6. It is concluded that the results are consistent with the hypothesis that isolated rat-liver microsomes are able to synthesize albumin but that this albumin is not released in a soluble form.

We would like to thank Professor F. Dickens, F.R.S., and Dr R. Rendi for their interest and advice and Mr H. E. H. Jones for performing the partial hepatectomies and preparing the antiserum. Dr J. Kohn of Queen Mary's Hospital, Roehampton, also provided helpful advice about his method of electrophoresis. The work was made possible by a grant to this Medical School by the British Empire Cancer Campaign.

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Metabolism of Polycyclic Compounds

15. THE CONVERSION OF NAPHTHALENE INTO A DERIVATIVE OF GLUTATHIONE BY RAT-LIVER SLICES*

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(Received 22 June 1959)

Naphthalene is converted by animals into a compound which is believed to be *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-*L*-cysteine (scheme I, III), which gives rise to 1-naphthylmercapturic acid (VI) on acidification (Boyland & Sims, 1958). Knight & Young (1958) found that benzene, an-

thracene and the monohalogenobenzenes were probably converted into analogous derivatives. The mode of formation of these acids in the body has not yet been established, but they may arise by the reaction of intermediates (such as epoxides) with an SH-containing compound. In the formation of mercapturic acids, cysteine, glutathione and the cysteine SH of tissue protein have all been cited

* Part 14: Sims (1959).

from time to time by various workers as the source of the cysteine residues. This earlier work has recently been reviewed by Barnes, James & Wood (1959), who have found that when a number of compounds which are known to give rise to mercapturic acids are fed to rats, the levels of glutathione in the livers decrease in amounts roughly commensurate with the quantities of mercapturic acids formed. On the other hand, the glutathione levels in the blood, kidney and intestine are unaffected.

The present work, in which naphthalene has been incubated *in vitro* with rat-liver slices, has shown that a substance which is believed to be *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione (I) is formed.

METHODS AND MATERIALS

Melting points are uncorrected.

S-(1-Naphthyl)glutathione (IV). This compound was prepared by a modification of the method of Parke & Williams (1951). Glutathione (4 g.) (which was known to contain a little of the oxidized form) in water (100 ml.) and acetic acid (5 ml.) was warmed to 50–60° with zinc dust (3 g.) for 15 min. and then kept at room temperature with occasional shaking for 3 hr. Cuprous oxide was added until no more would dissolve and the solution was cooled to 0° and stirred while 1-naphthylamine (2 g.) diazotized in $N-H_2SO_4$

(50 ml.) with $NaNO_2$ (1 g.) was slowly added. The mixture was stirred at 0° for 1 hr. and at room temperature for 1 hr. and was then kept overnight at 0°. The brown precipitate was filtered off and the filtrate was washed with ether (3 × 250 ml.). Activated charcoal (10 g.) was added to the aqueous layer and the charcoal was filtered off and washed with water (1 l.). The adsorbed material was eluted with methanol (500 ml.) containing 5% (v/v) of aq. NH_3 soln. (sp.gr. 0.88) and the solution was evaporated to small volume under reduced pressure. The residue was acidified with acetic acid and the precipitated solid was recrystallized from water to yield *S*-(1-naphthyl)glutathione (IV) (1.2 g.) as a cream-coloured powder, m.p. 225–226° (decomp.) (Found: C, 55.1; H, 5.4; N, 9.85; S, 7.2; $C_{20}H_{23}O_6N_2S$ requires C, 55.4; H, 5.35; N, 9.7; S, 7.4%). A little of the compound was hydrolysed by heating to 100° with 6*N*-HCl for 2 hr. and the products were examined on paper chromatograms with solvent 3 (Table 1). Glycine, glutamic acid and *S*-(1-naphthyl)cysteine were detected as ninhydrin-positive spots at R_F 0.23, 0.28 and 0.71 respectively.

S-(1-Naphthyl)-L-cysteine (V). This was prepared by the method of West & Mathura (1954), except that the compound was isolated from the reaction mixture by means of activated charcoal as outlined above. It crystallized from water in needles, m.p. 184° (decomp.), $[\alpha]_D^{25} + 6^\circ$ in 0.1*N*-NaOH (*c*, 1).

1-Naphthylmercapturic acid (VI). This was prepared by the acetylation of *S*-(1-naphthyl)-L-cysteine in 2*N*-NaOH with acetic anhydride and formed needles from water, m.p. 173°, $[\alpha]_D^{25} - 24^\circ$ in ethanol (*c*, 1).

