SUMMARY

1. Increased synthesis of actinomycins II and III occurred when sarcosine was added to the medium of growing cultures of *Streptomyces antibioticus*. The amount of actinomycins II and III formed depends, in part, on the concentration of sarcosine and on the time and the number of additions of this amino acid. Mycelial actinomycin, as well as that produced in the medium, contains considerable amounts of actinomycins II and III.

2. The effect observed with sarcosine is highly specific; compounds structurally and biochemically related were ineffective.

3. Exogenous L-proline reversed the effect of a given concentration of sarcosine; larger amounts of sarcosine abolished the activity of proline.

4. Incorporation of DL-pipecolic acid, a proline analogue, into the medium resulted in synthesis of several new actinomycins.

5. When washed suspensions of *S. antibioticus* were incubated in the presence of 1 mM-sarcosine, there was a fivefold increase in synthesis of actinomycin III, but no change in the percentage of actinomycin II.

6. The results obtained support the view that exogenous sarcosine and pipecolic acid compete with and replace endogenous proline in certain actinomycin peptides.

7. A generalized theory of actinomycin biogenesis is proposed.

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The Formation of Mercapturic Acids

3. N-ACETYLATION OF S-SUBSTITUTED CYSTEINES IN THE RABBIT, RAT AND GUINEA PIG*

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In the previous papers (Barnes, James & Wood, 1959; Bray, Franklin & James, 1959) evidence was given which supported the hypothesis that, in the formation of mercapturic acids *in vivo*, glutathione and glutathionase are involved in the synthesis of S-substituted cysteines. The subsequent acetylation of these compounds to mercapturic acids

remained to be examined. During this work it was observed that, although guinea-pig liver had greater glutathionase activity than had the livers of either the rabbit or the rat, the guinea pig excreted unexpectedly small amounts of mercapturic acids when given compounds which were readily converted into mercapturic acids by the rabbit and rat. A possible explanation of the failure of the guinea pig to excrete mercapturic acid

* Part 2. Bray, Franklin & James (1959).

would be a failure to acetylate *S*-substituted cysteines. This provided an additional reason for studying the acetylation stage in the synthesis of mercapturic acid with special reference to the guinea pig. Several workers have investigated the acetylation of *S*-substituted cysteines in the rat, e.g. Stekol (1938), Zbarsky & Young (1943), West, Mathura & Black (1951) and West & Mathura (1954), but there is little available information about the rabbit and none for the guinea pig.

While much work has been done on the acetylation of aromatic amino compounds *in vitro*, there have been few investigations of *N*-acetylation in aliphatic compounds. Gutmann & Wood (1951) reported the acetylation by rat liver and kidney slices of *S*-benzyl-DL-homocysteine and *S*-(*p*-bromophenyl)-L-cysteine. Chou & Soodak (1952) prepared extracts of pigeon liver which acetylated glucosamine and chondrosamine. Davidson, Blumental & Roseman (1957) showed that rabbit liver, kidney and muscle, dog kidney and human liver acetylated glucosamine 6-phosphate. Phillips & Anker (1957) showed that both rat liver and kidney could acetylate 3-cyclohexyl-L-alanine. The acetylation of some *S*-substituted cysteines and some other amino compounds has now been investigated *in vivo* and in liver and kidney slices of the rabbit, rat and guinea pig and the results suggest that, in general, the guinea pig does not acetylate aliphatic amino groups as readily as does the rabbit or the rat.

MATERIALS

All melting points are uncorrected. Elementary microanalyses were carried out by F. and E. Pascher, Bonn. Chlorine in *S*-(2:3:5:6-tetrachlorophenyl)-L-cysteine was determined by the flask method (see Macdonald, 1959) and titration with silver nitrate and dithizone (Archer, 1958).

Chlorobenzene, benzyl chloride, *p*-chlorobenzyl chloride, 1-bromobutane, 3:4-dichloronitrobenzene, 2:3:5:6-tetrachloronitrobenzene, D-glucosamine and *p*-aminobenzenesulphonamide were purchased. *N*-Acetyl-D-glucosamine, $[\alpha]_D^{25}$ in water +38±5° (c, 0.5), was prepared by acetylation of D-glucosamine with silver acetate (cf. Stacey, 1944). *S*-(*p*-Chlorophenyl)-L-cysteine, m.p. 193°, was prepared according to Parke & Williams (1951*a*). The other *S*-substituted cysteines were prepared from halogeno compounds and cysteine by a modification of the method of Suter (1895) as described by Bray, James & Thorpe (1958), who described the properties of *S*-benzyl-L-cysteine and *S*-(*p*-chlorobenzyl)-L-cysteine. *S*-(2-Chloro-4-nitrophenyl)-L-cysteine formed from aqueous ethanol pale-yellow needles, m.p. 182°, turning brown when kept in bright daylight; $[\alpha]_D^{25}$ +11±5° in 0.1*N*-KOH (c, 0.5); light-absorption max. in 0.1*N*-KOH 347 mμ (ε 9382), min., 275 mμ (Found: C, 39.4; H, 3.5; N, 9.7; Cl, 12.3; S, 11.1. C₉H₆O₄N₂ClS requires C, 39.1; H, 3.3; N, 10.1; Cl, 12.8; S, 11.6%). *S*-Butyl-L-cysteine formed from aqueous ethanol colourless needles, m.p. 246° (decomp.); $[\alpha]_D^{25}$ +24±5° in 0.1*N*-KOH (c, 0.8) (Found: C, 47.7; H, 7.8; N, 8.0; S, 18.5. Calc. for C₇H₁₄O₂NS: C, 47.4; H, 8.5; N, 7.9; S, 18.1%). Armstrong

