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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°

under reduced pressure to yield more solid material. The two solid fractions were combined and recrystallized from water. This gave 250 mg. of *S*-(*p*-chlorobenzyl)glutathione as a white amorphous powder which darkened progressively without melting on heating to 200° (Found: C, 47·5; H, 5·7; N, 9·3; Cl, 8·0; S, 7·6. $C_{17}H_{22}O_6N_3ClS$ requires C, 47·3; H, 5·1; N, 9·7; Cl, 8·2; S, 7·4%). After hydrolysis for 12 hr. with 6N-HCl at 100° it yielded *S*-(*p*-chlorobenzyl)-L-cysteine, glutamic acid and glycine, which were identified by paper chromatography as described below. *S*-(*p*-Chlorobenzyl)glutathione was also obtained from concentrated solutions of glutathione prepared from baker's yeast by the method of Schroeder, Collier & Woodward (1939). The yield was about 0·5 g. of *S*-(*p*-chlorobenzyl)glutathione from 2 lb. of yeast.

S-(p-*Chlorophenyl*)*glutathione*. This was prepared from diazotized *p*-chloroaniline and the cuprous mercaptide of glutathione by a method similar to that of Parke & Williams (1951) for phenylcysteines, except that the reaction mixture was kept overnight in the refrigerator. From 1 g. of glutathione 325 mg. of *S*-(*p*-chlorophenyl)-glutathione, m.p. 218° (decomp.), was obtained as a white amorphous powder (Found: C, 46·6; H, 5·3; N, 9·7; Cl, 8·4; S, 7·6. $C_{16}H_{20}O_6N_3ClS$ requires C, 46·0; H, 4·8; N, 10·0; Cl, 8·5; S, 7·7%).

S-(*p*-Chlorobenzyl)-L-cysteine, m.p. 202°, was prepared by a modification (Bray *et al.* 1958*b*) of the method of Suter (1895). *S*-(*p*-Chlorophenyl)-L-cysteine, m.p. 193°, was prepared by the method of Parke & Williams (1951).

Buffers. The following buffers were used. The pH was checked on a Cambridge pH meter with glass electrode. *For tissue slices*. Ringer–phosphate solution, pH 7·4, was prepared according to Umbreit, Burris & Stauffer (1949). *For homogenates*. 0·1M-Sodium phosphate buffers, pH 7·0 and pH 8·0, were as described by Britton (1932); sodium phosphate buffer with KCl + $MgSO_4$ was prepared according to Johnston & Bloch (1951), except that the sodium phosphate buffer used had pH 7·0 or 8·0 instead of 7·4.

Animals and tissues. Rats (about 200 g.), rabbits (about 2·5 kg.) and guinea pigs (about 500 g.) were killed by a sharp blow on the back of the head. The tissues were quickly removed and rapidly cooled in crushed ice. Slices (0·2–0·4 mm. thick) were cut by hand and immediately transferred to ice-cold Ringer–phosphate solution. Homogenates were prepared from 0·5 g. of tissue and 1 ml. of ice-cold buffer in a Potter–Elvehjem-type homogenizer with a Teflon pestle. The mixture was centrifuged at 2000 *g* for about 2 min. to remove larger particles and facilitate pipetting. The supernatant (homogenate) was added to an equal volume of a solution of the substrate in the chosen buffer in 100 ml. conical flasks fitted with a rubber bung with inlet and outlet tubes. The mixture was 'gassed' and shaken in a Warburg tank at 37° in an atmosphere of $O_2 + CO_2$ (19:1) or N_2 as appropriate.

The guinea pigs (about 500 g.) used for studying the excretion of mercapturic acid were maintained on a diet of rabbit pellets and water *ad lib.* during the experimental period. The compounds were administered with water by stomach tube. No toxic effects were observed except that 3:4-dichloronitrobenzene caused anorexia.

Glutathionase preparation. This was prepared from hog kidney which was frozen as soon as possible after the death of the animal and treated as described by Binkley & Olson (1951). Contrary to the experience of these workers it was found that reprecipitation of the enzyme reduced its activity. The preparation was, therefore, dried after the first precipitation with sodium acetate buffer (pH 4·0).