Scheme 1

N-Acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-*L*-cysteine (III). This was isolated as the ammonium salt from the urine of rabbits dosed with naphthalene as described previously (Boyland & Sims, 1958).

Paper chromatography

Descending chromatograms on Whatman no. 1 chromatography paper were developed for 18 hr. with the solvents listed in Table 1. The dried chromatograms were examined in u.v. light after exposure to NH_3 and were then sprayed with one of the following reagents: 1, diazotized *p*-nitroaniline (0.02% in 0.1*N*-HCl) followed by aq. 10% (w/v) Na_2CO_3 ; 2, 0.1*M*- $\text{K}_2\text{Cr}_2\text{O}_7$ -acetic acid (1:1) followed by 0.1*M*- AgNO_3 (Knight & Young, 1958); 3, a solution of ninhydrin (0.4%) in butanol saturated with water. Other chromatograms were sprayed with 2*N*-HCl and heated to 70° for 10 min. before being sprayed with reagent 1 above.

Tissue experiments

Slices of liver (approx. 5 g. wet wt.) from male adult rats were incubated in Ringer phosphate solution (Umbreit, Burris & Stauffer, 1949) saturated with naphthalene (final concn. approx. 0.3 mM) pH 7.4 (60 ml.) for 2 hr. at 37° with shaking. At the end of the incubation period the reaction mixture was decanted from the slices, which were washed once with water (30 ml.). The washings and the reaction mixture were combined and adjusted to pH 4.5 with acetic acid. The reaction mixture was centrifuged for 20 min. at 600 *g*, the supernatant was treated with a little activated charcoal and the charcoal filtered off and washed with water. The material obtained by the subsequent elution of the charcoal with methanol containing 0.2% (v/v) of aq. NH_3 soln. (sp.gr. 0.88) and evaporation under reduced pressure was examined on paper chromatograms. Kidneys from male adult rats were homogenized in 9 vol. of 0.154*M*-NaCl with a Potter & Elvehjem (1936)-type homogenizer with a Teflon pestle.

RESULTS

Identification of the new metabolite

Examination of the products from the incubation of naphthalene with liver slices on paper chromato-

grams showed the presence of a compound which formed a dark absorbent spot when the chromatograms were inspected in u.v. light, gave a blue on spraying with diazotized *p*-nitroaniline and aq. Na_2CO_3 , a mauve with ninhydrin and a positive test with the $\text{K}_2\text{Cr}_2\text{O}_7$ - AgNO_3 reagent. On two-dimensional chromatograms, run in either solvent system 1 or 2, which had been sprayed with aq. *N*-HCl and allowed to dry between the first and second developments, the compound could no longer be detected, but a second compound of different R_F was seen, which had a bright-orange fluorescence under u.v. light and reacted with ninhydrin and the $\text{K}_2\text{Cr}_2\text{O}_7$ - AgNO_3 reagent but not with diazotized *p*-nitroaniline. At the same time a spot identical with that of 1-naphthol was formed. The orange fluorescent spot was indistinguishable from synthetic *S*-(1-naphthyl)glutathione (IV) on paper chromatograms. The properties (apart from the ninhydrin reaction) of the original compound were similar to those of *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-*L*-cysteine (III), except that this compound forms 1-naphthylmercapturic acid on acidification (Boyland & Sims, 1958) and has a higher R_F in all the solvents used (see Table 1). These properties suggested that the new metabolite was *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione (I).

Approximately 25 g. of liver slices was incubated with Ringer phosphate saturated with naphthalene as described above. The product was prepared for paper chromatography and the solution applied as a 15 cm. long strip to the paper, which was then developed with solvent 1 (Table 1) for 18 hr. The chromatogram was dried and a test strip cut off and sprayed with diazotized *p*-nitroaniline and aq. Na_2CO_3 . The zone on the main chromatogram containing the metabolite was cut out and eluted with

Table 1. Paper chromatography of compounds related to *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione

Solvent systems: 1, butanol-propan-1-ol-aq. 2*N*- NH_3 soln. (2:1:1, by vol.); 2, butanol-acetic acid-water (2:1:1, by vol.); 3, the organic phase of butanol-acetic acid-water (5:1:4, by vol.). Time of run, 18 hr. All compounds gave positive reactions with the $\text{K}_2\text{Cr}_2\text{O}_7$ - AgNO_3 reagent.

