The Fate of Certain Organic Acids and Amides in the Rabbit

5. *o*- AND *m*-HYDROXYBENZOIC ACIDS AND AMIDES

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We have already reported (Bray, Ryman & Thorpe, 1947) upon the fate of *p*-hydroxybenzoic acid and its amide in the rabbit. The present paper gives the corresponding results for the *o*- and *m*-isomers. The changes which these compounds might undergo are theoretically the same as those for the *p*-isomer, i.e. the hydroxyl group may be conjugated with either glucuronic or sulphuric acids and the carboxyl group with glucuronic acid or glycine. In the case of the amides the extent of conjugation through the carboxyl group would depend upon the degree of hydrolysis of the carbamyl group. There is also the possibility of the introduction of a second hydroxyl group, comparable to the formation of protocatechuic acid from *p*-hydroxybenzoic acid or amide (Bray *et al.* 1947).

The extensive literature upon salicylic acid resulting from its clinical use in the treatment of rheumatic conditions was critically reviewed up to 1926 by Hanzlik (1926), who concluded that salicylic acid was excreted largely unconjugated, though some conjugation with glycine, to form salicyluric acid, might occur. The degree of this conjugation was variable in amount, depending to some extent on species; the evidence was most complete for man. There was no general agreement about glucuronide formation. It seemed probable that some destruction of salicylic acid occurred in the organism, and there was evidence that gentisic acid (2:5-dihydroxybenzoic acid) was formed by hydroxylation of salicylic acid. There has been general agreement among workers that salicylic acid is not conjugated with sulphate in man, dog or rabbit (e.g. Baumann & Herter (man and rabbit), 1877-8; Mosso (man and dog), 1889-90; Vogelius (man), 1880; Williams (rabbit), 1938), though Baumann & Herter claimed to have observed ethereal sulphate formation in the dog. The results of the comparatively few investigations described since 1926 have in general supported the conclusions in Hanzlik's (1926) review. Quick (1932*a, b*) found some evidence of glucuronic acid conjugation of salicylic acid in the dog, but very little conjugation with glycine was observed. He was, however, able to isolate salicyluric acid from human salicylate urine (Quick, 1933). Kapp & Coburn (1942) isolated salicylic acid (20%), salicyluric acid (55%) and gentisic acid (4-8%) from human salicylate urine, and in addition obtained a small amount of uraminosalicylic acid, apparently a complex of glycine, salicylic and gentisic acids, which was probably identical with a compound (uramin-salicylsäure) isolated by Baldoni (1908) from the urine of dogs dosed with salicylate. It is possible that these may

have been artifacts formed during isolation, but their formation and exact constitution have not been studied further. Kapp & Coburn (1942) obtained evidence of the conjugation of salicylic acid with glucuronic acid (25%), but no glucuronide could be isolated. Lutwak-Mann (1943) showed that rats can also hydroxylate salicylic acid to form gentisic acid.

Very few investigations of the metabolism of salicylamide have been reported. Bondzynski (1897) concluded that it was completely absorbed in man. Baumann & Herter (1877-8) and Baas (1889-90) found that it caused an increase in ethereal sulphate in the dog, and Williams (1938) found that 29-40% was excreted in this form by the rabbit. On the basis of the salicyluric acid which he could isolate, Baas claimed that 2-12% was hydrolyzed in the dog.

m-Hydroxybenzoic acid has been found to be conjugated with glycine and glucuronic acid in the dog (Quick, 1932*a, b*), with sulphate in man and dog (Baumann & Herter, 1877-8) and in the rabbit to the extent of 3-5% (Williams, 1938).

The only record of the metabolism of *m*-hydroxybenzamide is Williams's (1938) observation that 32-44% was excreted as ethereal sulphate by the rabbit.

In this study we sought to establish the degree of hydrolysis of the carbamyl group in *o*- and *m*-hydroxybenzamides by quantitative estimation of the excretion of various metabolites, and comparison of the results with those obtained after administering the corresponding acids. We were able to isolate 2- and 3-carbamylphenylglucuronides as metabolites of *o*- and *m*-hydroxybenzamides respectively, and to detect the formation of hydroxylation products from all four compounds, isolating gentisic acid from salicylate urine and gentisamide from salicylamide urine.

