Studies in Detoxication

18. A STUDY OF THE RELATION BETWEEN CONJUGATION AND DEAMINATION OF *p*-HYDROXYBENZYLAMINE AND RELATED COMPOUNDS IN THE RABBIT

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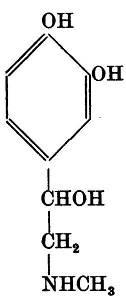
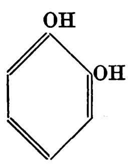
The present work was carried out as part of a programme of research on the inactivation of L-adrenaline (I) in the body. There are two views of the mode of inactivation of adrenaline: briefly, the first is that the inactivation involves monoamine oxidase which oxidatively deaminates adrenaline, and the second is that the inactivation takes place through conjugation of the hydroxyl groups of adrenaline to produce an inactive ethereal sulphate (cf. Richter, 1940; Richter & MacIntosh, 1941–2).

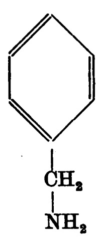
Our plan was to investigate the fate in the body of compounds containing the various structural elements of the adrenaline molecule. Our studies have included, for example, investigations on the metabolism of catechol (II) (Garton & Williams, 1948), *p*-hydroxybenzylamine (IV), *p*-hydroxybenzylmethylamine (V), protocatechuic acid (VI) and D-adrenaline (I) (Dodgson, Garton & Williams, 1947).



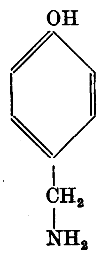
(I)



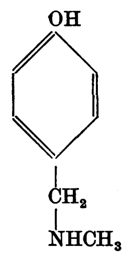
(II)



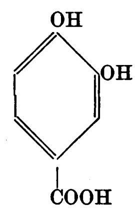
(III)



(IV)



(V)



(VI)

The fate of benzylamine (III) has been studied both *in vivo* and *in vitro*. It is oxidized *in vitro* by amine oxidase preparations from liver and intestinal tissue at about one tenth the rate at which tyramine is oxidized (Blaschko, Richter & Schlossman, 1937), and in the dog it is converted almost quantitatively into hippuric acid (Mosso, 1890), the mechanism of transformation (cf. Green, 1941) being probably as follows:

$$C_6H_5CH_2NH_2 \rightarrow C_6H_5CH{:}NH \rightarrow C_6H_5CHO \rightarrow C_6H_5COOH \rightarrow C_6H_5CONHCH_2COOH.$$

In *p*-hydroxybenzylamine (IV) two changes can take place *in vivo*, namely, conjugation of the OH group and oxidative deamination of the —CH_2NH_2 group, and it was our aim to find out how one process influenced the other. We shall show in this paper that conjugation of the OH group is related to the rate of deamination and oxidation of the —CH_2NH_2 group.

METHODS

Animals. Chinchilla rabbits, 2–3 kg. wt., on a diet of 50 g. Lever's cubes and water *ad lib.* were used throughout.

Analytical methods. Ethereal sulphate was determined by the Folin gravimetric method (cf. Williams, 1938), glucuronic acid by the method of Hanson, Mills & Williams (1944), and free and combined *p*-hydroxybenzoic acid by the method of Quick (1932) which is based on the bromination method of Day & Taggard (1928).

MATERIALS

A. *p*-Hydroxybenzoic acid, m.p. 213° (all melting points are uncorrected), was prepared by hydrolysis of ethyl *p*-hydroxybenzoate. B. *p*-Hydroxybenzaldehyde, m.p. 115°, was a purified commercial sample. C. *p*-Hydroxybenzylamine was fed as the monohydrate, m.p. 98°, and prepared according to Tiffeneau (1911).

D. *Preparation of* p-*hydroxybenzylacetamide* (N-*acetyl*-p-*hydroxybenzylamine*). This compound appears to be new. *p*-Hydroxybenzylamine (5 g.) was dissolved in a solution of 10 g. K_2CO_3 in 25 ml. water: 10 ml. acetic anhydride were added slowly with shaking, the temperature being maintained below 5°. An oil separated which solidified on standing. The solid was broken up, washed with cold water, dried and recrystallized from benzene. p-*Acetoxybenzylacetamide*, m.p. 62°, forms needles easily soluble in ethanol but insoluble in water (yield, 5 g.). (Found: C, 63·8; H, 6·45; N, 6·7. $C_{11}H_{13}O_3N$ requires C, 63·75; H, 6·3; N, 6·8%.) *p*-Acetoxybenzylacetamide (5 g.) was suspended in a solution of 5 g. KOH in 50 ml. water, and the mixture was refluxed gently until the solid dissolved. Heating was continued until no oil globules separated on cooling. The solution was now cooled and acidified with HCl. On standing at 0° long white needles of p-*hydroxybenzylacetamide* separated. It was recrystallized from hot water (yield 3·5 g.) and had m.p. 125°. It was slightly soluble in cold water but readily soluble in hot, and it gave a deep violet colour with $FeCl_3$. (Found: C, 65·55; H, 6·8; N, 8·6. $C_9H_{11}O_2N$ requires C, 65·4; H, 6·7; N, 8·5%.)