Determination of metabolites

Reduced glutathione. The method of Bhattacharya, Robson & Stewart (1955), depending on the activation of yeast glyoxalase by glutathione, was used.

Free glycine. The modification of Christensen, Riggs & Ray (1951) of the ninhydrin method of Alexander, Landwehr & Seligman (1945) was used.

Mercapturic acid in urine. *N*-Acetyl-*S*-(2-chloro-4-nitro)phenyl-L-cysteine was determined by the colorimetric method of Bray, James & Thorpe (1956). *N*-Acetyl-*S*-benzyl-L-cysteine, *N*-acetyl-*S*-(*p*-chlorobenzyl)-L-cysteine and *N*-acetyl-*S*-butyl-L-cysteine were determined by the nitroprusside method described by Bray *et al.* (1958*b*). *N*-Acetyl-*S*-(*p*-chlorophenyl)-L-cysteine, *N*-acetyl-*S*-(2:3:4:6-tetrachlorophenyl)-L-cysteine and *N*-acetyl-*S*-(2:3:5:6-tetrachlorophenyl)-L-cysteine were determined by the modification (Betts *et al.* 1955) of the iodometric titration method of Stekol (1936).

Determination of glutathionase activity of small intestine

The small intestine was excised from a rat immediately after death, and its contents were removed. The intestine was cut into small pieces and ground with sand in 25 ml. of phosphate buffer (pH 8·0). The mixture was then centrifuged for 15 min. at 2000 *g*. For assay of glutathionase, the supernatant (5 ml.) and substrate (30 μmoles in 5 ml. of water) were incubated at 37° with shaking for 4 hr. Then 10% (w/v) $ZnSO_4,7H_2O$ (5 ml.) and 0·5N-NaOH (5 ml.) were added. The mixture was filtered and the filtrate was diluted tenfold with water for determination of glycine.

Paper chromatography

Centrifuged homogenates were applied to the paper. Phenol red, phenylalanine and tyrosine were used as markers with the monophasic solvent mixture given in Table 1, which gives for the compounds examined the rates of flow, R_P, relative to phenol red. In addition to R_P values, *S*-(*p*-chlorobenzyl)glutathione and *S*-(*p*-chlorobenzyl)-L-cysteine were readily distinguished by the different colours given with the ninhydrin reagent. The corresponding *p*-chlorophenyl compounds could not be so distinguished. The approximate amounts of *S*-(*p*-chlorobenzyl)-L-cysteine and *S*-(*p*-chlorophenyl)-L-cysteine liberated were estimated by comparison of the areas and intensities of the spots obtained. The extracts were applied from an Agla syringe. The minimum amount of each compound which could be detected was about 5 μg.

RESULTS

Liberation of S-(p-*chlorobenzyl*)-L-*cysteine from* S-(p-*chlorobenzyl*)*glutathione*

In liver slices. Liver slices were incubated with 0·06M-*S*-(*p*-chlorobenzyl)glutathione in Ringer–phosphate buffer (pH 7·4) for 4 hr. at 37° with gas phase $O_2 + CO_2$ (19:1). After incubation the

medium, or a homogenate of the slices in the medium, was examined for *S*-(*p*-chlorobenzyl)-cysteine by paper chromatography. This compound was detected in the reaction mixture from slices of guinea-pig liver but not in those from rat liver. As shown below, rat liver is less active than guinea-pig liver in liberating *p*-chlorobenzylcysteine from the *p*-chlorobenzylglutathione.

In homogenates of rat liver. Rat-liver homogenates were incubated with *S*-(*p*-chlorobenzyl)-glutathione in buffer containing adenosine triphosphate (ATP) and L-cysteine. The results are shown in Table 2. Chromatographic analysis of the supernatant from the centrifuged digests showed the presence of *S*-(*p*-chlorobenzyl)-L-cysteine. Boiled homogenates gave negative results. If the mechanism of the process involves the transfer of the *p*-chlorobenzyl residue from the glutathione complex to cysteine, glutathione should be formed as the reaction proceeds (see equation 2). The digests contained L-cysteine as a possible acceptor for the *p*-chlorobenzyl residue in such a reaction. In two homogenates a 90–100 % fall in the level of glutathione occurred in both controls and experiments. While these results did not indicate a net formation of glutathione, there might have been formed a small amount of glutathione, which was masked by a relatively large loss due to other reactions, e.g. hydrolysis or oxidation. Attempts were made to eliminate the effect of oxidation by using a gaseous phase of pure nitrogen, but a considerable disappearance of glutathione still occurred in the absence of *S*-(*p*-chlorobenzyl)glutathione; this might have been due to hydrolysis by a glutathionase system similar to that described by Binkley & Olson (1951) and Johnston & Bloch (1951). Experiments were carried out with a medium containing, in addition to the usual constituents, glutathione, which is reported by

