The Fate of Certain Organic Acids and Amides in the Rabbit

4. THE AMINO BENZOIC ACIDS AND THEIR AMIDES

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Investigations of the metabolism of the aminobenzoic acids in mammals have suggested that the principal methods by which these compounds are conjugated *in vivo* are acetylation of the amino group and conjugation of the carboxyl group with glucuronic acid. For example, Muenzen, Cerecedo & Sherwin (1926) found that *m*- and *p*-aminobenzoic acids, but not anthranilic acid, were acetylated by man and the rabbit, although none of the acids was conjugated in this way by the dog. Acetylation of *p*-aminobenzoic acid has also been observed in the rabbit by Ellinger & Hensel (1914), Hensel (1914), and Harrow, Mazur & Sherwin (1933) and in man and the guinea pig by Zehender (1943). Mitsuba & Ichihara (1927) reported that anthranilic acid was the only aminobenzoic acid to cause an increased excretion of reducing substances in the dog or rabbit, although Quick (1932) showed that in the dog all three isomers were conjugated to a considerable extent with glucuronic acid.

Other methods of conjugation are, however, recorded in the literature. Mitsuba & Ichihara (1927) found that anthranilic acid, but not the *m*- or *p*-isomers, gave rise to an increased excretion of ethereal sulphate by the dog and rabbit. Without satisfactory evidence they attributed this increase to the formation of salicylic acid. Salkowski (1882) also observed no increase in ethereal sulphate excretion when *m*-aminobenzoic acid was given to dogs or rabbits and claimed to have isolated some *m*-aminohippuric acid from the urine. He also stated that some *m*-ureidobenzoic acid (*N*-phenylurea-3-carboxylic acid) was formed. Hildebrandt (1903) claimed that this type of conjugation occurred with all three isomers in the rabbit and mouse, and Mitsuba & Ichihara (1927) confirmed this for anthranilic acid in the dog and rabbit. Muenzen *et al.* (1926) however, denied the formation of ureido- and aminohippuric acids in man, dog or rabbit although they gave little detail to support their statement. Formation of other ureido compounds *in vivo* has been suggested by early workers, e.g. from taurine in man and dog (Salkowski, 1872, 1873) and from sulphanilic acid in the dog (Ville, 1892); but Schmidt & Allen (1920) were unable to detect the excretion of ureidotaurine as a metabolic end product of taurine

in man, and we have not obtained *p*-ureidobenzene-sulphonic acid as a metabolite of sulphanilic acid in the rabbit (unpublished). In the present work, although we did not deliberately search for ureido acids, we have not made any observation which suggests their formation. Several workers have recorded that the aminobenzoic acids are partly excreted unchanged, e.g. Salkowski (1882) (*m*-isomer), Hildebrandt (1903) (all isomers), Muenzen *et al.* (1926) (*o*-isomer only in man and rabbit, all isomers in dog).

The metabolism of the aminobenzamides has received little attention. The hypothesis (Woods, 1940; Fildes, 1940) that the bacteriostatic effect of sulphanilamide is due to its structural similarity to *p*-aminobenzoic acid which enables it to compete with that compound in certain essential metabolic processes in bacteria led to the expected observation (Hirsch, 1942) that *p*-aminobenzamide had similar bacteriostatic activity to that of sulphanilamide. In the work reported here we have compared the fate of the aminobenzamides in the rabbit with that of their respective acids. The extent of conjugation with acetic, glucuronic and sulphuric acids and the excretion of ether-soluble acid show that, unlike the carbamyl group in benzamide (Bray, Neale & Thorpe, 1946*a*), the same group in aminobenzamides is comparatively stable to hydrolysis. One conspicuous difference between the acids and amides is that the latter are oxidized to aminohydroxybenzamides which are at least to some extent conjugated with sulphuric acid, whereas the acids are not oxidized, the principal conjugates excreted being ester-type glucuronides. Some of these metabolites (or derivatives of them) have been isolated and identified. The aminohydroxybenzoic acids isolated* support the view put forward in an earlier paper (Bray, Ryman & Thorpe, 1947) that when oxidation