E. *Preparation of* p-*hydroxybenzylmethylamine*. The method of Tiffeneau (1911) for preparing this amine by heating anisyl chloride with methylamine in a sealed tube was found unsatisfactory owing to the formation of the tertiary di(*p*-hydroxybenzyl)methylamine. We therefore devised a new synthesis.

A solution of 9 g. anisylacetamide (m.p. 97°; Tiffeneau, 1911) in 100 ml. dry benzene was refluxed for 3 hr. with 4 g. finely divided sodamide. The solution, now containing the sodio derivative of anisylacetamide, was cooled and treated with 8 ml. methyl iodide. The mixture was gently refluxed for 10 min., then the NaI which separated was filtered off and the benzene removed by distillation *in vacuo*. The residual *anisylmethylacetamide* was a pale yellow oil (10 g.). This oil was refluxed with 100 ml. N-*n*-butanolic KOH for 2 hr. to remove the acetyl group. The resulting solution was cooled, made strongly acid with conc. HCl and then extracted with 3 × 25 ml. portions of water. The aqueous extract, which contained anisylmethylamine hydrochloride, was made alkaline with solid KOH and extracted with ether. After drying the ethereal solution with solid KOH and filtering, the anisylmethylamine was precipitated as the hydrochloride by passing a stream of dry HCl through the solution. The yield of hydrochloride was 5 g.; it was crystallized from ethanol-ether and had m.p. 165° (Tiffeneau (1911) gives m.p. 166°). Anisylmethylamine was converted into *p*-hydroxybenzylmethylamine hydrochloride (m.p. 185°) according to Tiffeneau (1911).

RESULTS

A. *Experiments with* p-*hydroxybenzoic acid*

(1) *Ethereal sulphate and glucuronic acid outputs.* The figures in Table 1 indicate that about one quarter of the *p*-hydroxybenzoic acid, fed at a dose level of 0·35 g./kg., is excreted conjugated through its hydroxyl group, and that the ratio glucuronic acid/ethereal sulphate is about 2. Our figures are a little higher than those obtained in a more detailed study by Bray, Ryman & Thorpe (1947) for doses of 0·25 g./kg., but our results lead to essentially the same conclusion, viz. that the extent of conjugation of *p*-hydroxybenzoic acid through its hydroxyl group is in the region of 20–30% of the dose.

(2) *Isolation of* p-*hydroxyhippuric acid.* According to Bray *et al.* (1947) about 20–30% of the dose of *p*-hydroxybenzoic acid is conjugated with glycine. We did not repeat this estimation, but isolated the conjugated product.

A total of 6 g. of *p*-hydroxybenzoic acid was fed to 3 rabbits and a 24 hr. urine (350 ml.) collected. The basic lead acetate precipitate of the urine was prepared in the usual manner. This was suspended in water and the Pb removed with H_2S. The filtrate from PbS was continuously extracted with ether for 2 hr. to extract *p*-hydroxybenzoic and *p*-hydroxyhippuric acids. On evaporating the ether a white crystalline residue remained. This residue was extracted with small portions of ether to remove *p*-hydroxybenzoic acid and there remained 300 mg. (3·6% of the dose) of *p*-hydroxyhippuric acid, which after recrystallization from water had m.p. 238° (Bray *et al.* (1947) and Fischer (1908) give m.p. 240°).

(3) *The glucuronide in* p-*hydroxybenzoic acid urine.* Quick (1932) has reported the isolation, from the urine of dogs receiving *p*-hydroxybenzoic acid orally, of *p*-glucuronosidobenzoylglucuronide, a substance which in virtue of its ester glucuronide link reduces alkaline copper reagents. We have been unable to isolate such a glucuronide from rabbit urine and our evidence indicates that, if such a glucuronide is formed, then it must be present in very small amounts (cf. Bray *et al.* 1947).

We found that *p*-hydroxybenzoic acid urines from rabbits were non-reducing, and conclude that the main glucuronide excreted is probably the non-reducing *p*-carboxyphenylglucuronide. We were, however, unable to isolate this substance or a derivative of it in a crystalline state. It was obtained from the basic lead acetate precipitate of *p*-hydroxybenzoic acid urine as a non-reducing yellowish gum. Attempts to form a triacetyl methyl ester did not result in crystalline material. The glucuronide gum (0·5 g.) was hydrolyzed by boiling with 15 ml. of 3–4 N-HCl for 0·5 hr. Ether extraction of the hydrolysate yielded only *p*-hydroxybenzoic acid, m.p. and mixed m.p. 213° (yield of pure material 0·1 g.).