Table 1. *Paper chromatography of* S-(p-*chlorobenzyl*)-L-*glutathione and reference compounds*

The descending method was used with phenol red as marker. The solvent mixture was *n*-butanol–water–acetic acid–butyl acetate (24:10:5:2, by vol.) and the detecting reagent 0·1 % ninhydrin in acetone containing 1 drop of collidine/100 ml. (Woiwod, 1949). The time of run was 16 hr., by which time the solvent front had moved off the paper. Rates of flow, R_P, are expressed relative to that of phenol red taken as 1·0.

Compound	R_P	Colour of detected spot
Glycine	0·30	Purple
L-Glutamic acid	0·43	Purple
L-Tyrosine	0·73	Purple
DL-Phenylalanine	0·87	Purple
Phenol red	1·0	Yellow
S-(*p*-Chlorobenzyl)glutathione	1·12	Purple
S-(*p*-Chlorobenzyl)-L-cysteine	1·31	Blue
S-(*p*-Chlorophenyl)glutathione	1·05	Purple
S-(*p*-Chlorophenyl)cysteine	1·22	Purple
Glutathione,* GSH	0·21	Purple
GSSG	0·07	Purple
	0·36	Purple

* Glutathione gives a purple colour with ninhydrin reagent when about 100 μg. is applied to the paper. (The other compounds listed above can be detected in amounts of about 5 μg.) The GSH used gave a faint spot (probably GSSG), R_P 0·07. The main spot from GSSG had R_P 0·07. The GSH spot (R_P 0·21) first appeared as a purple ring with a colourless centre, but after several hours the whole spot became purple.

Table 2. *Changes in glutathione levels and liberation of* S-(p-*chlorobenzyl*)-L-*cysteine from* S-(p-*chlorobenzyl*)*glutathione*

Medium: sodium phosphate buffer (pH 8·0 unless stated otherwise) with KCl and $MgSO_4$ to which was added, for each millilitre, L-cysteine hydrochloride (2·5 mg.), ATP (2·5 mg.) and, where indicated, *S*-(*p*-chlorobenzyl)glutathione (CBG) (2·5 mg.) or glutathione (GSH) (0·5 mg.). The homogenate was mixed with an equal volume of medium and incubated at 37° with continuous shaking. Values are means of duplicate determinations in close agreement.

Additions	Time of incubation (hr.)	Percentage fall in level of GSH	Relative intensity of *S*-(*p*-chlorobenzyl)-L-cysteine spot on chromatogram
	Gas phase: O_2+CO_2 (19:1)		
None	4	100, 90	—
CBG	4	90, 90	+ +, + +
	Gas phase: N_2		
None	4	80	—
CBG	4	85	+ +
CBG (pH 7·0)	4	75	+
CBG + GSH	4	20	Trace
CBG + GSH (pH 7·0)	4	18	-
None	2	40, 40	—
GSH	2	35, 25	—
GSH + CBG	2	27, 24	-, -
GSH (pH 7·0)	2	16, 12	—
GSH + CBG (pH 7·0)	2	16, 14	-, -

Johnston & Bloch (1951) to stimulate the glutathione-synthesizing systems in pigeon-liver homogenates and by Binkley & Olson (1951) to inhibit glutathionase in hog kidney. The reaction was studied also at pH 7·0, at which, according to Binkley & Olson (1951), the activity of the glutathionase system is diminished. Evidence of glutathione synthesis was not obtained, so that a transfer mechanism seemed unlikely. In experiments where the fall in the level of glutathione was diminished significantly, the release of *S*-(*p*-chlorobenzyl)-L-cysteine was also diminished and was often negligible. The addition of glutathione to the digests and the use of pH 7·0 diminished the fall in the level of glutathione. It seemed, therefore, likely that the disappearance of glutathione could be attributed to the action of glutathionase and that the appearance of *S*(*p*-chlorobenzyl)-L-cysteine was the result of a hydrolytic process.