* All oxidation products isolated in this work were acids. It is not known whether the actual compounds excreted are acids or amides. It is very probable that in the case of the *o*-isomer the amide is excreted, since the percentage oxidation is greater than the estimated degree of hydrolysis, but analytical data for the other isomers do not allow any similar deductions to be made. In this paper the term 'oxidation product' may apply to either acid or amide.

of a disubstituted aromatic compound is effected by the introduction of a hydroxyl group into the ring, the resulting phenol has a 1:3:4-configuration, although the isolation of 3-hydroxy-anthranilic acid as an oxidation product of *o*-aminobenzamide makes it clear that the hypothesis must be extended to include such compounds.

METHODS

Preparation of aminobenzamide. The amides used were prepared by reduction of the corresponding nitrobenzamides using FeSO_4 and NH_3 (Jacobs & Heidelberger, 1917). The nitroamides were prepared by the action of ammonia upon the acid chlorides.

Diet and feeding. The rabbits used were does of 2-3 kg. maintained on a constant diet of rabbit pellets (Bruce & Parkes, 1946), except in the quantitative study of *p*-aminobenzoic acid and amide when bran and oats were used (for details of administration of the diets see Bray *et al.* 1947). We have no reason to believe that the metabolism of these compounds differs in any essential way on the two diets, except for variations in the 'base line' values of some normal metabolites as described below.

Dosage. Owing to their toxicity, anthranilic acid and its amide were not given in doses exceeding 0.5 g./kg. The other acids and amides were not toxic at dose levels up to 1 g./kg. The compounds were administered by stomach tube, the acids being dissolved in NaHCO_3 solution and the amides suspended in water.

Estimation of ether-soluble acid. The method was essentially that previously described (Bray *et al.* 1946a). We found that all three acids could be quantitatively extracted from water or urine at pH 4. This pH was attained in the urines by adding to 10 ml. urine 10 ml. of a solution containing no ether-soluble material and made up by mixing 40 ml. $\text{m-Na}_2\text{HPO}_4$ with 52 ml. m-NaHSO_4 .

Estimation of diazotizable material. This was carried out according to the method of Bratton & Marshall (1939) as referred to previously (Bray *et al.* 1946b). Eckert (1943) states that in blood filtrates quantitative estimation of *p*-aminobenzoic acid requires 15-20 min. for diazotization and 30-60 min. for coupling. With our solutions we did not find any modification of the Bratton-Marshall procedure necessary. With the *o*-acid and amide, however, it was necessary to leave the coupled solution 22 hr. in order to attain maximum colour development. Kohl & Flynn (1941) state that a diazotization procedure using 3 hr. for colour development gives low values for anthranilic acid after hydrolysis, owing to the formation of an undiazotizable anhydride, anthranil, when solutions are heated at an acid reaction. We found that if 22 hr. were allowed for colour development, solutions of anthranilic or acetylanthranilic acids, which had been subjected to the usual hydrolysis procedure, gave values within 3% of the theoretical.

Anthranilic acid gives rise to a much more intense colour than an equivalent amount of the corresponding amide. To match 5.8 mg. of the former 10 mg. of the latter are needed. The conditions of hydrolysis used (1 hr. with 0.2N-HCl in a boiling water bath) are not sufficiently drastic to cause significant hydrolysis of the carbamyl group. It has already been reported (Bray, James, Ryman & Thorpe, 1948) that only 8% hydrolysis of the amide occurs on

heating with N-HCl in a boiling water bath for 1 hr. Solutions of *o*-aminobenzamide and its *N*-acetyl derivative subjected to the ordinary hydrolysis procedure gave quantitatively accurate results when compared with a standard untreated *o*-aminobenzamide solution. An amide standard was, therefore, always used in determinations on urines obtained after administration of *o*-aminobenzamide. It will be seen later that some hydrolysis of the carbamyl group occurs *in vivo*. The values obtained for the percentage of *o*-aminobenzamide excreted with a free amino group (Table 2) will therefore appear higher than they actually are. The oxidation products isolated after acid hydrolysis following the administration of *o*-aminobenzamide, 3- and 5-hydroxyanthranilic acids, give no appreciable colour upon diazotization and coupling under our conditions of estimation. This probably applies also to their amides, which are likely to be the compounds actually excreted. Stronger solutions of these acids (100-200 mg./100 ml.) give a violet colour on diazotization and coupling (see p. 440).