& Lewis (1951) give m.p. 242–244° (decomp.) and Stoll & Seebeck (1949) 220–230° (decomp.). *S*-(2:3:5:6-Tetrachlorophenyl)-L-cysteine formed from absolute ethanol colourless whetstones, m.p. 179°; $[\alpha]_D^{25}$ in 0.1*N*-KOH 0±7° (c, 0.7); light-absorption max. in 0.1*N*-KOH 287 mμ (ε 2480) (Found: C, 32.7; H, 2.1; N, 4.4; Cl, 42.0; S, 9.4. C₉H₄O₄NCl₄S requires C, 32.2; H, 2.1; N, 4.2; Cl, 42.4; S, 9.6%). The mercapturic acids required as reference compounds were prepared by acetylation of the *S*-substituted cysteines with acetic anhydride and NaOH as described by Zbarsky & Young (1943). *N*-Acetyl-*S*-butyl-L-cysteine formed from ether-light petroleum (b.p. 40–60°) colourless prisms, m.p. 67°; $[\alpha]_D^{25}$ –24±3° in ethanol (c, 0.6) (Found: C, 49.6; H, 7.9; N, 6.6; S, 14.8%; equiv. 204. C₉H₁₇O₃NS requires C, 49.3; H, 7.8; N, 6.4; S, 14.6%; equiv. 219). The properties of the other mercapturic acids used have already been described (e.g. Jaffe, 1879; Spencer & Williams 1950; Stekol, 1938; Bray *et al.* 1958; Bray, James & Thorpe, 1957; Betts, James & Thorpe, 1955; Bray, Hybs, James & Thorpe, 1953).

METHODS

Animals, diet and dosage

The rabbits (about 2.5 kg.) used and their diet were as previously described (Bray, Ryman & Thorpe, 1947). Guinea pigs (about 500 g.) had the same diet and rats (about 200 g.) were maintained on a diet of rat cubes and water *ad lib*. The compounds were administered with water by stomach tube. No toxic effects were observed except with *S*-(2-chloro-4-nitrophenyl)-L-cysteine in the guinea pig. This compound (dose 72 μmoles/100 g.) caused severe anorexia and death ensued after about 7 days. 3:4-Dichloronitrobenzene (dose 104 μmoles/100 g.) also caused some anorexia in the guinea pig but the animals recovered in 3 days.

Preparation of tissue slices

The animals were killed by a sharp blow on the back of the neck. The organs required were removed rapidly and kept in ice-cold Ringer phosphate buffer, pH 7.4, prepared according to Umbreit, Burris & Stauffer (1949). Tissue slices (thickness 0.2–0.4 mm.) were cut between ground-glass plates by a razor blade and transferred to ice-cold Ringer phosphate solution. When required they were dried on filter paper, then weighed (1–2 g.) into a conical flask containing 10 ml. of a solution of substrate and glucose in Ringer phosphate buffer which had previously been saturated with oxygen. The reaction mixture was incubated at 37° with continuous shaking.

Determination of metabolites in urine

Mercapturic acids were determined by the methods recorded by Bray *et al.* (1958) and Barnes *et al.* (1959). The validity of the determinations in the presence of the corresponding *S*-substituted cysteines (see Table 3) requires comment. *S*-Benzyl-L-cysteine and *S*-(*p*-chlorobenzyl)-L-cysteine do not react under the conditions used in the nitroprusside method for determining the mercapturic acids. The treatment with zinc hydroxide in the iodometric method entirely removes added *S*-(2:3:5:6-tetrachlorophenyl)-L-cysteine from urine. The intensity of the yellow colour given by *S*-(2-chloro-4-nitrophenyl)-L-cysteine under the conditions for the colorimetric method is

Table 1. R_F values and conditions for identification of metabolites by paper chromatography

Solvent mixtures were: A, butan-1-ol-water-acetic acid-butyl acetate (24:10:5:2, by vol.); B, butan-1-ol saturated with aq. 2N-NH₃ soln.; C, benzene-acetic acid-water (2:2:1, by vol.). The descending method was used, unless stated otherwise, on Whatman no. 4 or 3MM paper.