METHODS

Diet and dosing. The rabbits used were does of 2-3 kg. The diet of rabbit pellets already described (Bray *et al.* 1947) was used throughout this investigation. Salicylic acid and amide were appreciably toxic, and the maximum dose level used was 0.4 g./kg. The meta isomers were not toxic at dose levels up to 1.5 g./kg.

Methods of analysis. These were the same as those previously used (Bray *et al.* 1947), but in this investigation the ether-soluble acid was not fractionated.

Preparation of o- and m-hydroxybenzamides. Salicylamide was prepared by refluxing methyl salicylate with excess ammonia (sp.gr. 0.88) until there were no longer

two layers. On cooling, the reaction mixture deposited long needles which were recrystallized from water. The final product melted at 139°. Yield 90% of theory. The final traces of methyl salicylate were removed by heating at 100° under reduced pressure. *m*-Hydroxybenzamide was prepared in the same way as *p*-hydroxybenzamide (Bray *et al.* 1947). Yield: 38% of the ester used. M.p. 170°.

Gentisic acid. This reference compound was prepared by the method of Brunner (1907). M.p. 200–202°. Methyl gentisate, m.p. 87–88°, was prepared from it as described by Raistrick & Simonart (1933).

Gentisamide. This was prepared by refluxing methyl gentisate (1 g.) with ammonia sp.gr. 0.88 (10 ml.) for 2 hr. Concentration of the solution gave *gentisamide*, which on treatment with charcoal and recrystallization from water formed fine white needles, m.p. 217–218°. Yield 60% of theory. Found: C, 54.5; H, 4.63; N, 9.07. $C_7H_7O_3N$ requires C, 54.9; H, 4.58; N, 9.15%. *Gentisamide* gave an intense blue colour with $FeCl_3$, reduced ammoniacal $AgNO_3$, but gave a yellow, instead of pink, colour with Lutwak-Mann's (1943) test for gentisic acid.

RESULTS

Quantitative studies

Ether-soluble acid excretion. For the rabbits used in these studies the average normal excretion of ether-soluble acid (calculated as hippuric acid) ranged from 635 to 847 mg./day. Table 1 shows the percentage of doses of the four compounds under investigation, excreted as ether-soluble acid, calculated in all cases as hydroxybenzoic acid. No attempt was made to fractionate the ether-soluble acid, but since, as is described later, the amounts of glycine conjugates excreted appear to be negligible, the error introduced by assuming that the increase in acid excretion is entirely due to *o*- or *m*-hydroxybenzoic acids will be correspondingly small.

Excretion of reducing material. The average normal daily excretion of reducing substances (calculated as glucuronic acid) by the rabbits used in this investigation ranged from 135 to 219 mg., and the average hydrolyzed reducing values from 176 to 378 mg. Table 2 summarizes the results obtained.

Excretion of ethereal sulphate. The average normal daily excretion of ethereal sulphate ranged from 40 to 64 mg. Table 3 shows the percentage of doses of the compounds excreted conjugated with sulphate. The results for the acids agree with those obtained by Williams (1938), but those for the amides are much lower, viz. 13–16 and 10–19% as compared with 29–40 and 32–44% respectively. The diet in Williams's study consisted of bran, oats and cabbage, while in the present study pellets were given. This difference in diet is probably the reason for the divergence of the results obtained, since with *p*-hydroxybenzoic acid and amide, when the rabbits received the bran, oats and cabbage diet, the values

were obtained were in agreement with those of Williams (Bray *et al.* 1947); Braunstein, Parschin & Chalisowa (1931) have investigated the effect of diet on the metabolism of phenol, and concluded that rabbits on an acid-forming diet conjugate this compound to a greater extent than those on an alkali-forming diet. This is particularly marked in the case

Table 1. *Excretion of ether-soluble acid (calculated as hydroxybenzoic acid) after doses of o- and m-hydroxybenzoic acids and amides**

Rabbit no.	Approx. dose (g./kg.)	Percentage of acid excreted as ether-soluble acid	Percentage of amide excreted as ether-soluble acid
Ortho compounds			
117	0.25	76	2
135	0.25	84	0
145	0.25	80	0
147	0.25	98	0
117	0.5	93	0
135	0.5	88	0
145	0.5	79	0
147	0.5	81	0
Meta compounds			
119	0.25	78	—
130	0.25	76	—
140	0.25	57	—
143	0.25	69	—
119	0.5	94	—
130	0.5	64	9
140	0.5	87	19
143	0.5	92	14
119	1.0	87	—
119	1.0	101	11
130	1.0	107	10
140	1.0	97	19
143	1.0	95	4
143	1.0	—	6
119	1.5	97	0
130	1.5	99	3
140	1.5	97	8
143	1.5	96	3