The oxidation products isolated from *m*-aminobenzamide urine after acid hydrolysis likewise give no diazo reaction. The difference in colour intensity of the diazotized and coupled acid and amide is much less than with the *o*-isomer (9.4 mg. acid $\equiv$ 10 mg. amide), but is sufficient to require the use of an amide standard.

The colours developed from *p*-aminobenzoic acid and its amide are of equal intensity, but an amide standard was actually used in experiments in which *p*-aminobenzamide was given. This amide is only oxidized to a very small extent and the oxidation product (isolated as acid) gives no diazo reaction.

Estimation of reducing substances. This was carried out as previously (Bray *et al.* 1946b). The method does not give reliable results for ether-type glucuronide excretion if the amount of ester-type glucuronide excreted is high, as in the case of the aminobenzoic acids. Further, oxidation products may interfere if they are themselves reducing. Since the aminohydroxybenzoic acids isolated after the administration of *o*- and *m*-aminobenzamides are strongly reducing, values for these amides are probably unreliable. Values obtained with the *p*-amide can be accepted since it is only oxidized to a very slight extent.

Results of estimations of ester-type glucuronides formed from the aminobenzoic acids are probably somewhat low. Pure *p*-aminobenzoylglucuronide (see p. 439) gave values about 9% lower than the theoretical.

Estimation of ethereal sulphate. This was carried out as previously, by the method of Folin (1905-6).

EXPERIMENTAL AND RESULTS

Quantitative studies

Ether-soluble acids. The ether-soluble acid of the normal urine of rabbits on the bran, oats and cabbage diet ranged from 200 to 450 mg./day (calculated as hippuric acid) and on the pellet diet from 400 to 700 mg. Average values were 325 and 574 mg. respectively. The results obtained after administration of aminobenzoic acids and amides (calculated as aminobenzoic acid) are summarized in Table 1.

It should be noted that the ether-soluble acid values obtained represent only acid unconjugated with glucuronic acid since the glucuronides are not extracted by ether. Thus, the difference between the ether-soluble acid values for

Table 1. *Excretion of ether-soluble material (calculated as aminobenzoic acid) by rabbits after administration of aminobenzoic acids and amides*

Dose (g./kg.)	Percentage of dose excreted as ether-soluble acid								
	o-Compound			m-Compound			p-Compound		
	Rabbit no.	Acid	Amide	Rabbit no.	Acid	Amide	Rabbit no.	Acid	Amide
0.25	131	60	36	—	—	—	133	45	29
	135	61	27	—	—	—	125	29	10
	137	79	38	—	—	—	137	20	Nil
	144	69	31	—	—	—	139	26	10
0.5	131	29	4	99	49	10	133	42	28
	131	15	19	99	52	—	135	41	11
	135	33	28	117	59	16	137	30	6
	137	42	5	117	62	—	139	24	10
	137	52	10	139	43	10	—	—	—
	—	—	—	139	47	—	—	—	—
	144	18	26	142	47	10	—	—	—
	144	26	19	142	45	—	—	—	—
	144	38	Nil	—	—	—	—	—	—
	—	—	—	—	—	—	—	—	—
1.0	—	—	—	99	55	7	133	49	19
	—	—	—	117	61	8	135	32	12
	—	—	—	117	—	9	137	47	10
	—	—	—	139	37	6	139	24	13
	—	—	—	139	46	—	—	—	—
	—	—	—	142	39	10	—	—	—
	—	—	—	142	50	—	—	—	—
	—	—	—	—	—	—	—	—	—

acid and amide does not give a true idea of the hydrolysis of the carbamyl group, unless the fact that some of the acid is excreted as glucuronide is taken into account (cf. Table 3).