B. *Experiments with* p-*hydroxybenzaldehyde*

(1) *Ethereal sulphate and glucuronic acid outputs.* Table 2 shows that about 40% of the aldehyde is excreted conjugated through its hydroxyl group and

Table 1. *The ethereal sulphate and glucuronic acid conjugations of* p-*hydroxybenzoic acid in the rabbit*

		Dose		Ethereal sulphate as SO_3		Glucuronic acid		Dose excreted as		
Rabbit no.	Wt. (kg.)	(mg.)	(mg./kg.)	Mean normal value (mg./day)	Increase after dosing (mg.)	Mean normal value (mg./day)	Increase after dosing (mg.)	Sulphate (%)	Glucuronide (%)	Total conjugation (%)
68	2·9	1002	345	19·9	43·8	186·5	239·8	7·7	17·0	24·7
82	2·9	999	344	25·1	47·8	195·3	141·4	8·8	10·1	18·9
84	2·85	1009	253	16·6	66·2	148·6	377·1	12·1	26·6	38·7

the glucuronic acid/ethereal sulphate ratio is about 3·5. The extent of total conjugation is greater than with *p*-hydroxybenzoic acid, the glucuronic acid conjugation of the aldehyde being twice that of the acid, although the sulphate conjugations are the same (cf. Williams, 1938). Since *p*-hydroxybenzaldehyde is largely transformed *in vivo* into *p*-hydroxybenzoic acid (Quick, 1932; Dakin, 1910), it is highly probable, in view of the higher glucuronic acid excretion provoked by the aldehyde, that some *p*-hydroxybenzaldehyde is conjugated with glucuronic acid before its aldehyde group becomes oxidized. In an earlier paper on vanillin (Sammons & Williams, 1941 *a*) it was shown that the aldehyde glucuronide could be detected in urine in small amounts during the early hours after dosing.

Table 2. *The ethereal sulphate and glucuronic acid conjugations of* p-*hydroxybenzaldehyde in the rabbit*

		Dose		Ethereal sulphate as SO_3		Glucuronic acid		Dose excreted as		
Rabbit no.	Wt. (kg.)	(mg.)	(mg./kg.)	Mean normal value (mg./day)	Increase after dosing (mg.)	Mean normal value (mg./day)	Increase after dosing (mg.)	Sulphate (%)	Glucuronide (%)	Total conjugation (%)
68	2·75	753	274	14·9	52·7	177·5	391·7	10·7	32·7	43·4
82	2·8	752	269	20·2	45·3	173·8	396·5	9·2	33·1	42·3
84	2·75	751	273	23·9	36·8	197·6	388·1	7·7	32·5	40·2

Table 3. *The ethereal sulphate and glucuronic acid conjugations of* p-*hydroxybenzylamine in the rabbit*

		Dose		Ethereal sulphate as SO_3		Glucuronic acid		Dose excreted as	
Rabbit no.	Wt. (kg.)	(mg.)	(mg./kg.)	Mean normal output (mg./day)	Increase after dosing (mg.)	Mean normal output (mg./day)	Increase after dosing (mg.)	Ethereal sulphate (%)	Glucuronide (%)
91	2·5	624	250	38·1	55·5	—	—	15·7	—
92	2·5	644	258	23·6	80·8	—	—	22·1	—
93	3·0	798	270	37·3	83·9	—	—	18·5	—
99	2·7	663	250	—	—	225·9	406·6	—	44·6
101	2·7	680	252	—	—	221·9	321·8	—	34·4
104	2·5	622	250	—	—	184·0	323·5	—	38·4
							Average	18·8	39·4
							Average total conjugation		58·2

(2) *Isolation of* p-*glucuronosidobenzaldehyde-2:4-dinitrophenylhydrazone. p*-Hydroxybenzaldehyde (8 g.) was fed to 4 rabbits, and the urine was collected for the following 5 hr. The urine gave a red colour with naphthoresorcinol and HCl in the cold, thus indicating that either *p*-hydroxybenzaldehyde or an *O*-conjugated aldehyde was being excreted (colour reaction of Sammons & Williams, 1941 *b*). The urine (300 ml.) was filtered first through cotton wool and then through filter paper, and to it was added 15 ml. of conc. HCl followed by a solution of 1 g. 2:4-dinitrophenylhydrazine in 15 ml. conc. HCl and 100 ml. ethanol. The mixture was kept at 0° overnight, during which time an orange-red precipitate separated. More ethanol was added and the precipitate was filtered and washed with dilute HCl, water and finally ethanol (yield 0·3 g.). The solid was extracted with hot ethanol, and on cooling the extracts there was deposited a reddish orange microcrystalline solid, m.p. 166° (yield 125 mg.). The compound gave positive tests for glucuronic acid, and after acid hydrolysis it gave a red colour for *p*-hydroxybenzaldehyde in the Sammons & Williams (1941 *b*) colour reaction. These tests and elementary analysis indicated it to be p-*glucuronosidobenzaldehyde-2:4-dinitrophenylhydrazone*. (Found: C, 44·4; H, 4·2; N, 11·6. $C_{19}H_{18}N_4O_{11}.2H_2O$ requires C, 44·4; H, 4·3; N, 10·9%.) A sample of this compound had been prepared by one of us in another investigation and it had m.p. 165° and mixed m.p. with the above sample 166°. (Found: C, 44·5; H, 4·4; N, 11·3%.)