* All values in Tables 1, 2 and 3 are calculated neglecting the formation of gentisic acid which is excreted conjugated with sulphuric acid (Likhatscheff, 1895–6) and probably also with glucuronic acid. The fact that gentisic acid itself is reducing is also ignored.

of ethereal sulphate formation. Injection of phenol (500 mg.) into rabbits on the 'acid' diet caused an increase in ethereal sulphate excretion of 82 mg. above the normal value, while with rabbits on the 'alkaline' diet, the corresponding increase was only 5 mg. The increase on a 'mixed' diet was 22 mg. Our observations are in accordance with these findings, inasmuch as the diet used by Williams and by us in our earlier investigation was an 'acid' diet and the pellet diet in this study 'alkaline'.

Table 2. *Percentage of doses of o- and m-hydroxybenzoic acids and amides excreted as ester- and ether-type glucuronides**

Rabbit no.	Approx. dose (g./kg.)	Percentage of acid excreted as		Percentage of amide excreted as	
		Ester glucuronide	Ether glucuronide	Ester glucuronide	Ether glucuronide
		Ortho compounds			
117	0.25	8	5	8	71
135	0.25	7	1	7	83
145	0.25	0	0	3	69
147	0.25	0	12	7	82
117	0.5	14	11	5	64
135	0.5	12	15	4	58
145	0.5	10	14	7	76
147	0.5	15	16	1	71
Meta compounds					
130	0.25	14	24	13	56
135	0.25	8	22	14	66
138	0.25	13	10	17	53
140	0.25	8	19	22	63
130	0.5	6	10	0	32
136	0.5	6	—	—	—
138	0.5	13	—	0	39
140	0.5	5	14	1	30
140	0.5	3	—	—	—
143	0.5	—	8	0	40
119	1.0	6	6	—	—
130	1.0	4	8	1	47
130	1.0	—	—	0	—
136	1.0	7	11	—	—
138	1.0	7	9	1	35
140	1.0	5	1	2	25
143	1.0	—	—	1	22
143	1.0	—	—	0	15
130	1.5	5	1	0	21
136	1.5	6	0	—	—
138	1.5	10	5	0	27
140	1.5	4	0	1	23
143	1.5	—	—	1	29

* See footnote to Table 1.

*Qualitative studies**Isolation of metabolites of salicylic acid and salicylamide*

Gentisic acid (2:5-dihydroxybenzoic acid). Urine (24 hr.) was collected from 40 rabbits which had received 40 g. salicylic acid as its sodium salt, and hydrolyzed by boiling for 20 min. with 0.2 vol. conc. HCl. It was then continuously extracted with ether for 48 hr., and the ethereal extract thoroughly washed with NaHCO₃ solution. The aqueous extract thus obtained was acidified with 5N-H₂SO₄ and extracted with ether. After removal of ether the ether-soluble material was dissolved in water, and the resulting solution extracted with chloroform until all the salicylic acid was removed as indicated by the FeCl₃ colour given by successive extracts. The aqueous solution then gave an intense deep blue colour with FeCl₃ and reduced Benedict's solution and ammoniacal AgNO₃. On evaporation a yellow syrup remained which yielded fine white needles,

m.p. 198–201°. After recrystallization from ether-light petroleum the m.p. was 202–203°. Yield 1.5–2.0 g. from 40 g. salicylic acid. The identity of this product with gentisic acid (m.p. 200–202°) was established by the mixed m.p. with an authentic specimen. (Found: C, 54.4; H, 3.95. Calc. for C₇H₆O₄: C, 54.6; H, 3.9 %.) The methyl ester (m.p. 87–89°) mixed with methyl gentisate (m.p. 87–88°) gave m.p. 87–89°.

When a similar procedure was applied to urine from rabbits which had received salicylamide, about 5 mg. of gentisamide (m.p. 215–216°) were isolated. Its identity was confirmed by mixed m.p. determination, colour with FeCl₃, response to Lutwak-Mann's test and reduction of ammoniacal AgNO₃.