The nature of the ether-soluble acid was investigated in the case of *p*-aminobenzoic acid. The ether extract from the urine of six rabbits which had each received 2 g. of the acid yielded 4 g. pure *p*-aminobenzoic acid. No other metabolite, e.g. *p*-aminohippuric acid, was detected. Owing to the difficulty of obtaining and estimating *o*- and *m*-aminohippuric acids the ether-soluble acid from the *o*- and *m*-isomers was not examined. Quick (1932) was unable to devise any satisfactory procedure for estimation or identification of aminohippuric acids in urine. It is theoretically possible that the apparently low results for anthranilic acid, at 0.5 g./kg. dose level, may be due to the formation of a conjugate which is not estimated by the method used.

Diazotizable material. Table 2 shows the results obtained for excretion of diazotizable material after administration of the three aminobenzoic acids and their amides. The values obtained directly on the urine, and given under the heading 'free', represent the sum of free acid or amide plus acid conjugated as ester-type glucuronide, if present; other substances which might contribute are conjugates of the acids with glycine (aminohippuric acids) or *O*-conjugates of aminohydroxybenzoic acids or amides with sulphuric or glucuronic acids formed as a result of oxidation. The aminohydroxybenzoic acids which have been identified as oxidation products (and presumably their amides) do not diazotize and couple under the conditions of estimation. Whilst it is not definitely established that the *O*-conjugates diazotize and couple, it is possible that they do. We find, for example, that *o*-aminophenylglucuronide diazotizes and couples, although *o*-aminophenol itself does not. Available evidence

(see Table 5) suggests that the contribution of *O*-conjugates to the total of 'free' diazotizable material would be of significant magnitude only in the case of *o*-aminobenzamide. The additional diazotizable material obtained after hydrolysis of the urine is given under the heading 'acetyl' and can be assumed to represent acetamidobenzoic acid or amide except in the case of *o*-aminobenzamide. Here hydrolysis of an *O*-conjugate could cause a decrease in diazo value large enough to mask an increase due to deacetylation. We actually found that the hydrolyzed values never exceeded the unhydrolyzed, but since we were unable to isolate any *o*-acetamidobenzamide we have recorded 'Nil' values in Table 2.

The fact that neither anthranilic acid nor its amide appear to be acetylated in the rabbit may account for the greater toxicity of these compounds as compared with the other isomers. The values obtained for the percentage of 'free' amide excreted are certainly high since some amide is hydrolyzed to acid which gives a more intense colour on diazotization and coupling. It is not possible to calculate the true values with accuracy without knowing whether *o*-aminohippuric acid is present, the full extent of amide hydrolysis (i.e. whether any *o*-aminobenzoylglucuronide has been formed), and the diazotization and coupling properties of the *O*-conjugates. Table 4 shows that the sulphate conjugation of *o*-aminobenzamide corresponds to 26–40% of the dose. This is presumably due to 3- and 5-hydroxyanthranilic acids or their amides. A further small amount of these compounds may be excreted as ether glucuronide.

In the case of *m*-aminobenzamide there is a clear indication that acetylation occurs. The values for free amide excreted can be taken as reasonably accurate since the difference in colour intensity of the diazotized and coupled

Table 2. Excretion of diazotizable material, free and acetylated (calculated as aminobenzoic acid or amide) by rabbits after administration of aminobenzoic acids and amides