Compounds	R_F			Fluorescence	Colour	
	Solvent 1	Solvent 2	Solvent 3		With diazotized <i>p</i> -nitroaniline and Na_2CO_3	With ninhydrin
<i>S</i> -(1:2-Dihydro-2-hydroxy-1-naphthyl)-glutathione	0.04	0.47	0.36	Dark-absorption	Blue	Mauve
<i>S</i> -(1:2-Dihydro-2-hydroxy-1-naphthyl)- <i>L</i> -cysteine	0.31	0.63	0.53	Dark-absorption	Blue	Mauve
<i>N</i> -Acetyl- <i>S</i> -(1:2-dihydro-2-hydroxy-1-naphthyl)- <i>L</i> -cysteine	0.37	0.84	0.82	Dark-absorption	Blue	—
<i>S</i> -(1-Naphthyl)glutathione	0.07	0.69	0.53	Orange	—	Mauve
<i>S</i> -(1-Naphthyl)- <i>L</i> -cysteine	0.46	0.75	0.69	Orange	—	Mauve
1-Naphthylmercapturic acid	0.51	0.86	0.87	Orange	—	—

methanol containing 0.2% of aq. NH_3 soln. (sp.gr. 0.88). The solution was evaporated to a small volume and the residue applied as a 5 cm. long strip to a chromatogram which was then developed with solvent 2 (Table 1). The metabolite was located on the dried chromatogram by means of its dark absorption in u.v. light, and this portion of the chromatogram was cut out and eluted, and the eluate evaporated as before. The material thus obtained was chromatographed on a two-dimensional chromatogram with solvent 3 (Table 1), the chromatogram being sprayed with N-HCl and allowed to dry before the second development with solvent 3. The spot with an orange fluorescence under u.v. light was cut out and the material eluted with methanol-aq. NH_3 soln. as before. In this way a solution of the acid-decomposition product of the metabolite free from contamination by other substances (particularly amino acid-containing substances) was obtained. The methanol was distilled off and the residue heated to 100° with HCl (0.5 ml.) for 3 hr. The solution was evaporated to dryness, the residue was dissolved in water (0.5 ml.) and the solution again evaporated to dryness. The residue was treated with 1-fluoro-2:4-dinitrobenzene as described by Sanger (1945) and the material thus obtained examined on paper chromatograms with the conditions and solvents of Phillips (1958). By this means, three dinitrophenyl derivatives were detected, identical with those of glycine, glutamic acid and *S*-(1-naphthyl)-L-cysteine.

Enzymic decomposition

A solution (100 ml.) of *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione (I), obtained from incubation of 5 g. of liver slices as indicated above, except that the elutions were carried out with Ringer phosphate, was incubated with kidney homogenate (10 ml.) at 37° for 2 hr. and treated with charcoal. Examination of the products on paper chromatograms showed that the glutathione derivative (I) was no longer present in the reaction mixture. Instead a compound was present which showed as a dark absorbent spot in u.v. light and gave the colour reactions of *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione, but which differed from this compound in its R_f values in all the solvents used. On two-dimensional chromatograms with solvent 2, which were sprayed with N-HCl and dried between the first and second developments, the original compound could no longer be detected, but a compound which was indistinguishable from *S*-(1-naphthyl)-L-cysteine (V) was detected together with 1-naphthol. The above evidence suggests that the new compound is *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine (II).

When a solution of this metabolite, obtained by

elution of the appropriate portion from a paper chromatogram with Ringer phosphate (100 ml.), was incubated with liver slices (approx. 5 g.) its acetylated derivative (III) could be identified in the reaction mixture. Furthermore when a solution of *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione in boiled liver extract, obtained by boiling the supernatant from the incubation of liver slices with naphthalene, was incubated with kidney homogenate both *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine and *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine were identified by paper chromatography.

S-(1-Naphthyl)glutathione (2 mg.) in Ringer phosphate (100 ml.) and rat-kidney homogenate (10 ml.) were incubated together at 37° for 2 hr. The product, separated from the reaction mixture with charcoal as before, was shown on paper chromatograms to be indistinguishable from *S*-(1-naphthyl)-L-cysteine.