The yield of the hydrazone corresponded to about 2–3% (6% in one case) of the aldehyde fed. None of the conjugated aldehyde could be detected in the urines collected later than 5 hr. after feeding.

C. *Experiments with* p-*hydroxybenzylamine*

(1) *The ethereal sulphate and glucuronic acid conjugations.* The results are given in Table 3 which shows that about 58% of the *p*-hydroxybenzylamine is excreted as *O*-conjugates and that the ratio glucuronic acid/ethereal sulphate is roughly 2. The glucuronic acid conjugation (39%) is slightly higher than that for *p*-hydroxybenzaldehyde (33%), but the ethereal sulphate conjugation (19%) is double that of both *p*-hydroxybenzoic acid and aldehyde (9%). These results could be interpreted as meaning that the amine is partly sulphated before deamination, but conjugated with glucuronic acid after conversion to the aldehyde but before conversion to the acid.

(2) *Isolation of* p-*hydroxybenzoic and* p-*hydroxyhippuric acids*. Three rabbits were each given 2 g. *p*-hydroxybenzylamine with water by stomach tube. The 24 hr. urine (390 ml.) was acidified with 40 ml. of conc. HCl, filtered through glass wool and then extracted with ether continuously for 3 hr. The extract was evaporated to a syrup which crystallized on adding 10–15 ml. of water. The crystals were filtered off, and after two recrystallizations from water yielded white plates of *p*-hydroxybenzoic acid, m.p. 212° and mixed m.p. 213°. The yield after recrystallization was 1 g. or 17 % of the dose of amine. This material represents the free acid excreted.

The combined *p*-hydroxybenzoic acid was obtained by continuous ether extraction for 3 hr. of the residual urine, which had been brought to pH 1 and boiled under reflux for 1 hr. The yield of combined acid (m.p. and mixed m.p. 213°) was 1·1 g. or about 18 % of the dose of amine.

For the isolation of *p*-hydroxyhippuric acid, a 24 hr. urine, after the feeding of 6 g. of *p*-hydroxybenzylamine, was acidified and extracted with ether as described above. The ether extract was evaporated and the residue extracted with small portions of ether. Most of the residue dissolved, leaving a small residuum of *p*-hydroxyhippuric acid (100 mg., or 1 % of the dose of amine). On recrystallization from hot water the acid formed plates, m.p. and mixed m.p. 238°. These isolation experiments account for nearly 40 % of the amine fed.

an equal volume of conc. HCl (Sammons & Williams, 1941 *b*). The colour increases in intensity in the course of 1 hr. and thereafter it fades to a dirty brown colour. This colour reaction suggests that a conjugate of *p*-hydroxybenzaldehyde is being excreted. An attempt was made, after feeding 8 g. of the amine, to isolate the aldehyde as a 2:4-dinitrophenylhydrazone according to the method already described under the section B (2) on *p*-hydroxybenzaldehyde. A small amount (15 mg.) of a hydrazone was obtained, but we were unable to identify it as *p*-glucuronosidobenzaldehyde-2:4-dinitrophenylhydrazone. The material melted indefinitely, but gave a deep purple colour in dilute NaOH similar to that given by the authentic aldehyde derivative. It also gave the Tollens reaction for glucuronic acid.

In another experiment a 24 hr. urine, after feeding 6 g. of the amine, was collected and the basic lead acetate precipitate was prepared in the usual manner. The Pb was removed with H_2S and the excess H_2S in the filtrate removed by aeration. The filtrate gave a pale red colour in the Sammons & Williams test, but after gentle hydrolysis with HCl a strong red colour was obtained indicating the presence of *p*-hydroxybenzaldehyde. Attempts to prepare an identifiable 2:4-dinitrophenylhydrazone on half of the filtrate failed. The other half was hydrolyzed by boiling for 1 hr. with an equal volume of conc. HCl. The dark solution was cooled, decolorized with charcoal and filtered. This solution gave an intense red colour in the Sammons & Williams reaction, an intense Tollens reaction and reduced Benedict's reagent. It was extracted with ether to remove any *p*-hydroxybenzaldehyde present. The ether extract was taken to dryness and the residue taken up in 10 ml. ethanol. This ethanolic solution gave a very intense red colour in the Sammons & Williams test, and was treated with 2:4-dinitrophenylhydrazine in ethanol containing H_2SO_4. A small red precipitate, m.p. 270° (after recrystallization from ethanol-benzene), separated. We were unable, however, to identify it definitely as *p*-hydroxybenzaldehyde-2:4-dinitrophenylhydrazone (m.p. 280°) for the quantity obtained was too small for micro-analysis.