		Percentage of dose excreted as diazotizable material															
		o-Compound				m-Compound				p-Compound							
Dose (g./kg.)	Rabbit no.	Acid		Amide		Rabbit no.	Acid		Amide		Rabbit no.	Acid		Amide			
		Free	Acetyl	Free	Acetyl		Free	Acetyl	Free	Acetyl		Free	Acetyl	Free	Acetyl		
0.25	131	75	Nil	33	Nil	—	—	—	—	—	103	87	9	22	46		
	131	87	Nil	—	—	—	—	—	—	—	103	62	9	25	76		
	135	81	Nil	41	Nil	—	—	—	—	—	103	77	4	—	—		
	137	69	—	36	Nil	—	—	—	—	—	113	81	21	20	64		
	144	59	Nil	31	Nil	—	—	—	—	—	113	80	13	16	68		
	—	—	—	—	—	—	—	—	—	—	113	—	—	15	73		
	—	—	—	—	—	—	—	—	—	—	121	83	9	24	81		
	—	—	—	—	—	—	—	—	—	—	121	67	14	23	78		
	—	—	—	—	—	—	—	—	—	—	121	—	—	21	77		
	—	—	—	—	—	—	—	—	—	—	125	73	16	19	60		
	—	—	—	—	—	—	—	—	—	—	125	67	4	—	—		
	—	—	—	—	—	—	—	—	—	—	133	—	—	14	81		
	—	—	—	—	—	—	—	—	—	—	137	—	—	12	62		
	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
0.5	131	69	Nil	50	Nil	99	70	10	23	41	103	87	1	37	45		
	131	—	—	45	Nil	99	85	12	27	35	103	67	15	28	85		
	135	68	Nil	48	—	99	82	23	—	—	113	71	18	35	51		
	137	75	Nil	48	—	99	92	9	—	—	113	75	2	28	45		
	144	71	Nil	45	Nil	117	78	Nil	24	42	113	78	14	—	—		
	144	67	Nil	39	Nil	117	80	14	—	—	121	62	7	28	58		
	—	—	—	—	—	117	76	19	—	—	121	83	21	31	55		
	—	—	—	—	—	139	83	13	52	10	121	72	14	—	—		
	—	—	—	—	—	139	100	Nil	50	10	125	70	13	38	41		
	—	—	—	—	—	139	94	4	—	—	125	76	17	—	—		
	—	—	—	—	—	142	74	11	50	6	—	—	—	—	—		
	—	—	—	—	—	142	86	7	52	9	—	—	—	—	—		
	—	—	—	—	—	142	82	4	54	2	—	—	—	—	—		
	—	—	—	—	—	142	—	—	37	14	—	—	—	—	—		
	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
1.0	—	—	—	—	—	99	96	4	38	42	103	44	16	36	24		
	—	—	—	—	—	99	—	—	41	30	103	57	1	—	—		
	—	—	—	—	—	117	90	9	30	43	103	49	Nil	—	—		
	—	—	—	—	—	117	81	17	25	41	113	51	1	42	37		
	—	—	—	—	—	139	100	Nil	66	6	113	41	3	—	—		
	—	—	—	—	—	142	70	Nil	49	6	121	41	3	34	35		
	—	—	—	—	—	142	79	2	48	5	121	53	2	—	—		
	—	—	—	—	—	—	—	—	—	—	121	62	1	—	—		
	—	—	—	—	—	—	—	—	—	—	125	61	5	39	38		
	—	—	—	—	—	—	—	—	—	—	125	86	8	—	—		

acid and amide is of the same order as the experimental error of the diazo method. As with the *o*-isomer, the metabolite formed by oxidation would probably not contribute to the diazotizable material. The amounts formed, however, are much smaller and will be of correspondingly less significance. The *m*-acid is acetylated to only a small extent. The oxidation product of *p*-aminobenzamide would also probably not contribute to the diazotizable material.

Table 2 also shows that different rabbits may have widely different powers of acetylation. Rabbits 139 and 142 are consistently poor acetylators compared with 135 and 137. It has been our experience that rabbits which are not good acetylators are much less resistant to toxic amino compounds.

Reducing substances. The average base line values were 209 mg./day on bran, oats and cabbage and 185 mg. on the pellet diet. The results obtained after administration of

aminobenzoic acids are given in Table 3. It will be seen that there is evidence of conjugation for all three isomers. The glucuronide excretion after administration of amides could be reliably estimated only in the case of the *p*-amide which is oxidized to only a very slight extent. The formation of considerable amounts of reducing oxidation products from the *o*- and *m*-isomers invalidates the glucuronide estimation.

Ethereal sulphate. The average base line values for SO₃ were 21 mg./day on the bran, oats and cabbage diet and 41 mg. on the pellet diet. The results obtained after administration of aminobenzoic acids and amides are summarized in Table 4. Increases in ethereal sulphate excretion were observed only after giving the amides. We did not confirm the observation of Mitsuba & Ichihara (1927) that anthranilic acid causes an increase in ethereal sulphate excretion.