None of the above products was detected in the reaction mixture when naphthalene was incubated with boiled liver slices, nor were they formed by liver slices in the absence of naphthalene. Boiled kidney homogenate was unable to effect the hydrolysis of the glutathione derivative (I). In the experiments in which naphthalene was incubated with liver slices, other naphthalene metabolites were detected on paper chromatograms run in solvent 1 (Table 1) by means of the colour reactions with diazotized *p*-nitroaniline and aq. Na_2CO_3 described by Sims (1959). These included *trans*-1:2-dihydro-1:2-dihydroxynaphthalene, 1-naphthylsulphate, 1-hydroxy-2-naphthyl sulphate and *trans*-1:2-dihydro-2-hydroxy-1-naphthylglucosiduronic acid.

DISCUSSION

Although the problem of mercapturic acid excretion by animals has been studied over many years by different workers, there is little agreement about the mechanism by which these acids are formed, although some reaction of the foreign compound with the SH groups of cysteine, glutathione or tissue protein seems probable. There are two aspects to this problem: the mechanism of the reaction of the foreign compound with SH groups and the nature of the residue of which the SH group is part. Barnes *et al.* (1959) have divided compounds which are known to give rise to mercapturic acids into types according to the group which is displaced by *N*-acetylcysteine in the formation of these acids. Apart from the aromatic hydrocarbons and the halogenobenzenes, the compounds contain groups which could react *in vitro* under suitable conditions with *N*-acetylcysteine to form mercapturic acids. The aromatic hydrocarbons and the halogenobenzenes possess no such reactive

groups, but the formation of reactive intermediates such as epoxides (Boyland, 1950; Boyland & Sims, 1958), which then react with SH-containing compounds, could explain the formation of compounds such as *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-*L*-cysteine (VI) in the body. Benzene, anthracene and the halogenobenzenes give similar acid-labile metabolites (Knight & Young, 1958).

There is little doubt that the product formed when naphthalene is metabolized by liver slices is related to the product in the urine of animals dosed with naphthalene and differs from it in having a glutathione residue in place of an acetylcysteine residue. If, as seems likely, the product from the urine has the structure (III) (Boyland & Sims, 1958), then the product from the liver slices is *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione (I) and this structure is confirmed by the formation of *S*-(1-naphthyl)glutathione (IV) with cold acid and of glycine, glutamic acid and *S*-(1-naphthyl)-*L*-cysteine (VI) with hot acid. Other amino acids were not detected. The product formed by the enzymic hydrolysis of the glutathione derivative (I) by kidney homogenates is also clearly related to the product from the urine and, since it gives *S*-(1-naphthyl)-*L*-cysteine (V) on acid decomposition, it is presumed to be *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-*L*-cysteine (II). This compound is acetylated by liver slices to yield *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-*L*-cysteine (III), identical with that from the urines of naphthalene-treated animals. Neither the glutathione derivative (I) nor the cysteine derivative (II) has been detected in these urines: evidently only the acetyl derivative is excreted. It is generally accepted that the final stage in mercapturic acid synthesis involves an acetylation of the amino group of the substituted cysteine. West & Mathura (1954), for example, found that *S*-substituted cysteines are converted by animals into the mercapturic acids, and *S*-(*p*-bromophenyl)-*L*-cysteine is acetylated by liver and kidney slices (Gutmann & Wood, 1951). In experiments in which [¹³¹I]iodobenzene was fed to rats, Mills & Wood (1956) found that liver, kidney and intestine contained *S*-(*p*-iodophenyl)-*L*-cysteine, whereas only the mercapturic acid was excreted in the urine. When mercapturic acids are fed to animals large proportions are excreted unchanged (cf. Marsden & Young, 1958). Stekol (1941) showed that *S*-(*p*-bromobenzyl)glutathione is excreted as *p*-bromobenzylmercapturic acid when fed to rats, and Roberts & Warwick (1958) showed that *S*-ethylglutathione is converted into ethylmercapturic acid by these animals.