In a second experiment with salicylamide urine only a very small amount of crystalline material m.p. 170–180° was obtained. It gave a similar blue colour with FeCl₃ and contained only 1.54% N. There was not sufficient material for further investigation. The product may be a complex similar

to the 'uraminosalicylic acid' isolated by Baldoni (1908) and by Kapp & Coburn (1942) which contained 3.7-4% N. It is, however, perhaps more reasonable to suggest that the complex from salicylamide might be a mixture of gentisic acid and gentisamide.

Table 3. Percentages of doses of *o*- and *m*-hydroxybenzoic acids and amides excreted conjugated with ethereal sulphate*

Rabbit no.	Approx. dose (g./kg.)	Percentage of acid excreted as ethereal sulphate	Percentage of amide excreted as ethereal sulphate
Ortho compounds			
145	0.25	0	11
147	0.25	0	14
117	0.5	0	12
135	0.5	0	19
Meta compounds			
130	0.25	6	21
140	0.25	2	16
136	0.5	3	—
138	0.5	1	—
140	0.5	—	16
143	0.5	—	20
119	1.0	2	—
130	1.0	3	—
138	1.0	—	10
140	1.0	1	—
143	1.0	—	9
143	1.0	—	14
130	1.5	3	11
140	1.5	1	9

* See footnote to Table 1.

2-Carbamylphenylglucuronide. This was isolated from salicylamide urine by the method described for 4-carbamylphenylglucuronide (Bray *et al.* 1947). The barium salt could not be obtained crystalline but the glucuronide itself crystallized readily. Yield 2 g. from 12 g. amide, m.p. 175-176° (decomp.); $[\alpha]_D^{25.5} - 84^\circ$ (water; c, 1). It did not reduce Benedict's solution. (Found: C, 49.8; H, 4.9; N, 4.3; glucuronic acid (after 5 min. hydrolysis with 2N-HCl) 59.02; equivalent (by titration) 315. $C_{15}H_{15}O_8N$ requires C, 49.9; H, 4.8; N, 4.5; glucuronic acid 62.0%; equivalent 313.)

Attempted isolation of salicyluric acid (*o*-hydroxyhippuric acid). As already mentioned, there has been considerable disagreement about the conjugation of salicylic acid with glycine in animals, although it may be accepted that this occurs to some extent in man. We made several attempts to isolate salicyluric acid from rabbits' salicylate urine by fractionation of the ether-soluble acid by means of various solvents (e.g. toluene, light petroleum), but in every case we were able to isolate only unchanged salicylic acid and hippuric acid. That the failure of

the rabbit to form salicyluric acid was not due to a lack of glycine in the diet was evident from the fact that we were still unable to isolate salicyluric acid from the urine of rabbits which had been given glycine (0.3 g.) along with the salicylic acid (1 g.). The reliability of our technique was shown by the ease with which we isolated salicyluric acid from the urine of children under salicylate therapy, using toluene for fractionation of the ether-soluble acid. The product had m.p. 168-170° (mixed m.p. with hippuric acid 151°) and contained 6.79% N. (Calc. for $C_9H_7O_4N$: N, 7.18%.)

Isolation of metabolites of *m*-hydroxybenzoic acid and amide

Gentisic acid. It is probable that this compound is a metabolite of both acid and amide. The $FeCl_3$ colour given by products isolated from hydrolyzed *m*-hydroxybenzoic acid and amide urines was deep blue, very similar to that given by gentisic acid, but it could not be assumed that this was the hydroxylation product, since 2:3-hydroxybenzoic acid, which is a theoretically possible metabolite, also gives a blue colour with $FeCl_3$ and has a similar m.p. (204°). The intensity of the colour given by products from *m*-hydroxybenzoic acid urine suggested that the amount formed was too small to encourage the hope of isolation. Attempts to isolate a dihydroxybenzoic acid from *m*-hydroxybenzamide urine were made by methods similar to that described in the previous section.

