 150 g. well-fed rats, whereas our dosage of chlorobenzene was 169 μmoles/100 g. orally to 200 g. fasting rats. Our dose caused a fall in the level of total glutathione to 15% of the initial value. The dose given by Binet & Wellers was so large as to produce serious toxic effects since they state that after 6–10 days 'la mortalité...devient sensible'. At our dose levels the animals showed no toxic symptoms and there was no mortality amongst those used only for collection of urine. The most conspicuous difference in results, apart from the more prolonged effect of the dose given by Binet & Wellers, was that in our experiments no significant change in the level of glutathione in the kidney was observed. In general our experiments were not continued beyond 6 hr. after dosage, but with 3:4:5-trichloronitrobenzene we did note that 18 hr. after dosage the GSH level in liver was higher than the initial level (see Table 4).

Simkin & White (1957) have shown that the turnover rate for free glycine in liver can be calculated from the depletion of free glycine in the liver and the rate of excretion of hippuric acid after the administration of the hippuric acid precursor, benzoic acid, by the formula $\mathbf{v} = v_h g_1/(g_1 - g_2)$, where $\mathbf{v}$ is the turnover rate, v_h the constant rate of formation of hippuric acid and g_1 and g_2 the steady free-glycine levels in the liver before and after the dose. If the liver glutathione is the immediate source of cysteine for mercapturic acid formation, then a similar formula, $\mathbf{v} = v_m g_1/(g_1 - g_2)$, where v_m is the constant rate of formation of mercapturic acid and g_1 and g_2 are the steady glutathione levels in the liver before and after the dose, would be expected to yield results for the turnover rate for glutathione in the liver comparable with the value found by other methods. For calculation of the turnover rates given in Table 8 the lowest values of Table 4 were used for g_2 and the corresponding control values for g_1. At these times the rate of excretion of the mercapturic acid was maximum and constant (Fig. 1). The mean turnover rates for GSH and for GSH + GSSG were 42 and 43 mg./100 g. of liver/hr. respectively (= 4·4 and 4·5 mg. of S/100 g./hr.).

The turnover rate for GSH in rabbit and rat liver was investigated by Waelsch & Rittenberg (1942) with [^{15}N]glutamic acid. They calculated a half-life period of 2–4 hr. Anderson & Mosher (1951) estimated that maximum incorporation of ^{35}S from [^{35}S]cystine into liver GSH in the rat took place in less than 3 hr. From the formula $t^{\frac{1}{2}} = 0{\cdot}7\ g/\mathbf{v}$ the turnover rates for GSH from Table 5 correspond with half-life periods of 2·1–4·0 (mean 3·1) hr. and those for GSH + GSSG with 2·0–3·8 (mean 3·1) hr., which are in good agreement with the results of Waelsch & Rittenberg (1942). For these calculations g, the normal level of glutathione, has been taken as 176 mg./100 g. of liver, the mean of the control values of Table 4. Although the values obtained for turnover rates cannot be regarded as precise they provide, in the absence of more precise data, a reasonable basis for the conclusion that the turnover rate for glutathione in liver would be sufficient to sustain the synthesis of the mercapturic acids. It should, however, be mentioned that Henriques *et al.* (1955) estimated that the half-life for glutathione in the rabbit was 7–9 hr., indicating a much slower turnover rate than that given by Waelsch & Rittenberg (1942), but Henriques *et al.* (1955) did not investigate the turnover rate in rats.

Butyl chloral hydrate did not significantly affect the level of glutathione in liver but caused a considerable fall in the level of glutathione in blood. The effect of butyl chloral hydrate therefore does not resemble that of a mercapturic acid precursor.

Table 8. *Turnover rates for glutathione in rat liver calculated from falls in level during formation of mercapturic acids*

The turnover rates $\mathbf{v}$ have been calculated from the formula $\mathbf{v} = v_m g_1/(g_1 - g_2)$. Values used for v_m are from Table 3, those for g_2 are the lowest values from Table 4 and those for g_1 those for the controls (water only) corresponding to the time period of the value taken for g_2. It has been assumed that the liver is 4% of the body weight.

Precursor	Turnover rate (mg./100 g. of liver/hr.) calculated from: GSH	GSH + GSSG
Chlorobenzene	32	32
Benzyl chloride	49	50
p-Chlorobenzyl chloride	45	—
1-Bromobutane	59	63
3:4-Dichloronitrobenzene	31	33
3:4:5-Trichloronitrobenzene	42	—
2:3:4:6-Tetrachloronitrobenzene	34	36

Benzyl alcohol, however, caused a significant fall in the liver glutathione in 4 hr., with a recovery to the normal level in 6 hr. This may be due to the utilization of glycine for conjugation with the benzoic acid formed by oxidation from the alcohol, since almost the whole of a dose of benzyl alcohol is excreted by the rat as hippuric acid (unpublished result). 2:4:6-Trichloronitrobenzene, which forms neither a mercapturic acid to any appreciable extent nor a glycine conjugate, does not affect the level of glutathione in the liver. The results in this paper suggest that, when the mercapturic acids are formed from precursors, there is a fall in the level of glutathione in the liver, but not in blood, kidney or intestine, which is roughly commensurate with the amount of mercapturic acid formed. This suggests that an early stage in the formation of mercapturic acid might be the combination of the precursor with glutathione in the liver. The possibility that the mercapturic acid precursor combines with some other cysteine-containing compound, which breaks down to form the substituted cysteine and is itself reconstituted at the expense of cysteine from glutathione, has not been excluded.