Compound	R_F value	Time of run (hr.)	Solvent mixture
S-Butyl-L-cysteine	0.73	7	A
	0.35	16	B
N-Acetyl-S-butyl-L-cysteine	0.92	7	A
	0.46	16	B
Unidentified metabolite of bromobutane	0.20*	16	B
S-(2:3:5:6-Tetrachlorophenyl)-L-cysteine	0.60*	14	A
	0.44	16	B
N-Acetyl-S-(2:3:5:6-tetrachlorophenyl)-L-cysteine	0.95	14	A
	0.60	16	B
Unidentified metabolite of S-(2:3:5:6-tetrachlorophenyl)-L-cysteine	0.13	14	A
	0.05	16	B
2:3:5:6-Tetrachlorothiophenol	0.75†	4	B
	1.0†	4	C
S-(2-Chloro-4-nitrophenyl)-L-cysteine	0.5†	16	A
S-Benzyl-L-cysteine	0.63	7	A
	0.22	16	B
N-Acetyl-S-benzyl-L-cysteine	0.93	7	A
	0.41	16	B

* Streaks.

† Ascending method.

‡ Decomposes in solvent B.

less than 10% of that given by the mercapturic acid. The determination of the mercapturic acids corresponding to these four S-substituted cysteines can be regarded as valid.

Under the more drastic conditions of hydrolysis used for determination of butylmercapturic acid by the nitroprusside method (Barnes *et al.* 1959) S-butylcysteine gave about 50% of the colour given by an equivalent amount of butylmercapturic acid. Treatment of rabbit, rat or guinea-pig urine with zinc hydroxide (as in the iodometric method) before the alkaline hydrolysis caused a loss of added butylmercapturic acid not exceeding 10%, but with S-butylcysteine the loss was $40 \pm 10\%$ from rabbit urine and $70 \pm 5\%$ from rat and guinea-pig urine. Determinations by the nitroprusside method with and without treatment with zinc hydroxide should therefore reveal the presence of appreciable amounts of S-butylcysteine in urine. By the iodometric method only 12% of added S-(p-chlorophenyl)-L-cysteine was recovered from urine treated with zinc hydroxide so that the presence of small amounts of this compound would not influence the result for N-acetyl-S-(p-chlorophenyl)-L-cysteine significantly.

Ethereal sulphate was determined by the method of Folin (1905-06). Glucuronic acid was determined by the modified naphthoresorcinol method described by Bray, Humphris, Thorpe, White & Wood (1952). Anilines were determined as described by Bray, James & Thorpe (1956).

Determination of unabsorbed material

To eliminate the possibility that low results might be caused by poor absorption of the compounds administered, guinea-pig faeces were examined for unabsorbed material where a method was available. 3:4-Dichloronitrobenzene and 2:3:5:6-tetrachloronitrobenzene were determined in the steam-distillate of the faeces as described by Bray *et al.* (1953, 1956). The 24 hr. samples of faeces passed im-

mediately before the dose of S-(p-chlorophenyl)-L-cysteine and for the 2 days afterwards were separately finely ground and extracted with 500 ml. of 0.1N-NaOH. The light-absorption of a centrifuged sample of the extract, suitably diluted, was measured. A calibration curve was constructed by measuring the difference in the absorption at 258 and 310 m μ of solutions made by the addition of known amounts of S-(p-chlorophenyl)cysteine to a similar extract of normal faeces. The same method was used for S-(2-chloro-4-nitrophenyl)-L-cysteine except that the absorptions were measured at 350 m μ and 410 m μ .

Paper chromatography

The procedure was as described by Bray *et al.* (1956, 1957). Additional solvent mixtures used and R_F values are given in Table 1. Mercapturic acids and S-substituted cysteines were detected by the potassium dichromate-silver nitrate reagent of Knight & Young (1958) and by ninhydrin.

Determination of acetylation by tissue slices

p-Aminobenzenesulphonamide as substrate. A sample (1 ml.) of the medium was removed and deproteinized by the addition of 10% (w/v) ZnSO₄·7H₂O (5 ml.) and 0.5N-NaOH (5 ml.). The precipitate was removed by centrifuging and 2 ml. of the supernatant was added to 3 ml. of water and the amino compounds were determined by the method of Bratton & Marshall (1939). The intensity of colour was measured in a Spekker photoelectric absorptiometer with a Chance glass filter OG1.

D-Glucosamine as substrate. The procedure of Morgan & Elson (1934) was adapted. The mixture of slices and medium was homogenized with a Potter-Elvehjem type homogenizer with a polytetrafluoroethylene (Teflon) pestle. Duplicate samples (4 ml.) of the homogenate were

mixed with absolute ethanol (5 ml.) and filtered after 30 min. Samples (2 ml.) of the filtrate were transferred to test tubes graduated at 10 ml. Then 0.1 ml. of 0.5N-Na₂CO₃ was added and the mixture was heated in a boiling-water bath for exactly 5 min. The tube was fitted with a 'cold finger' to reduce evaporation. To the cooled mixture 6 ml. of acetic acid and 1 ml. of *p*-dimethylaminobenzaldehyde reagent (2 g. in 95 ml. of acetic acid and 5 ml. of conc. HCl) were added. The volume was made up to 10 ml. with acetic acid and the mixture was kept in the dark for not less than 45 min. before the extinction was measured in a Spekker photoelectric colorimeter with a Chance filter OG1. Calibration curves were prepared from *N*-acetyl-D-glucosamine in water. The average recovery of *N*-acetyl-D-glucosamine added to liver slices in six control experiments was 71 % (range 66–75 %). This method is more sensitive than that described by Chou & Soodak (1952); see Table 2.