The urine excreted by 14 rabbits, each of which had received *m*-hydroxybenzamide (3 g.), was hydrolyzed and extracted with ether as previously described. The ether-soluble material thus obtained was dissolved in water, and the solution extracted with chloroform, which removed most of the *m*-hydroxybenzoic acid present. The aqueous solution was then evaporated to dryness, the residue dissolved in ether, and this solution extracted with $NaHCO_3$ solution. The extract was treated as for the isolation of gentisic acid. In this way more *m*-hydroxybenzoic acid, together with some hippuric acid, was crystallized. Recrystallization of the remaining solid from glacial acetic acid gave 100 mg. colourless needles, m.p. 184-185°, which gave an intense blue colour with $FeCl_3$. Further recrystallizations from water failed to raise the m.p. to that of gentisic acid. Other attempts at isolation yielded similar products whether purification was by fractional crystallization or sublimation *in vacuo* at 140°. Analysis indicated the presence of about 1.6% N, an amount similar to that of the product obtained in one experiment from salicylamide (see above). (Found: C, 57.3; H, 4.32; N, 1.6. Calc. for $C_7H_5O_4$: C, 54.5; H, 3.9%.) We were unable to obtain a nitrogen-free product, even after a further period of heating with conc. HCl. That the substance

contained gentisic acid (or amide) is suggested by the fact that, on heating with pumice powder, a distillate was obtained which was identified as hydroquinone by mixed m.p. and mixed m.p. of the acetyl derivatives. Gentisic acid (or amide) is the only dihydroxybenzoic acid (or amide) which would give hydroquinone under these conditions. 2:3-Dihydroxybenzoic acid would yield catechol. The distillate gave only the feeble fugitive blue-green colour with FeCl_3 typical of hydroquinone and no suggestion of the stable and definite colours given by catechol or resorcinol. We conclude, therefore, that gentisic acid (or amide) is a metabolite of *m*-hydroxybenzamide, although its isolation and positive identification is prevented by the formation of a nitrogen-containing complex.

3-Carbamylphenylglucuronide. This compound was isolated by the method used for the other isomers. Crude barium salt (56 g.) was obtained after the administration of 27 g. *m*-hydroxybenzamide. Recrystallization gave a *product* m.p. 270° (decomp.), $[\alpha]_D^{25.5} - 59^\circ$ (water; *c*, 1). (Found: N, 3.12. $\text{C}_{13}\text{H}_{15}\text{O}_6\text{N}_2\text{Ba}$ requires N, 3.35 %.) The *glucuronide* itself forms plates m.p. $163-164^\circ$ (decomp.), $[\alpha]_D^{25.5} - 67^\circ$ (water; *c*, 1). Yield 11 g. (Found: C, 49.1; H, 5.0; N, 4.3; glucuronic acid, 64.6; equivalent (by titration) 339. $\text{C}_{13}\text{H}_{15}\text{O}_6\text{N}$ requires C, 49.9; H, 4.8; N, 4.5; glucuronic acid, 62.0 %; equivalent 313.)

Attempted isolation of *m*-hydroxyhippuric acid. We could not isolate this glycine conjugate from *m*-hydroxybenzoic acid urine by the method used successfully for the *p*-isomer (Bray *et al.* 1947). Unconjugated *m*-hydroxybenzoic acid and hippuric acid were readily isolated. We were still unable to isolate *m*-hydroxyhippuric acid after administering glycine (1 g.) to the rabbit along with the *m*-hydroxybenzoic acid (3 g.). It is of interest to note that *m*-hydroxyhippuric acid has not been claimed as a metabolite of *m*-hydroxybenzoic acid in the rabbit, but only in man and the dog. We have been unable to confirm the formation of this conjugate in man. Following Quick's (1932*b*) procedure, *m*-hydroxybenzoic acid and hippuric acid, but no *m*-hydroxyhippuric acid, were isolated from the urines of two human subjects who had each ingested *m*-hydroxybenzoic acid (3 g.) with glycine (1 g.). Our investigation of *m*-hydroxyhippuric acid was rendered more difficult by our failure to prepare it. The literature contains only two accounts of its preparation, in both cases from *m*-aminohippuric acid (Griess, 1868; Conrad, 1877). Neither provides unequivocal evidence for its existence. We were unable to obtain it also by Fischer's (1908, 1909) carbomethoxy method which is satisfactory for the *o*- and *p*-isomers. It should be noted that the claims of neither Baumann & Herter (1877-8) nor Quick (1932*b*) to have identified this compound as a meta-

bolite of *m*-hydroxybenzoic acid are supported by comparison with a synthetic specimen.

DISCUSSION

Table 4 summarizes the results of all our investigations of the metabolism of hydroxybenzoic acids and amides. It is evident that there are considerable differences between the fates of each acid and its amide. The degrees of hydrolysis of the amides are: ortho, 4-7; meta, 4-16; para, 0-7 %. These values are comparable with those obtained from *in vitro* studies with liver extracts (Bray, James, Ryman & Thorpe, 1948), i.e. ortho, 0; meta, 1; para, 6 %. The presence of a hydroxyl group stabilizes the carbamyl group to a considerable extent, for benzamide itself is readily hydrolyzed to benzoic acid (Bray, Neale & Thorpe, 1946).