Table 4. *Free and conjugated acid* (*calculated as* p-*hydroxybenzoic acid*) *excreted by rabbits receiving* p-*hydroxybenzylamine*

			Acid excreted (calculated as *p*-hydroxybenzoic acid)												Total acid − free acid = conjugated acid
			Blank value on urine before hydrolysis	Free acid					Blank value on urine after hydrolysis	Total acid					
		Dose of amine		Day 1		Day 2		Total		Day 1		Day 2		Total	
Rabbit no.	Wt. (kg.)	(mg.)	(mg.)	(mg.)	(% of dose)	(mg.)	(% of dose)	(% of dose)	(mg.)	(mg.)	(% of dose)	(mg.)	(% of dose)	(% of dose)	(%)
91	2·5	624	136·5	163	26·7	115	18·8	45·5	118·6	456·3	74·7	114·8	18·8	93·5	48
92	2·5	644	133·1	260·3	41·3	53·3	8·5	49·8	122·6	494·7	78·5	110·8	17·6	96·1	46·3
93	3·0	798	174·1	139·6	17·9	101	12·6	30·5	143·6	498	63·7	119·5	15·3	79·0	48·5

(3) *Quantitative determination of free and combined* p-*hydroxybenzoic acid*. The results are given in Table 4 which show that about 90 % of the dose of *p*-hydroxybenzylamine is excreted as *p*-hydroxybenzoic acid. The figures for free *p*-hydroxybenzoic acid include also any *p*-hydroxyhippuric acid, and give, therefore, a measure of the amount of *p*-hydroxybenzylamine excreted as acidic compounds carrying a free phenolic hydroxyl group, i.e. about 40%. The amount of *p*-hydroxybenzylamine excreted as *O*-conjugates is by this method about 47·5 %, which is somewhat lower than that found by summation of the glucuronic acid and ethereal sulphate conjugations, 58·1 %, given in Table 3. The values obtained by these two methods are, however, of the same order.

(4) *Detection of* p-*hydroxybenzaldehyde derivatives*. The urine collected up to 6 hr. from rabbits after their receipt of doses of *p*-hydroxybenzylamine gives a faint red colour when treated with a little naphthoresorcinol in ethanol and

We conclude from these experiments that it is very probable that a small amount of conjugated *p*-hydroxybenzaldehyde is excreted after *p*-hydroxybenzylamine has been fed to rabbits, but we have been unable finally to prove this by actual isolation of the aldehyde.

(5) *Detection of nuclear oxidation of* p-*hydroxybenzylamine* in vivo. Bray *et al.* (1947) have shown that *p*-hydroxybenzoic acid is oxidized to a small

extent to protocatechuic acid. They were unable to isolate this acid after feeding *p*-hydroxybenzoic acid, but did so when *p*-hydroxybenzamide was fed. It is therefore possible that, since *p*-hydroxybenzylamine is mainly converted to *p*-hydroxybenzoic acid, the latter may also be slightly oxidized to protocatechuic acid.

p-Hydroxybenzylamine (12 g.) was fed to 6 rabbits and a 24 hr. urine (600 ml.) collected. The urine was acidified with HCl and extracted continuously with ether for 3 hr. Removal of the ether left a crystalline mass, which on recrystallization from water yielded *p*-hydroxybenzoic acid, m.p. 214°. The mother liquors gave no green colour, under the appropriate conditions, with $FeCl_3$, indicating the absence of any free protocatechuic acid. The urine was now made strongly acid with conc. HCl and boiled for 1 hr. It was then cooled and extracted continuously for 3 hr. with ether. Removal of the ether and recrystallization of the residue from water yielded *p*-hydroxybenzoic acid. The mother liquor now gave an intense green colour with $FeCl_3$, which turned blue, violet and finally red on adding $NaHCO_3$. We were unable to isolate crystalline protocatechuic acid from this mother liquor, but there is little doubt that it was present.

We therefore conclude that *p*-hydroxybenzylamine is converted to a very small extent to a catechol derivative which is probably conjugated protocatechuic acid.

(6) *Observations on the glucuronide of* p-*hydroxybenzylamine urine*. The glucuronide of *p*-hydroxybenzylamine urine was obtained via the basic lead acetate precipitate as a gum. Attempts to form crystalline derivatives such as salts or the triacetyl methyl ester were not successful (see section A (3)). The gum was a non-reducing acidic substance, and on acid hydrolysis yielded *p*-hydroxybenzoic acid, m.p. 213°.

We conclude that the glucuronide excreted after feeding *p*-hydroxybenzylamine is largely *p*-carboxyphenylglucuronide.

D. *Experiments with* p-*hydroxybenzylacetamide*

(1) *Ethereal sulphate and glucuronic acid excretion.* Table 5 shows that about 70–80% of *p*-hydroxybenzylacetamide is excreted as *O*-conjugates, the glucuronic acid/ethereal sulphate ratio being about 5.