S-(*p*-Chlorobenzyl)-L-cysteine and *S*-butyl-L-cysteine as substrates. The mixture of slices and medium was homogenized as described above and 0.5 % uranyl acetate (10 ml.)

was mixed with the homogenate. After 30 min. the mixture was filtered and 13 ml. of the filtrate was acidified (pH 1.0) with conc. HCl and continuously extracted with ether for 6 hr. The extract was concentrated to 5 ml., transferred to a boiling tube with three washings with 5 ml. of ether and evaporated to dryness. Water (5 ml.) was added and mercapturic acid was determined by the nitroprusside method described by Bray *et al.* (1958). The mean recovery of *p*-chlorobenzylmercapturic acid added to liver slices in six experiments was 68 ± 4 %. That for the butylmercapturic acid was 63 % (range 56–68 %).

RESULTS

Absorption of 3:4-dichloronitrobenzene, 2:3:5:6-tetrachloronitrobenzene, S-(*p*-chlorophenyl)-L-cysteine and *S*-(2-chloro-4-nitrophenyl)-L-cysteine by the guinea pig and rat

No 3:4-dichloronitrobenzene or 3:4-dichloroaniline was detected in the faeces excreted by two guinea pigs during 72 hr. after dosage with 3:4-dichloronitrobenzene. In two experiments with 2:3:5:6-tetrachloronitrobenzene 14 and 22 % of the dose were found in the faeces. In similar experiments with rats the values were 19 and 28 %. No unchanged material was detected in the faeces of two guinea pigs which had each received 75 mg. of *S*-(*p*-chlorophenyl)-L-cysteine; since the method of analysis would have detected 10 % of the dose in the faeces, it is probable that at least 90 % was absorbed provided that no breakdown of the compound took place in the alimentary canal. Analysis of the faeces of two guinea pigs dosed with *S*-(2-chloro-4-nitrophenyl)-L-cysteine failed to reveal

Table 2. *Comparison of methods for determination of acetylglucosamine*

For experimental details see Chou & Soodak (1952) and the Methods section. The amounts of acetylglucosamine indicated were in 10 ml. of the final solution in the cuvette.

Acetyl-glucosamine present (μg.)	Spekker reading	
	Method of Chou & Soodak	Present method
500	0.215	0.505
400	0.165	0.430
250	0.105	0.312
100	0.03	0.150
50	0.01	0.085
0	0.01	0.01

Table 3. *Acetylation of S-substituted L-cysteines by the rabbit, rat and guinea pig*

Results are expressed as percentages of the dose excreted as mercapturic acid and are 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. For validity of results for *S*-*p*-chlorophenyl-L-cysteine, *S*-butyl-L-cysteine and *S*-(2:3:5:6-tetrachlorophenyl)-L-cysteine see Methods and Discussion.

Compound administered	Rabbit		Rat		Guinea pig	
	Dose (μmoles/100 g.)	Acetylation (% of dose)	Dose (μmoles/100 g.)	Acetylation (% of dose)	Dose (μmoles/100 g.)	Acetylation (% of dose)
<i>S</i> -(<i>p</i> -Chlorophenyl)-L-cysteine	173	51 (38–69) ⁶	108	37 (34–42) ³	65	4 (0–12) ³
<i>S</i> -Benzyl-L-cysteine	95	45 (35–55) ³	118	56 (52–62) ³	71	13 (11–14) ³
<i>S</i> -(<i>p</i> -Chlorobenzyl)-L-cysteine	82	43 (34–50) ³	123	80 (65–100) ³	73	3 (2–5) ³
<i>S</i> -Butyl-L-cysteine	114	39 (30–47) ³	142	61 (37–69) ⁸	85	31* (22–43) ⁶
<i>S</i> -(2-Chloro-4-nitrophenyl)-L-cysteine	72	62 (56–67) ⁴	127	57 (47–63) ³	72	9 (6–11) ³
<i>S</i> -(2:3:5:6-Tetrachlorophenyl)-L-cysteine	—	—	75	9 (6–13) ³	45	65* (42–90) ⁶

* No mercapturic acid could be isolated from these urines and it is doubtful whether any of this acid was excreted (see text).

any unchanged material in one animal and gave 0.8 % of the dose in the second, so that subject to the above proviso this compound seems to be almost completely absorbed.