The conversion of the glutathione derivative (I) into the cysteine derivative (II) by kidney homogenate could have been brought about either by the loss of glycine and glutamic acid or by the

replacement of the glutathione moiety by cysteine. The former reaction, which could involve either a hydrolysis or a transfer of the amino acids to acceptors, seems the more likely. With glutathione itself, cleavage can take place either by hydrolysis or by transfer and these reactions are carried out by kidney and, according to some workers, by liver. Binkley & Nakamura (1948) found, for example, that only rat kidney converted glutathione into cysteinylglycine whereas other tissues could break down the latter compound. Revel & Ball (1959) showed that of 12 rat tissues, including liver, only kidney and pancreas could transfer the glutamyl group of glutathione. On the other hand, Neubeck & Smythe (1944) found that guinea-pig liver could hydrolyse glutathione with the production of cysteine and Cliffe & Waley (1959) found that the nuclear fraction of rabbit liver could catalyse both the hydrolysis and transepeptidation of glutathione. Bray, Franklin & James (1959) found that guinea-pig-liver slices and homogenates of guinea-pig, rat and rabbit liver would convert *S*-(*p*-chlorobenzyl)-glutathione into *S*-(*p*-chlorobenzyl)-*L*-cysteine, whereas in the present work the conversion of naphthalene into *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-*L*-cysteine (II) via the glutathione derivative (I) could not be effected by liver slices alone, even on prolonged incubation, although it might be that small amounts of the decomposition products would not be detected by the methods used. It seems probable therefore that, in animals treated with naphthalene, the glutathione derivative (I) is formed in the livers and is broken down to the cysteine derivative (II) in the kidneys. The conversion of this into the mercapturic acid (III) could take place in either of these organs. Mills & Wood (1954) showed that the products from the incubation of [¹³¹I]iodobenzene with rat liver (and kidney) slices were mainly iodophenol derivatives, although a little mercapturic acid was formed, whereas in intact animals this acid is predominant. In later experiments (Mills & Wood, 1956) small amounts of *S*-(*p*-iodophenyl)-*L*-cysteine were also found. It is possible that in these experiments, in which the labelled metabolites were isolated by extraction from acidified solutions and purified and identified by the use of carrier acids, a glutathione derivative similar to the one found in the present work was also formed but not detected.

The conversion of naphthalene into the glutathione derivative (I) could have occurred by the direct action of an intermediate on glutathione, and the work of Barnes *et al.* (1959) suggests that this might be so. On the other hand, Mills & Wood (1956) isolated a protein apparently containing radioactive material when the rat was fed [¹³¹I]-iodobenzene, so that protein (or large peptide) SH groups may be involved. If this is so, then either

the protein must contain glutathione residues which are eliminated after the reaction with the intermediate or a cysteine derivative is eliminated which then reacts with glycine and glutamic acid to form the glutathione derivative. If it is cysteine which reacts with the intermediate, then a similar synthesis of the glutathione derivative must be envisaged. Glutathione can be formed in the body by a two-step addition of glutamic acid and glycine to cysteine (Meister, 1957), but analogous reactions seem unlikely to occur in the biosynthesis of the naphthalene derivative; substituted dipeptides such as γ -glutamyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine have not been encountered, whilst the incubation of *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine (II) with liver slices did not yield *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione (I). At the moment therefore it seems likely that glutathione is the peptide involved in mercapturic acid synthesis, but the possibility that reaction with a larger peptide or a protein may occur is under investigation.

Even if the main reaction is with glutathione, it is possible that some reaction with protein SH groups occurs, but that the substituted cysteine residues are not subsequently eliminated from the protein. It may be that the polynuclear hydrocarbons, which are not excreted as mercapturic acids (Crabtree, 1946; Gutmann & Wood, 1950), behave in this way. Alternatively, it may be that these compounds form glutathione derivatives, similar to the one described in this paper, which cannot then be split in the body and are therefore not eliminated.

SUMMARY

1. *S*-(1-Naphthyl)glutathione, *S*-(1-naphthyl)-L-cysteine and 1-naphthylmercapturic acid have been synthesized.

2. Slices of livers of male rats convert naphthalene into a compound which is almost certainly *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione and which yields *S*-(1-naphthyl)glutathione when treated with acid at room temperature.

3. Rat-kidney homogenates convert *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione into a compound believed to be *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine and which forms *S*-(1-naphthyl)-L-cysteine on treatment with acid at room temperature. Kidney homogenates also

hydrolyse *S*-(1-naphthyl)glutathione to *S*-(1-naphthyl)-L-cysteine.

4. *S*-(1:2-Dihydro-2-hydroxy-1-naphthyl)-L-cysteine is acetylated by liver slices or kidney homogenate and boiled liver extract to *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine.

We thank Miss P. Simson for advice on the identification of the amino acids. This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

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