(2) *The isolation of unchanged* p-*hydroxybenzylacetamide.* A rabbit was fed 2 g. of *p*-hydroxybenzylacetamide and a 24 hr. urine collected. The urine was made acid to Congo red and continuously extracted with ether for 3 hr. The ether was removed and the residue recrystallized (charcoal) from hot water. The crystals (25 mg.) obtained were identified as *p*-hydroxybenzylacetamide, m.p. and mixed m.p. 125°. No trace of *p*-hydroxybenzoic acid was found, and there was no evidence of deacetylation.

(3) *The glucuronide of* p-*hydroxybenzylacetamide.* Each of 3 rabbits was fed 1 g. of the amide and a 6 hr. urine (110 ml.) collected. Very little glucuronide was excreted after the first 6 hr. The basic lead acetate precipitate was prepared in the usual manner and the Pb removed by H_2S. The filtrate from PbS was treated with charcoal, filtered and concentrated to 25 ml. Addition of ethanol to this solution precipitated the glucuronide as an amorphous solid which became gummy on standing. It was therefore redissolved by addition of more water, and the whole was evaporated under reduced pressure to a hard dry amorphous powder (1·5 g.). It could not be induced to crystallize.

The powder was shaken for 10 min. with an ethereal solution of diazomethane (from 1·5 g. of nitrosomethylurea). It formed a gum on the sides of the flask and the mixture was kept at room temperature overnight. During this time the colour of the diazomethane had disappeared, and a further quantity of ethereal diazomethane together with 5 ml. of ethanol were added. The mixture was kept for 48 hr., then decanted from any solid material and taken to dryness at 40°. The residue did not crystallize. It was therefore dissolved in 5 ml. pyridine and 5 ml. acetic anhydride and kept overnight at room temperature. The mixture was then poured into 50 ml. of water and the solution neutralized with solid Na_2CO_3. On standing for 5–6 days, the solution deposited rosettes of fine needles. These were collected and recrystallized from acetone-water (yield 100 mg.). The product, p-*acetamidomethylphenyl triacetylglucuronide methyl ester*, formed rosettes of needles, m.p. 174°, and showed $[\alpha]_D^{20^\circ}$ − 29·05° (c = 1·8 in acetone). (Found: C, 54·95; H, 5·9; N, 3·2. $C_{22}H_{27}O_{11}N$ requires C, 54·9; H, 5·65; N, 2·9%.)

E. *Experiments with* p-*hydroxybenzylmethylamine*

(1) *Ethereal sulphate and glucuronic acid conjugations.* Table 6 shows that about 60% of the amine fed is excreted as *O*-conjugates, the ratio glucuronide/ethereal sulphate being about 8. It is to be noted that the sulphate conjugation is relatively low (7%) and is only a third of that of

Table 5. *The ethereal sulphate and glucuronic acid conjugation of* p-*hydroxybenzylacetamide in the rabbit*

		Dose		Ethereal sulphate as SO_3		Glucuronic acid		Dose excreted as		
Rabbit no.	Wt. (kg.)	(mg.)	(mg./kg.)	Mean normal value (mg./day)	Increase after dosing (mg.)	Mean normal value (mg./day)	Increase after dosing (mg.)	Sulphate (%)	Glucuronide (%)	Total conjugation (%)
99	2·7	610	230	32·2	46·5	202	481	15·7	67·0	82·7
101	2·7	630	233	26·8	21·7	184	657	7·1	88·7	95·8
104	2·5	504	201	35·3	31·7	220	361	13·1	61·0	74·1
110	3·0	795	253	16·3	57·8	153	475	15·7	53·2	68·9
111	3·2	747	234	15·3	51·8	96	465	14·3	52·0	66·3
112	2·9	745	261	15·5	42·2	134	272	11·7	39·1	50·8

p-hydroxybenzylamine (19%). This result is similar to that found for D-adrenaline (Dodgson *et al.* 1947). On the other hand, the glucuronic acid conjugation of this amine (54%) is higher than that of p-hydroxybenzylamine (39%), and is approaching that of p-hydroxybenzylacetamide (60%).

Table 6. *The ethereal sulphate and glucuronic acid conjugations of* p-*hydroxybenzylmethylamine hydrochloride in the rabbit*

Rabbit no.	Wt. (kg.)	Dose (mg.)	Dose (mg./kg.)	Ethereal sulphate as SO_3: Mean normal value (mg./day)	Ethereal sulphate as SO_3: Increase after dosing (mg.)	Glucuronic acid: Mean normal value (mg./day)	Glucuronic acid: Increase after dosing (mg.)	Dose excreted as Sulphate (%)	Dose excreted as Glucuronide (%)
109	2·5	601	245	28·3	19·7	130	360	7·1	52·4
115	2·6	603	232	27·6	22·2	134	342	8·0	49·6
116	2·6	604	232	19·8	18·9	127	323	6·8	46·7
110	3·0	1474	499	17·5	35·6	137	979	5·2	59·4
111	2·8	1474	526	13·3	54·8	127	1100	7·9	64·1
112	3·2	1500	476	14·2	31·9	62·4	850	4·7	50·5