N-Acetylation of S-substituted L-cysteines by rabbit, rat and guinea pig in vivo

The results obtained are given in Table 3. From the urine of one rabbit 45 % of the dose of *S*-(2-chloro-4-nitrophenyl)-L-cysteine was isolated by the method of Barnes *et al.* (1959) as *N*-acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine, m.p. 191° alone and in admixture with an authentic specimen; light-absorption max. 345 m μ (ϵ 10 530). Similarly, from rat urine 17 % of the dose was isolated as the mercapturic acid, m.p. and mixed m.p. 190°; light-absorption max. 345 m μ (ϵ 10 700). No mercapturic acid was isolated from the urine of guinea pigs dosed with *S*-(2-chloro-4-nitrophenyl)-L-cysteine. Butylmercapturic acid was determined in the urines of animals given bromobutane or *S*-butylcysteine with and without treatment of the urine with zinc hydroxide. The agreement between the two determinations (Table 4) suggests that little, if any, *S*-butylcysteine is excreted.

Acetylation of p-aminobenzene sulphonamide by the guinea pig in vivo

To confirm that the guinea pigs used would acetylate an aromatic amine, three guinea pigs were each given 50 mg. of *p*-aminobenzene sulphonamide. Analysis of urine collected during the next 48 hr. showed that 25 ± 5 % of the dose was excreted as free *p*-aminobenzene sulphonamide and 56 ± 6 % of the dose was acetylated.

N-Acetylation by tissue slices

The results obtained for *N*-acetylation of amino compounds by tissue slices are summarized in

Table 5. Since there is some doubt about the validity of the results obtained in the determination of butylmercapturic acid, experiments with liver slices similar to those of Table 5 were carried out with *S*-benzylcysteine and *S*-butylcysteine, in each of which the combined contents of three flasks (total 30 ml.) which had been incubated for 4 hr. were filtered through cotton wool. A sample of the filtrate was applied to Whatman no. 3MM paper and developed with solvent mixtures *A* and *B*. The remainder of the filtrate was acidified with HCl to pH 1.0 and continuously extracted with ether. The ether extract was examined by paper chromatography as described above. The unchanged cysteines were readily detected on the paper chromatograms from the filtrates of the liver slices of all three species. With *S*-benzylcysteine the mercapturic acid was readily detected in the ether extracts from liver slices of the rat and rabbit but only traces were present in those of the guinea pig. With *S*-butylcysteine the mercapturic acid was readily detected in the ether extracts from rat slices but only traces were found in those from the guinea pig and from the rabbit.

Metabolism of 3:4-dichloronitrobenzene and S-(2-chloro-4-nitrophenyl)-L-cysteine in the guinea pig

The metabolism of these compounds was of particular interest since *S*-(2-chloro-4-nitrophenyl)-L-cysteine, which would be expected to be an intermediary metabolite of 3:4-dichloronitrobenzene, was very toxic to the guinea pig. Quantitative analysis of 24 hr. urine of guinea pigs which had received 3:4-dichloronitrobenzene (104 μ moles/100 g.) showed the presence of mercapturic acid corresponding to an average of 3 % of the dose (range 2–5 % in five experiments), ethereal sulphate to 12 % (7, 17 %) and 3:4-dichloroaniline

Table 4. *Effect of zinc hydroxide treatment on results for butylmercapturic acid*

Mercapturic acid was determined by the nitroprusside method in urines with (B) and without (A) treatment with zinc hydroxide. Doses of bromobutane were 190, 175 and 108 μ moles/100 g. for rabbits, rats and guinea pigs respectively; those of *S*-butyl-L-cysteine were 114, 142 and 85 μ moles/100 g. respectively. Results are expressed as means with ranges in parentheses; superior figures indicate the number of experiments.

Compound administered	Treatment of urine	Mercapturic acid excreted (% of dose)		
		Rabbit	Rat	Guinea pig
Bromobutane	A	34 (30–37) ⁵	60 (47–72) ⁵	22* (19–28) ⁴
		32 (27–36) ⁵	57 (43–63) ⁵	18* (13–21) ⁴
	B	38 (35–39) ³	61 (37–69) ⁵	36† (28–43) ³
		37 (32–40) ³	57 (36–69) ⁵	35† (28–39) ³
<i>S</i> -Butyl-L-cysteine	A			
	B			

* See footnote to Table 6.

† See footnote to Table 3.