SUMMARY

1. The possibility that glutathione may be involved in the formation of mercapturic acids *in vivo* has been examined.

2. The levels of glutathione in the liver fell when mercapturic acid precursors were given to rats; this fall was roughly commensurate with the amount of mercapturic acid formed.

3. No decrease in the glutathione levels in blood, kidney and intestine were observed in rats given mercapturic acid precursors. There was no significant change in rabbit blood under the same conditions.

4. The injection of glutathione into the caudal vein of the rat did not affect the rate of excretion of mercapturic acid.

5. Values are given for the amounts of mercapturic acid excreted by rats given various mercapturic acid precursors.

6. Turnover rates for liver glutathione calculated from the maximum rates of excretion of mercapturic acids and the levels of liver glutathione before and after administration of precursors are in agreement with those reported by other workers.

7. Butyl chloral hydrate, which is not a mercapturic acid precursor, caused no fall in the level of glutathione in liver but a pronounced fall in the level in blood.

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The Formation of Mercapturic Acids

2. THE POSSIBLE ROLE OF GLUTATHIONASE

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Barnes, James & Wood (1959) reported results which could be explained by the hypothesis that the first stage in the overall process of the formation of mercapturic acid from a precursor *in vivo* is the formation of an *S*-substituted glutathione from glutathione in the liver. If such a compound is an intermediate in the formation of mercapturic acid, it would be expected that enzymes of the liver would convert it into mercapturic acid. The effect of liver preparations on a conjugate of this type has, therefore, been examined. The compound chosen for study was *S*-(*p*-chlorobenzyl)glutathione, which may be an intermediate in the formation of *N*-acetyl-*S*-(*p*-chlorobenzyl)-L-cysteine from *p*-chlorobenzyl chloride *in vivo*. The percentage of a dose of *p*-chlorobenzyl chloride converted into this mercapturic acid in the rabbit is 23 (Bray, James & Thorpe, 1958*b*) and in the rat 37 (Barnes *et al.* 1959); so that livers of these animals would be expected to contain the necessary enzymes in sufficient amounts. The subsequent stages envisaged were the breakdown of the *S*-(*p*-chlorobenzyl)glutathione to yield *S*-(*p*-chlorobenzyl)-cysteine and the acetylation of *S*-(*p*-chlorobenzyl)-cysteine to yield *N*-acetyl-*S*-(*p*-chlorobenzyl)-L-cysteine. This paper is concerned with the breakdown of the glutathione complex. This might be achieved either by a hydrolytic reaction, e.g.

S-(*p*-chlorobenzyl)glutathione → glutamic acid + *S*-(*p*-chlorobenzyl)cysteine + glycine (1)

or by a transfer reaction, e.g.

S-(*p*-chlorobenzyl)glutathione + cysteine → glutathione + *S*-(*p*-chlorobenzyl)cysteine (2)

If the mechanism is a hydrolytic one, it might be catalysed by glutathionase, which is known to occur in liver (Neubeck & Smythe, 1944; Johnston & Bloch, 1951). A similar suggestion has been made by Binet & Wellers (1951).

EXPERIMENTAL

Materials and Methods

All melting points are uncorrected. Elementary microanalyses were carried out by F. and E. Pascher, Bonn.

Chlorobenzene, benzyl chloride, *p*-chlorobenzyl chloride, 1-bromobutane, 3:4-dichloronitrobenzene and 2:3:5:6-tetrachloronitrobenzene were purchased. 2:3:4:6-Tetrachloronitrobenzene was prepared as described by Betts, James & Thorpe (1955).

S-(p-*Chlorobenzyl)glutathione*. This was prepared from glutathione (cf. Stekol, 1941). A solution of glutathione (Boehringer und Söhne, G.m.b.H., Mannheim) (0·43 g. in 100 ml. of water) was made just alkaline to litmus with 2·5N-NaOH. Then 5 ml. of 2·5N-NaOH and 2 ml. of an ethanolic solution of *p*-chlorobenzyl chloride (0·8%) were added. Alternate additions of alkali and chloride solution (2 ml. of each) were made, with shaking, until 20 ml. more of each had been added over 15 min. Excess of chloride was removed by extraction of the reaction mixture with ether and the aqueous phase was acidified to pH 6·0 with 2N-HCl and left at 0° overnight. The granular precipitate was filtered off and the filtrate was concentrated at 50°