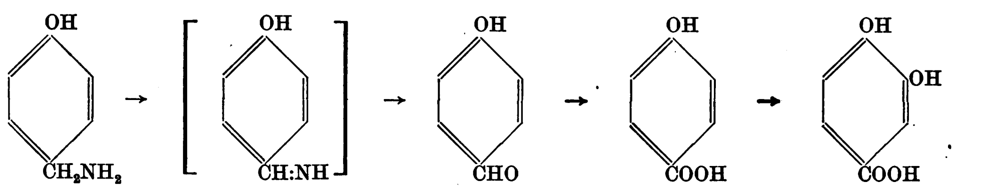
(2) *Isolation of* p-*hydroxybenzoic acid after feeding* p-*hydroxybenzylmethylamine.* Each of 3 rabbits received 1·5 g. of the amine hydrochloride and a 24 hr. urine (1100 ml.) was collected. From 400 ml. of the acidified urine 0·1 g. of p-hydroxybenzoic acid, m.p. 214°, was isolated by ether extraction. The extracted urine was strongly acidified with conc. HCl and refluxed for 1 hr. Ether extraction of the hydrolyzed urine yielded 0·1 g. p-hydroxybenzoic acid, m.p. 214°.

Thus 45% of the amine fed was accounted for by isolation as p-hydroxybenzoic acid, half of the acid occurring in the urine as an O-conjugate and the other half free. This experiment indicates that p-hydroxybenzylmethylamine is excreted largely as free and conjugated p-hydroxybenzoic acid.

DISCUSSION

The ethereal sulphate and glucuronic acid conjugations of the five compounds studied are summarized in Table 7 (p. 302).

The fate of p-hydroxybenzylamine in the rabbit can be expressed as follows:



Traces excreted as an O-conjugate

39% as glucuronide; 19% as sulphate; 40% as the free acid and p-hydroxy hippuric acid

Traces excreted conjugated

The main metabolites are free p-hydroxybenzoic acid, its ethereal sulphate and ether glucuronide. Small but isolable amounts of p-hydroxyhippuric acid and traces of conjugated p-hydroxybenzaldehyde and conjugated protocatechuic acid are also excreted.

The metabolism of p-hydroxybenzylamine involves two separate mechanisms: (a) deamination followed by oxidation of the $—CH_2NH_2$ group, and (b) conjugation of the phenolic hydroxyl group. It is interesting, therefore, to consider how the one mechanism affects the other. The deamination process can be blocked by acetylating the amino group as in p-hydroxybenzylacetamide, and from the figures (see Table 7) for the conjugation of this amide it is clear that when deamination does not take place there is a high O-conjugation (73%) particularly with glucuronic acid (60%).

p-Hydroxybenzylmethylamine is intermediate between p-hydroxybenzylamine and p-hydroxybenzylacetamide in the extent of its conjugation, and this suggests that it is more slowly deaminated than the primary amine. Its rate of deamination is such as to allow a considerable amount of O-conjugation to take place. This result indicates that secondary amines of the type $R.CH_2NHCH_3$ are more slowly deaminated *in vivo* than the corresponding primary amines, $R.CH_2NH_2$. This conclusion receives support from the earlier findings of

Ewins & Laidlaw (1910) and of Alles & Heegaard (1943). Ewins & Laidlaw showed that tyramine (*p*-hydroxyphenylethylamine) was readily converted to *p*-hydroxyphenylacetic acid in the perfused dog liver, whereas *p*-hydroxyphenylethyl methylamine formed the acid at a much slower rate. Hordenine (*p*-hydroxyphenylethyldimethylamine) only formed traces of *p*-hydroxyphenylacetic acid after prolonged perfusion. Alles & Heegaard studied *in vitro* the rate of deamination by amine oxidase of derivatives of some sympathomimetic amines, and found the *N*-monomethyl derivatives to be more slowly deaminated than the parent amines. Our results suggest that some idea of the rate of the *in vivo* deamination of phenolic amines may be obtained from a determination of their glucuronic acid conjugations; the possible application of this suggestion to adrenaline is obvious.

Table 7. *The* O-*conjugation of* p-*hydroxybenzylamine and related compounds in the rabbit*

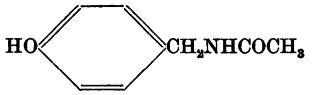
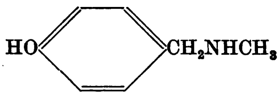
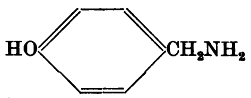
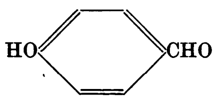
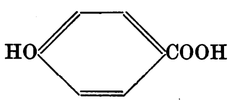
Compound	% of compound excreted as		Total	Extent of conversion to
	Ethereal sulphate	Glucuronide	*O*-conjugation (% of dose)	*p*-hydroxybenzoic acid
	13	60	73	Nil
	7	54	61	Probably complete
	19	39	58	Complete
	9	33	42	Complete
	9	18	27	—

Table 7 shows that the glucuronic acid conjugations of the compounds studied form a regular series, but this is not true for the sulphate conjugation. However, sulphate conjugation is complicated by the fact that it occurs in the intestine as well as in the liver (Marenzi, 1931; Arnoldt & De Meio, 1941), whereas the glucuronic acid conjugation occurs only in the liver. It is not possible, therefore, to comment at this stage on the sulphate conjugation of these compounds.