(free plus combined) to 5% (4–5% in three experiments). The corresponding average values for the rabbit (dose 208 μ moles/100 g.) were 45, 12 and 22% (Bray *et al.* 1957). Anorexia prevented satisfactory determination of glucosiduronic acid. Thus only 20% of the dose was accounted for as these three metabolites in the guinea pig, compared with 79% in the rabbit, which excreted an additional 13% of the dose as glucosiduronic acid. It is unlikely that the guinea pig excreted a large amount of glucosiduronic acid since the naphthoresorcinol values for the urines were usually lower than those from the urine passed before the dose was given. Neither 3:4-dichloronitrobenzene nor 3:4-dichloroaniline was detected in the faeces. 3:4-Dichloroaniline was detected by paper chromatography in ether extracts A and B and 5-amino-2:3-dichlorophenol in extracts A, B, C and D of the urine. (For preparation of extracts A, B, C and D and methods of identification see Bray *et al.* 1956, 1957.) The presence of 2:3-dichloro-5-nitrophenol was also shown since the elution of one of the spots and reduction of the eluate gave 5-amino-2:3-dichlorophenol. No *o*-aminophenols were detected. *S*-(2-Chloro-4-nitrophenyl)-L-cysteine was not detected in a butanol extract of urine adjusted to pH 4.0.

The substituted cysteine added to normal urine could be detected by this procedure. Examination of urines from guinea pigs which had received *S*-(2-chloro-4-nitrophenyl)-L-cysteine revealed the presence of at least five diazotizable compounds, none of which gave the indophenol reaction for *p*-aminophenols. Neither mercapturic acid nor the substituted cysteine was detected. Parke & Williams (1951*b*) reported that 24% of administered *S*-phenyl-L-cysteine was excreted unchanged by the rabbit.

Qualitative examination of urines

Butylmercapturic acid was readily detected on paper chromatograms of the urine excreted after the administration of bromobutane to the rabbit and rat, but only traces were found in the urine of a similarly treated guinea pig. No *S*-butylcysteine was detected on paper chromatograms from any of the urines by the reagent of Knight & Young (1958), but traces were detected by ninhydrin in the urine of rabbits and guinea pigs but not in that of rats. The unidentified metabolite (see Table 1) was detected in urines of the three species.

Butylmercapturic acid and traces of *S*-butylcysteine were detected by paper chromatography

Table 5. *Acetylation of amino compounds by tissue slices*

The basic medium, B, consisted of 10 ml. of Ringer phosphate buffer, pH 7.4, containing 2.5 mg. of substrate and 20 mg. of glucose. Medium A was the same except that 27.2 mg. of sodium acetate ($\text{CH}_3\cdot\text{CO}_2\text{Na}\cdot 3\text{H}_2\text{O}$) was added. After addition of tissue slices the system was incubated with continuous shaking for 4 hr. at 37° with O_2 as gas phase. Duplicate 1 ml. samples were withdrawn for analysis at 0 and 4 hr. The results shown are for liver slices. Similar experiments with 2 g. of kidney slices gave negative results except that a trace of acetylation of *S*-(*p*-chlorobenzyl)-L-cysteine was detected with rat kidney. Values are the means of duplicate determinations in close agreement. A values immediately below B values were obtained from experiments in which the same sample of liver was used.

Digest	Wt. of slices (g.)	Percentage of substrate acetylated		
		Rabbit	Rat	Guinea pig
Substrate: <i>p</i> -aminobenzene sulphonamide				
B	1	17, 14, 14	19, 12, 9, 8	7
A	1	20	26	13
B	2	39	38	19
A	2	44, 76, 47	39	21, 26
Substrate: D-glucosamine				
B	1	< 1, < 1	8, 6, 8	0, 0
A	1	< 1, < 1	14, 9, 13	0, 0
B	2	10, 7, 5	25	< 1, < 1
A	2	10, 9, 5	30	< 1, < 1
Substrate: <i>S</i> -(<i>p</i> -chlorobenzyl)-L-cysteine				
B	2	9, 10	9, 8	< 1, < 1
A	2	12, 10	9, 8	< 1, < 1
Substrate: <i>S</i> -butyl-L-cysteine				
B	2	11*	5	11, 5*
A	2	17*	6	13, 9*

* Very little of this appears to be true mercapturic acid; see text for results of paper chromatography of end products of similar experiments.

in the urines of rabbits and rats to which *S*-butylcysteine had been given but they were not detected in the urine of guinea pigs similarly treated. The unidentified metabolite was detected in the urine of all three species.

Neither the unchanged compound nor the corresponding mercapturic acid was detected on paper chromatograms of the urine of guinea pigs to which *S*(2:3:5:6-tetrachlorophenyl)-L-cysteine had been administered. An unidentified metabolite (see Table 1) was detected. The mercapturic acid was not detected in the ethereal extract of acidified urine. On acidification of alkali-hydrolysed urine obtained from three guinea pigs which had each received 75 mg. of *S*(2:3:5:6-tetrachlorophenyl)-L-cysteine a precipitate formed. This was separated and recrystallized from aqueous acetone, when 10 mg. of impure crystals, m.p. 75°, were obtained which gave a positive Folin-Ciocalteu test and a spot which was indistinguishable from 2:3:5:6-tetrachlorothiophenol on paper chromatograms. Recrystallization from aqueous acetone yielded crystals, m.p. 130–134°, admixture with which did not depress the m.p. of the presumed disulphide (see Bray *et al.* 1953).