In liver homogenates of guinea pig and rabbit. The results are given in Table 3. Advantage was taken of the fact that glutathionase is inhibited by phenol red and bromosulphalein (Binkley & Olson, 1951). The results show that these two compounds inhibit both the fall in the level of glutathione and the release of *S*-(*p*-chlorobenzyl)-L-cysteine in the preparations used. This observation provides further evidence that glutathionase may be involved in the formation of *S*-(*p*-chlorobenzyl)-L-cysteine from *S*-(*p*-chlorobenzyl)glutathione.

Table 3. *Effect of glutathionase inhibitors on liberation of* S-(p-*chlorobenzyl*)-L-*cysteine from* S-(p-*chlorobenzyl*)*glutathione and on changes in glutathione levels in guinea-pig and rabbit-liver homogenates*

Medium: 0·1 M-sodium phosphate buffer (pH 8·0) to which was added, for each millilitre, *S*-(*p*-chlorobenzyl)-glutathione (2·5 mg.) and, where indicated, glutathione (GSH) (0·5 mg.), phenol red (2 mg.) or bromosulphalein (2 mg.). The homogenate was mixed with an equal volume of medium and incubated with continuous shaking at 37° for 2 hr. Gas phase: N_2. Values are means of duplicate determinations.

Additions	Percentage fall in glutathione level	Intensity of *S*-(*p*-chlorobenzyl)-L-cysteine spot
	Guinea pig	
None	96	+ + +
GSH	96*	+ + +
Phenol red	26	+
Bromosulphalein	45	+
	Rabbit	
None	77	+ +
GSH	43	+
Phenol red	64	−
Bromosulphalein	69	−

* Guinea-pig liver is a richer source of glutathionase than is rabbit liver and this result presumably indicates that the amount of GSH added in this experiment was insufficient to inhibit the action of glutathionase.

Liberation of glycine from S-(p-*chlorobenzyl*)-*glutathione by liver homogenates*

Although paper chromatography gave satisfactory comparative estimates of the amounts of *S*-(*p*-chlorobenzyl)-L-cysteine liberated, a more accurate estimate of the breakdown of glutathione was desirable. If the formation of *S*-(*p*-chlorobenzyl)-L-cysteine involves complete hydrolysis of the tripeptide, glutamic acid and glycine should be liberated at the same time. According to Fodor, Miller & Waelsch (1953), free glutamic acid is not produced in homogenates, but is transferred to acceptors, e.g. other amino acids. Free glycine is released, however, and this can be readily determined quantitatively. The results obtained (Table 4) show that, with all three tissues, the conditions under which additional glycine is liberated are similar to those in which the liberation of *S*-(*p*-chlorobenzyl)-L-cysteine occurs. The relatively high control values are presumably due to the hydrolysis of glutathione present normally in the tissues. The inhibitory effect of phenol red again suggests the participation of glutathionase.

Two experiments were carried out with homogenates of guinea-pig liver and *S*-(*p*-chlorophenyl)-glutathione. Under the conditions described in

Table 4. *Effect of phenol red on liberation of glycine from* S-(p-*chlorobenzyl*)*glutathione by liver homogenates*

Medium: 0·1 M-sodium phosphate buffer (pH 8·0) to which was added, where indicated, for each millilitre, *S*-(*p*-chlorobenzyl)glutathione (CBG) (2·5 mg.) and phenol red (2 mg.). The homogenate was mixed with an equal volume of medium and incubated at 37° with continuous shaking for 4 hr. Gas phase: N_2. Values are means of duplicate determinations. Initial concentrations of glycine were 166–225 μg./ml. for guinea-pig liver, 48–62 for rat liver and 48 for rabbit liver.