The investigation reported here raises several points of interest. The formation of gentisic acid as a hydroxylation product of salicylic acid and of gentisamide from salicylamide is in accordance with the general rule put forward in a previous paper (Bray, Lake, Neale, Thorpe & Wood, 1948). The fact that salicylic acid is hydroxylated to a greater extent than salicylamide is, however, unusual, since in most other cases, e.g. the aminobenzoic acids and amides, the opposite is true. A large number of so-called 'detoxication' reactions which foreign compounds undergo in the organism may be interpreted as occurring as a result of the formation of 'centres for conjugation' in the molecule, i.e. groups which can be conjugated with sulphuric, glucuronic and acetic acids, or with glycine. Thus, in compounds which already possess carboxyl and hydroxyl groups, conjugation is usually the principal mechanism, although a relatively small amount of hydroxylation occurs in some instances (e.g. *p*-hydroxybenzoic acid). If a 'potential' centre for conjugation is present, a possible reaction is its conversion to an 'actual' centre, e.g. the oxidation of a methyl group, as in acetotoluidides (Jaffe & Hilbert, 1888; Bray & Thorpe, 1948), the hydrolysis of a carbamyl group to carboxyl as in benzamide (Bray *et al.* 1946), or the reduction of an aromatic nitro group to amino as in *m*-nitrobenzaldehyde (Cohn, 1893). If, however, the 'potential' centre is not converted in this way, or converted to only a limited extent, e.g. as when the carbamyl group is stabilized by the presence of another group, as in the hydroxy- or amino-benzamides (Bray, Lake, Neale, Thorpe & Wood, 1948), a new centre may be introduced by hydroxylation even if, as in *p*-hydroxybenzamide, one is already present though here the extent of hydroxylation is small. It was, therefore, unexpected to find that salicylamide was hydroxylated to a lesser extent than salicylic acid. The factor which determines the extent of these reactions is probably the

Table 4. *Metabolites of hydroxybenzoic acids and amides in rabbit*

Compound administered	Approx. dose (g./kg.)	Average percentage of dose excreted as				Approx. % hydrolysis of amide
		Ether soluble acid	Ester glucuronide	Ether glucuronide	Ethereal sulphate	
<i>o</i> -Hydroxybenzoic acid*	0.25	85	4	5	0	—
	0.5	85	3	14	0	—
<i>o</i> -Hydroxybenzamide*	0.25	1	6	76	13	7
	0.5	0	4	67	16	4
<i>m</i> -Hydroxybenzoic acid*	0.25	70	11	19	4	—
	0.5	84	7	11	2	—
	1.0	97	6	7	2	—
	1.5	97	6	2	2	—
<i>m</i> -Hydroxybenzamide*	0.25	—	16	60	19	16†
	0.5	14	0	36	18	14
	1.0	10	1	28	11	11
	1.5	4	0	25	10	4
<i>p</i> -Hydroxybenzoic acid‡	0.1	66	16	19	4	—
	0.25	65	5	10	7	—
	0.5	75	4	6	7	—
	1.0	73	6	12	5	—
	1.5	64	7	8	5	—
<i>p</i> -Hydroxybenzamide‡	0.1	2	0	15	35	2
	0.25	0	0	15	23	0
	0.5	7	0	15	17	7
	1.0	3	1	24	17	4
	1.5	5	1	22	14	6

* Diet: pellets.

† This value is low since it does not include ether-soluble acid excretion.

‡ Diet: bran, oats and cabbage (Bray *et al.* 1947).

availability of suitable enzyme systems to which the compound under consideration is acceptable. It would seem that the configuration of salicylamide is even less acceptable to the enzyme responsible for hydroxylation than is salicylic acid. It may be noted that *o*-substituted compounds are often metabolized to a smaller extent than the corresponding *m*- and *p*-isomers (e.g. sulphate conjugation of hydroxybenzoic acids, acetylation of aminobenzoic acids). The formation of gentisic acid as a hydroxylation product of *m*-hydroxybenzoic acid or its amide would be in accordance with our general rule.