DISCUSSION

Tables 3 and 6 show that the guinea pig forms mercapturic acids less readily than does the rabbit or the rat. By the method of determination used 1-bromobutane and *S*-butyl-L-cysteine appeared to yield about 19 and 31 % respectively of the dose as mercapturic acid but only traces of butylmercapturic acid could be detected in the urines. Grenby & Young (1959) detected propylmercapturic acid in the urine of guinea pigs dosed with

1-bromopropane. The results in Table 4 suggest that insignificant amounts of *S*-butylcysteine were excreted after the administration of either 1-bromobutane or *S*-butylcysteine; only traces of *S*-butylcysteine were detected on paper chromatograms. The analytical results could be accounted for by the presence in the urines of a compound (or compounds) which yields 1-mercaptobutane on hydrolysis with sodium hydroxide but which is neither *S*-butyl-L-cysteine nor butylmercapturic acid. The guinea pig also appeared to excrete a high proportion of the dose of *S*-(2:3:5:6-tetrachlorophenyl)-L-cysteine as mercapturic acid but again no mercapturic acid was isolated from the urine, although *N*-acetyl-*S*-(2:3:5:6-tetrachlorophenyl)-L-cysteine has been easily isolated on many occasions from rabbit urine. Neither the mercapturic acid nor the *S*-tetrachlorophenylcysteine could be detected by paper chromatography of guinea-pig urine. As *S*-(2:3:5:6-tetrachlorophenyl)cysteine added to urine is completely removed by the zinc hydroxide used in the iodometric method it is unlikely that the excretion of unchanged material accounts for the high iodine uptake of the hydrolysed guinea-pig urine. The tetrachlorothiophenol obtained on alkaline hydrolysis of the urine is presumably derived from an unidentified metabolite which is not formed to any significant extent when 2:3:5:6-tetrachloronitrobenzene is administered. By contrast, an unidentified sulphur-containing metabolite is excreted when either bromobutane or *S*-butylcysteine is given to guinea pigs. A possible metabolite which would account for the observed results would be the corresponding deaminated cysteine, e.g. *S*-butyl-3-mercaptopyruvic acid and *S*-(2:3:5:6-tetrachlorophenyl)-3-mercaptopyruvic acid. Keto acids of this type

Table 6. *Excretion of mercapturic acid by the rabbit, rat and guinea pig after administration of precursors*

Precursor administered	Mean percentage of dose excreted as mercapturic acid		
	Rabbit	Rat	Guinea pig
Naphthalene	4 ⁽¹⁾	12 ⁽²⁾	0.6 ⁽³⁾
Chlorobenzene	20 ⁽⁴⁾	18 ⁽⁵⁾	1 ⁽⁶⁾
Benzyl chloride	49 ⁽⁷⁾	27 ⁽⁸⁾	4 ⁽⁶⁾
<i>p</i> -Chlorobenzyl chloride	23 ⁽⁷⁾	37 ⁽⁸⁾	5 ⁽⁶⁾
1-Bromobutane	45 ⁽⁸⁾	44 ⁽⁵⁾	19 ^{(6)*}
3:4-Dichloronitrobenzene	45 ⁽⁸⁾	19 ⁽⁵⁾	3 ⁽⁶⁾
2:3:4:6-Tetrachloronitrobenzene	37 ⁽⁹⁾	32 ⁽⁶⁾	2 ⁽⁶⁾
2:3:5:6-Tetrachloronitrobenzene†	14 ⁽¹⁰⁾	11 ⁽⁵⁾	0 ⁽⁶⁾

(1) By isolation, Bourne & Young (1934).

(2) By isolation, Young (1947).

(3) By isolation, Corner & Young (1954).

(4) Spencer & Williams (1950).

(5) Barnes, James & Wood (1959).

(6) Bray, Franklin & James (1959).

(7) Bray, James & Thorpe (1958).

(8) Bray, James & Thorpe (1957).

(9) Betts, James & Thorpe (1955).

(10) Bray, Hybs, James & Thorpe (1953).

* The true value is probably very small since no butylmercapturic acid could be detected in the urines by paper chromatography (see text).

† Only 34 % of a dose of this compound is absorbed by the rabbit, 76 % by the rat and 82 % by the guinea pig.

have been identified as metabolites of *S*-benzylcysteine and *S*-benzylhomocysteine by Shen & Lewis (1946). Roberts & Warwick (1958), however, concluded that ethylmercaptopyruvic acid was not a metabolite of ethyl methanesulphonate.