Additions	Increase in glycine concentration (μg./ml.) over initial concentration		
	Guinea pig	Rat	Rabbit
None (control)	145, 111, 97	76, 39	65
CBG	262, 362, 270	153, 72	100
Phenol red	—, —, 67	—, 0	20
CBG + phenol red	—, —, 169	—, 5	40

Table 4 the control values were 72 and 67 and the values when *S*-(*p*-chlorophenyl)glutathione was added were 388 and 266 μg. of glycine/ml. respectively.

Glutathionase activity of small intestine

The extent of hydrolysis of glutathione and of *S*-(*p*-chlorobenzyl)glutathione by extracts of rat small intestine was determined from the glycine liberated. The results, expressed as percentage of substrate hydrolysed under the conditions given in the Experimental section, were 60 and 55 % for glutathione and 65 and 60 % for *S*-(*p*-chlorobenzyl)-glutathione. (Each value is the mean of duplicate determinations.) Examination of digests from *S*-(*p*-chlorobenzyl)glutathione by paper chromatography revealed the presence of *S*-(*p*-chlorobenzyl)-L-cysteine.

Table 5. *Liberation of glycine from glutathione and from* S-(p-*chlorobenzyl*)*glutathione by a preparation of glutathionase*

Substrates consisted of 10 ml. of 0·1 M-sodium phosphate buffer (pH 8·0) containing either 25 mg. of *S*-(*p*-chlorobenzyl)glutathione or 17·8 mg. of glutathione. Phenol red (20 mg.) and glutamine (20 mg.) were added as indicated. Tubes were incubated with 15 mg. of glutathionase preparation at 37°. Duplicate 1 ml. samples were withdrawn at intervals for analysis and the mean results are given. There was no free glycine at the start of any experiment. In the experiments with *p*-chlorobenzylglutathione additional samples were withdrawn and centrifuged. Portions (30 μl.) of the supernatant were spotted on sheets for analysis by paper chromatography.

Additions to substrate	Glycine liberated (μg./ml. of digest) 1 hr.	2 hr.	4 hr.	Relative intensity of *p*-chlorobenzyl-cysteine spot
	Glutathione			
None	38	64	113	–
Phenol red	38	48	62	–
Glutamine	54	140	257	–
	S-(*p*-Chlorobenzyl)glutathione			
None	78	153	265	+ + +
Phenol red	48	86	172	+
Glutamine	161	262	359	+ + + +

Experiments with a glutathionase preparation

The ability of the glutathionase preparation to liberate *S*-(*p*-chlorobenzyl)-L-cysteine from *S*-(*p*-chlorobenzyl)glutathione was first established by paper chromatography. For quantitative experiments, however, it was more satisfactory to assess its activity in terms of the liberation of glycine. Typical results are shown in Table 5. The inhibition by phenol red of the liberation of glycine from both glutathione and *S*-(*p*-chlorobenzyl)glutathione again supports the hypothesis that glutathionase is involved in mercapturic acid formation. The enhancement of the hydrolytic activity of glutathionase by glutamine is also very marked (Table 5). This property of glutathionase was noted by Binkley & Olson (1951), but we could not demonstrate it in slice and homogenate preparations, possibly because of the presence of glutaminase. It is of interest that *S*-(*p*-chlorobenzyl)-glutathione was more readily hydrolysed than is glutathione itself.

Formation of S-(p-*chlorobenzyl*)*glutathione from* p-*chlorobenzyl chloride* in vivo

A rabbit was given 0·5 g. of *p*-chlorobenzyl chloride and was killed 2 hr. later. The liver was removed and homogenized. The supernatant from the centrifuged homogenate was examined by paper chromatography with *S*-(*p*-chlorobenzyl)-cysteine and *S*-(*p*-chlorobenzyl)glutathione as reference compounds. In addition to slow running material, spots of R_F 1·1 and 1·3 corresponding to the two reference compounds in position and colour were observed. This result was obtained in several experiments on paper sheets, but attempts to achieve unequivocal identification by separation of these compounds on a larger scale on columns have so far been unsuccessful.

Excretion of mercapturic acids by the guinea pig

The amounts of mercapturic acid excreted by the guinea pig after administration of precursors are given in Table 6. The results are much lower than those obtained from the rat and rabbit under similar conditions (see Barnes *et al.* 1959).