With regard to the nature of the hydroxylation products derived from the hydroxybenzoic acids and amides it is of interest to refer to an investigation of Dakin & Herter (1907). It is well known that hydrogen peroxide under various conditions can hydroxylate benzene derivatives, giving in many cases products identical with those formed as metabolites of the original compound, e.g. benzene gives phenol, catechol and hydroquinone (e.g. Cross, Bevan & Heiberg, 1900; Baernstein, 1946). Dakin & Herter found that hydrogen peroxide oxidized ammonium salicylate to 2:3-dihydroxybenzoic acid and the ammonium salts of both the *m*- and *p*-isomers to protocatechuic acid (3:4-dihydroxybenzoic acid). (In spite of several attempts we failed to obtain 2:3-dihydroxybenzoic acid in this way, although we had no difficulty in isolating protocatechuic acid by oxidizing *m*- or *p*-hydroxybenzoic

acid using Dakin & Herter's directions.) 2:3-Dihydroxybenzoic acid has not been reported as a metabolite of salicylic acid, and we were unable to detect the formation of protocatechuic acid from *m*-hydroxybenzoic acid, although we showed it to be a metabolite of both *p*-hydroxybenzoic acid and its amide (Bray *et al.* 1947). We are also investigating the effect of hydrogen peroxide on the hydroxybenzamides, and hope to extend the study to a wider comparison of *in vitro* and *in vivo* hydroxylation.

SUMMARY

1. The metabolism of *o*- and *m*-hydroxybenzoic acids and their amides in the rabbit has been investigated.

2. A large proportion (85%) of salicylic acid is excreted as ether-soluble acid, 3–4% as ester glucuronide and 5–14% as ether glucuronide: the corresponding values for salicylamide are 0–1, 4–6 and 67–76%. Salicylic acid does not affect ethereal sulphate excretion, but 13–16% of salicylamide is excreted conjugated with sulphuric acid.

3. The percentages of *m*-hydroxybenzoic acid excreted as ether-soluble acid, ester glucuronide, ether glucuronide and ethereal sulphate are 70–97, 6–11, 2–19 and 2–4 respectively. The corresponding values for *m*-hydroxybenzamide are 4–14, 0–16, 25–60 and 10–19%.

4. From the above results it is calculated that the degree of hydrolysis of the carbamyl group of salicylamide is 4-7 and of *m*-hydroxybenzamide 4-16%.

5. 2- and 3-carbamylphenylglucuronides have been isolated as major metabolites of salicylamide and *m*-hydroxybenzamide respectively.

6. Gentisic acid (4-5%) has been isolated as a metabolite of salicylic acid and gentisamide of salicylamide.

7. Evidence has been obtained of the formation of gentisic acid (or amide) as a metabolite of *m*-hydroxybenzoic acid and amide.

8. Glycine conjugates of *o*- and *m*-hydroxybenzoic acids were not detected.

9. The fact that salicylic acid is hydroxylated to an extent greater than is salicylamide is discussed.

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Growth Factors for *Lactobacillus casei*

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We have described elsewhere (Chattaway, Happold, Lythgoe, Sandford & Todd, 1943; Chattaway, Happold & Sandford, 1944) the presence of a growth factor for *Corynebacterium diphtheriae gravis*, sub-type Dundee, in the amyl alcohol insoluble fraction obtained by extracting liver residues with this solvent at pH 2, and the separation of this factor from material stimulating the growth of *Lactobacillus casei* at pH 3, the *Lb. casei* factors being in the cresol extract.

The present paper describes the further concentration and separation of the factors promoting acid production by *Lb. casei* (Chattaway, Dolby & Happold, 1944), and Factor 3 of Dolby, Happold & Sandford (1944), and considers their relationships to

factors reported as stimulatory for this organism by other workers. Among these factors are fractions from liver, fermentation residues and yeast, which have been shown to be members of the pteroyl-glutamic acid (PGA) group of components, differing from one another in having one, three or seven glutamyl residues respectively (Angier, Boothe, Cosulich, Fahrenbach, Hultquist, Hutchings, Kuh, Mowat, Northey, Seager, Semb, Sickels, Smith, Stokstad, Subbarow & Waller, 1945, 1946; Bloom, Calkins, O'Dell & Pfiffner, 1946). The material known as folic acid on the other hand (Mitchell, Snell & Williams, 1941) is probably different from these, as also is the '*Streptococcus lactis* R' factor (Keresztesy, Riekes & Stokes, 1943) which has been shown