It is unlikely that absorption difficulties could account for the failure of the guinea pig to excrete mercapturic acid, since even with 2:3:5:6-tetrachloronitrobenzene about 82 % of the dose appears to be absorbed. The corresponding values for the rabbit and the rat are 34 % (Bray *et al.* 1953) and 76 % respectively. The results of the experiments with tissue slices (Table 5) also indicate that D-glucosamine and *S*-(*p*-chlorobenzyl)-L-cysteine were acetylated to a significant extent by liver slices from the rabbit and rat but not by those from the guinea pig. *p*-Aminobenzene sulphonamide was, and *S*-butylcysteine appeared to be, readily acetylated by liver slices of all three species although it is doubtful whether more than a trace of mercapturic acid was actually formed from *S*-butylcysteine by guinea-pig or rabbit liver. The results obtained from liver slices therefore are in accord with the results obtained *in vivo* except for the failure to detect significant formation of butylmercapturic acid in rabbit-liver slices. The reason for this finding has not been discovered.

The guinea pig provides another example of a species difference in acetylation. It is well known (e.g. Marshall, Cutting & Emerson, 1937; Krebs, Sykes & Bartley, 1947) that the dog excretes only a small proportion of administered aminoaryl sulphonamide as acetyl derivatives. Although the guinea pig readily acetylates *p*-aminobenzene sulphonamide it appears to have difficulty in acetylating the aliphatic amines examined. This may be due to the specificity of the enzyme responsible for transferring the acetyl group from acetyl-coenzyme A to the amine. Examples of such specificity have been given by Tabor, Mehler & Stadtman (1953), who obtained a pigeon-liver preparation which would acetylate *p*-aminobenzoic acid, *m*-nitroaniline, *o*-phenylenediamine, *o*- and *m*-toluidine, *o*- and *p*-bromoaniline and *o*- and *p*-anisidine but not *o*-aminobenzoic acid, *p*-nitromethylaniline or kynurenine. Davidson *et al.* (1957) obtained a preparation from *Neurospora crassa* which acetylated glucosamine 6-phosphate, but not glucosamine, galactosamine, *p*-nitroaniline or amino acids. The results of Krebs *et al.* (1947) suggest that the apparent inability of the dog to acetylate aromatic amines may be due to an active deacetylation process which occurs simultaneously. This deacetylating system is inactive in the intact rabbit. Experiments are in progress to test whether the apparent failure of the guinea pig to acetylate aliphatic amino compounds can be attributed to a deacetylation process.

None of the results reported in this and the two earlier papers (Barnes *et al.* 1959; Bray *et al.* 1959) provides an objection to the hypothesis that for the compounds examined mercapturic acids are formed by a three-stage process consisting of: (1) the conjugation of the precursor with glutathione, (2) the hydrolysis of the glutathione conjugate by glutathionase to an *S*-substituted cysteine, glycine and glutamic acid and (3) the acetylation of the *S*-substituted cysteine to mercapturic acid. Reactions 2 and 3 can occur in the liver of the rabbit and rat, but direct proof that reaction 1 can occur is desirable before the hypothesis can be regarded as established. The comparative failure of the guinea pig to excrete mercapturic acids is probably not due to a failure of reactions 1 or 2 but rather to a defect in reaction 3, since even when *S*-substituted cysteines are administered the guinea pig excretes relatively small amounts of the dose as mercapturic acid.

SUMMARY

1. The *N*-acetylation of some *S*-substituted cysteines by the rabbit, rat and guinea pig *in vivo* and their tissues *in vitro* has been studied.

2. Unlike the rabbit and the rat, the guinea pig did not excrete as mercapturic acid a large proportion of a dose of any of the six *S*-substituted cysteines examined.

3. The extent of acetylation of *S*-(*p*-chlorobenzyl)cysteine and *S*-butylcysteine *in vitro* with liver slices was comparable with that obtained *in vivo*. *p*-Aminobenzene sulphonamide was readily acetylated by the guinea pig and by slices of guinea-pig liver.

4. The failure of the guinea pig to excrete considerable amounts of mercapturic acid from precursors appears to be due to difficulty in *N*-acetylation of *S*-substituted cysteines.

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Kinetic Studies on Rabbit-Muscle Myokinase

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Myokinase (adenylate kinase) catalyses the reaction:

Highly purified preparations of this enzyme have been obtained from rabbit muscle by Noda & Kuby (1956, 1957*a, b*) and Callaghan (1956, 1957*a, b*), and the preparation of the last-named author, which appears to contain a single protein component, has been used for the present studies of the mechanism of myokinase action. Several possible mechanisms by which myokinase might catalyse transphosphorylation among adenosine nucleotides were considered by Whittam, Bartley & Weber (1955). The enzyme may accept the terminal phosphate of adenosine triphosphate (ATP) or adenosine diphosphate (ADP), thus forming an intermediate phosphorylated protein by the reactions:

or may act by forming an enzyme-nucleotide complex according to the following scheme:

A further possibility is expressed by the reactions:

By use of the purified enzyme, the hypothetical phosphorylated enzyme intermediate was tested for directly by incubation with ATP of comparable molar concentration in the region of the Michaelis constant (K_m) value for the substrate; in most experiments of this kind, the ATP was labelled with ^{32}P in the β - and γ -phosphate groups. Kinetic studies were also undertaken to determine the affinity of the enzyme for its substrates, and a special study of the reverse reaction was made to