Table 6. *Excretion of mercapturic acid by the guinea pig*

Results are expressed as percentages of the dose, given as means with ranges in parentheses; superior figures indicate the number of experiments. Samples of urine were collected until mercapturic acid could no longer be detected (usually 24 hr.).

Compound administered	Dose (μmoles/100 g.)	Mercapturic acid excreted
Chlorobenzene	200	<1 (<1, <1)[2]
Benzyl chloride	185	4 (2–6)[4]
p-Chlorobenzyl chloride	135	5 (0–8)[3]
1-Bromobutane	108	19 (11–23)[5]
3:4-Dichloronitrobenzene	104	3 (2–5)[5]
2:3:4:6-Tetrachloronitrobenzene	96	2 (0–4)[3]
2:3:5:6-Tetrachloronitrobenzene	96	0[3]

DISCUSSION

Experiments of earlier workers on the possible role of glutathione in the formation of mercapturic acids have been briefly reviewed by Barnes *et al.* (1959). Stekol (1940, 1941) showed that oral administration of *S*-benzylglutathione and *S*-(*p*-bromobenzyl)glutathione to rats led to the excretion of *N*-acetyl-*S*-benzyl-L-cysteine and *N*-acetyl-*S*-(*p*-bromobenzyl)-L-cysteine respectively in urine. Our results with rat intestine suggest that glutathione and its derivatives are likely to be extensively hydrolysed in the intestine before absorption and thus cast doubt on the value of Stekol's observations as evidence for the participation of glutathione in mercapturic acid formation. The results reported in this and the preceding paper (Barnes *et al.* 1959) are compatible with the view that stages in the formation of mercapturic acids *in vivo* are the combination of the precursor with glutathione in the liver and subsequent hydrolysis of the glutathione complex by glutathionase to *S*-substituted cysteine, glycine and glutamic acid. The existing evidence does not preclude the possibility that conjugation of a precursor of mercapturic acid may occur with a precursor of glutathione rather than with glutathione itself. Such a reaction could account for the failure of intravenously administered glutathione to increase the amount or rate of excretion of mercapturic acid (Barnes *et al.* 1959). Whether or not a mechanism of mercapturic acid formation involving glutathione is likely to be the only mechanism, applying to all types (see Barnes *et al.* 1959) of precursors of mercapturic acids, cannot yet be decided.

The results in the present paper are of particular interest in connexion with species differences. Neubeck & Smythe (1944) found that guinea-pig liver was a richer source of glutathionase than was rabbit or rat liver and gave the relative activities as 100:17:2–5, the last being within the experimental error of the determination. Binkley & Nakamura (1948) found that rat-liver homogenates were inactive in this respect. Our results confirm the observation of Neubeck & Smythe (1944) that guinea-pig liver is the richest in glutathionase activity, but indicate that the activity of rat liver is of the same order as that of rabbit and is certainly not negligible. The activity of rabbit and rat liver was such as might have been expected if glutathionase were involved in the formation of mercapturic acids *in vivo*; both animals excrete these acids readily when given precursors. On the same basis it would have been expected that the guinea pig would be at least as well equipped to form mercapturic acids. From the results in Table 6, however, it is clear that this is not generally true; the guinea pig in fact appears to excrete barely significant amounts of mercapturic acid from most of the precursors examined. This abnormality of the guinea pig has been observed by Corner & Young (1954), who isolated only 0·6% of naphthylmercapturic acid from the urine of guinea pigs to which naphthalene had been given. From the urine of rabbits, rats and mice similarly dosed the yields of naphthylmercapturic acid were 4, 12 and 6% respectively (Bourne & Young, 1934; Young, 1947). A possible explanation of this species difference would be that the failure of the guinea pig to excrete appreciable amounts of mercapturic acid is due, not to failure of glutathionase to hydrolyse the *S*-substituted glutathione, but to failure to acetylate the *S*-substituted cysteine liberated (Bray, Franklin & James, 1958*a*).