The similarity between the extent of the glucuronic acid conjugation of *p*-hydroxybenzylamine (39%) and of *p*-hydroxybenzaldehyde (33%) suggests that after being fed the amine is largely converted to the aldehyde in the wall of the gut, before being conjugated with glucuronic acid in the liver. Furthermore, this conjugation is virtually complete before the aldehyde is oxidized to *p*-hydroxybenzoic acid, for the glucuronic acid conjugation of this acid (18%) is much lower than that of the aldehyde. The sulphate conjugation of the amine (19%), on the other hand, is much higher than that of the aldehyde (9%), and this may indicate that some sulphate conjugation of the amine occurs in the wall of the gut before it is deaminated to the aldehyde.

SUMMARY

1. The metabolic fates in the rabbit of *p*-hydroxybenzylamine, *p*-hydroxybenzylmethylamine, *p*-hydroxybenzylacetamide, *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid have been compared.

2. The main metabolic products of *p*-hydroxybenzylamine are free *p*-hydroxybenzoic acid (*c*. 40%) and the glucuronide (39%) and ethereal sulphate (19%) of *p*-hydroxybenzoic acid. Small amounts of conjugated *p*-hydroxybenzaldehyde, *p*-hydroxyhippuric acid and conjugated protocatechuic acid are also excreted.

3. *p*-Hydroxybenzylmethylamine is also converted into *p*-hydroxybenzoic acid derivatives, but in this case the glucuronide formed is 54% of the dose whereas the sulphate is 7%.

4. *p*-Hydroxybenzylacetamide is not deacetylated *in vivo* and does not produce derivatives of *p*-hydroxybenzoic acid in the urine. It is mainly transformed (60%) into *p*-acetamidomethylphenylglu-

curonide which has been isolated and characterized as its triacetyl methyl ester. p-Hydroxybenzylacetamide and its O-acetyl derivative have been prepared and described for the first time.

5. p-Hydroxybenzaldehyde is largely converted into the glucuronide (33%) and sulphate (9%) of p-hydroxybenzoic acid. A small amount (2–3%) is, however, excreted as p-glucuronosidobenzaldehyde which has been isolated as a 2:4-dinitrophenylhydrazone.

6. p-Hydroxybenzoic acid is the least conjugated of all the compounds studied here and forms only 18% glucuronide and 9% ethereal sulphate.

7. The results indicate that with phenolic amines of the type studied here the extent of glucuronic acid conjugation is inversely proportional to the rate of deamination, the conjugation being highest where no deamination takes place. The significance of this conclusion is discussed.

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Further Observations on the Proteolytic Enzymes in Rat Skin

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The object of this communication is to extend knowledge of the skin proteinase or 'dermoproteinase' previously described by Beloff & Peters (1945) and of its distinction from other proteolytic enzymes present in rat skin (see especially Fruton, 1946).

It was shown that the dermoproteinase will digest casein, serum globulin, serum albumin and myogen, and that it does not fall within the known groups of digestive proteinases by virtue of its failure to digest N-benzoyl-L-arginine amide and carbobenzyloxy-L-tyrosylglycine amide, the typical synthetic substrates for trypsin and chymotrypsin; Beloff & Peters (1945) also showed that this proteinase activity could be separated from other peptidases present by differential extraction of the skin.

The experimental results to be presented here will be considered under the separate headings (1) of the effect of methods of extraction upon the enzymes present, (2) of the character of the individual enzymes.

METHODS

Preparation of skin

(*a*) Acetone-dried rat skin was made as described previously (Beloff & Peters, 1945), 0·3 g. of dried skin being equivalent to 1·0 g. of fresh skin. Extracts were prepared so that 5 ml. of extract were equivalent to 0·3 g. of dried skin; either 5% (w/v) aqueous KCl or 5–6% (w/v) aqueous KNO_3 (see below) were used for the extraction of the proteinase.

(*b*) Fresh skin extracts were made by the technique of Fruton (1946). About 30 g. of skin (from two rats), carefully cleaned, were cut into small pieces and stirred with 100 ml. 2% (w/v) NaCl solution in the Waring Blendor for 8–10 min. A further 100 ml. of 2% NaCl were then added, and the whole stirred slowly for 3 hr. at room temperature. The pulpy mass of skin was strained off through muslin, and the extract filtered through a Whatman no. 41 filter paper. Certain comparisons between the extracts obtained by the two methods are given below.