SUMMARY

1. The effect of liver preparations and of glutathionase on *S*-(*p*-chlorobenzyl)glutathione has been studied.

2. *S*-(*p*-Chlorobenzyl)glutathione was hydrolysed to *S*-(*p*-chlorobenzyl)cysteine by slices of guinea-pig liver, by homogenates of guinea-pig, rat and rabbit liver and by a preparation of glutathionase from hog kidney.

3. Glycine was liberated simultaneously and its determination provided a convenient method for the quantitative study of the reaction.

4. The results obtained are compatible with the hypothesis that the hydrolysis of *S*-(*p*-chlorobenzyl)glutathione to *S*-(*p*-chlorobenzyl)cysteine is a stage in the formation of mercapturic acid from *p*-chlorobenzyl chloride *in vivo*.

5. *S*-(*p*-Chlorobenzyl)glutathione was readily hydrolysed by extracts of rat small intestine.

6. Although guinea-pig liver is richer than is rabbit or rat liver in glutathionase, the guinea pig does not in general form as large amounts of mercapturic acid from various mercapturic acid precursors as do the other two species.

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Hormonal Factors Affecting Glucose 6-Phosphatase Activity

1. EFFECT OF HYPOPHYSECTOMY AND REPLACEMENT THERAPY IN THE RAT

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Cope & Marks (1934) observed that in hypophysectomized animals the liver glycogen appeared to be less responsive to glycogenolytic stimuli than that in normal animals. The present investigation was undertaken to ascertain whether in the hypophysectomized rat there is a diminution in liver glucose 6-phosphatase activity which might contribute to such a sluggishness in glycogenolysis, and further whether any diminution in glucose 6-phosphatase observed to follow removal of the pituitary gland in the rat could be prevented or cured by treatment with hormones.

METHODS

Animals. White rats of the Wistar strain, bred in the Laboratory, were used. Unless otherwise stated, they consumed a stock diet *ad lib.* to such an extent that their body weight rose by 35–40 g. each week. In some experiments pair-feeding was carried out. By this is meant that a pair of animals, one control and one experimental, chosen to be comparable in body weight, were fed the same amount of food each day. If one animal ate less than the other on any day, both were given the smaller amount the following day. In some experiments the principle of pair-feeding was extended to three or four comparable animals, the amount consumed by the animal eating least being fed to all.

Hypophysectomy was carried out by the parapharyngeal route. In any animal in which signs of incomplete hypophysectomy had been observed, such as incomplete cessation of growth or failure of the gonads to atrophy, completeness of removal of the gland was checked *post mortem* by visual examination of the sella turcica. If necessary the results for the animal were discarded. Alloxan diabetes was induced in intact rats by the intraperitoneal injection of 40 mg. of alloxan/kg. body wt. Only rats which exhibited frank glycosuria were used in the experiments. Some of the alloxan-diabetic rats were subsequently hypophysectomized.

Hormone preparations. Growth hormone was prepared in the Laboratory by a method essentially that of Wilhelmi, Fishman & Russell (1948). For injection it was dissolved in 0·9% NaCl soln. at a concentration of 5 mg./ml., and 0·2 ml. of this solution (1 mg. of hormone) was administered daily by the subcutaneous route to rats under treatment. Cortisol, suspended in 0·9% NaCl soln. at a concentration of 5 mg./ml., was administered subcutaneously at a rate of 1 mg./rat/day. Sodium-L-thyroxine was dissolved in 0·9% NaCl soln. by the addition of NaOH to give a concentration of 0·5 mg. of thyroxine/ml. Of this solution 0·2 ml. (equivalent to 0·1 mg.) was subcutaneously administered each day to a rat under treatment with this hormone.

Estimation of glucose 6-phosphatase. Glucose 6-phosphatase activity was measured in a tissue homogenate. The rat was stunned, decapitated and bled, and the needed organ removed and chilled in ice. A weighed sample of the organ (200–400 mg.) was homogenized in ice-cold 0·02M-acetate–borate–cacodylate buffer, pH 6·5 (Plumel, 1948; de Duve, Berthet, Hers & Dupret, 1949), in a Potter–Elvehjem glass homogenizer. Enzyme activity was determined by the method of de Duve *et al.* (1949) but with a final substrate concentration of 0·04M. The total volume of 0·6 ml. used

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