Table 3. *Excretion of reducing substances (calculated as glucuronic acid) by rabbits after administration of aminobenzoic acids and amides*

Percentage of dose excreted as glucuronide (ester or ether type)								
Dose (g./kg.)	o-Compound		m-Compound		p-Compound			
	Rabbit no.	Acid (ester type)	Rabbit no.	Acid (ester type)	Rabbit no.	Acid (ester type)	Amide	
							(Ester type)	(Ether type)
0.25	131	44	—	—	103	—	0	0
	131	65	—	—	113	8	0	0
	144	43	—	—	113	24	—	—
	144	47	—	—	121	—	8	0
	—	—	—	—	125	—	6	0
0.5	131	54	99	4	103	19	9	0
	144	43	99	17	113	22	10	1
	144	62	117	7	121	30	1	1
	—	—	117	8	125	14	3	0
	—	—	117	8	125	13	—	—
	—	—	139	4	—	—	—	—
	—	—	142	7	—	—	—	—
1.0	—	—	99	6	103	—	6	0
	—	—	99	8	113	21	7	0
	—	—	117	6	121	14	3	0
	—	—	117	7	125	15	6	0
	—	—	117	7	—	—	—	—
	—	—	139	7	—	—	—	—
	—	—	139	8	—	—	—	—
	—	—	142	6	—	—	—	—
	—	—	142	7	—	—	—	—
	—	—	142	10	—	—	—	—
	—	—	—	—	—	—	—	—

Table 4. *Excretion of ethereal sulphate by rabbits after administration of aminobenzoic acids and amides*

Percentage of dose excreted as ethereal sulphate									
Dose (g./kg.)	o-Compound			m-Compound			p-Compound		
	Rabbit no.	Acid	Amide	Rabbit no.	Acid	Amide	Rabbit no.	Acid	Amide
0.1	—	—	—	—	—	—	103	0	—
	—	—	—	—	—	—	113	0	—
0.25	131	0	26	—	—	—	103	—	2
	144	0	32	—	—	—	113	0	—
	—	—	—	—	—	—	121	0	—
0.5	131	0	33	—	—	—	125	0	2
	144	0	40	99	0	—	121	—	1
1.0	—	—	—	99	0	6	103	—	5
	—	—	—	99	0	7	113	—	2
	—	—	—	117	0	5	121	0	1
	—	—	—	142	0	7	125	—	1

Qualitative studies

Isolation of m- and p-acetamidobenzamides

In the case of *m*- and *p*-aminobenzamides, the *N*-acetyl derivatives could be isolated by continuous extraction with ether from the urine of rabbits to which the free amides had been administered by stomach tube (dose level 1 g./kg.). The material thus obtained was dissolved in water, treated with charcoal and recrystallized from water. The products obtained were identical with authentic specimens of *m*-acetamidobenzamide (m.p. 221°) and *p*-acetamidobenzamide (m.p. 274°), which were prepared by the action of acetic anhydride on the respective amides. This is direct

evidence that in these compounds the carbamyl group is not completely hydrolyzed in the rabbit. Similar evidence in the case of *o*-aminobenzamide is provided by the fact that, although the acetyl derivative could not be isolated by ether extraction, the amide itself was. Table 2 shows that this amide is not acetylated *in vivo*.

Isolation of ester type glucuronides as metabolites of acids

The method of isolation was essentially as previously described (Bray *et al.* 1947). Since the compounds being isolated were ester glucuronides and consequently less stable than those of the ether type, the time taken during isolation was reduced to a minimum.

Table 5. *Excretion of metabolites of aminobenzoic acids and amides by the rabbit*

Dose (g./kg.)	Isomer	Com- pound	Percentage of dose excreted as					Estimated average hydrolysis (%)
			Ether soluble acid	Ester glucuronide	Free NH ₂ derivative	Acetyl NH ₂ derivative	Ethereal sulphate	
0.25	Ortho	Acid	60-79	43-65	59-81	0	0	33*
		Amide	27-38	—	31-41	0	26-32	
0.5		Acid	15-52	43-62	67-75	0	0	12*
		Amide	0-28	—	39-50	0	33-40	
0.5	Meta	Acid	43-62	4-17	70-100	0-23	0	12*
		Amide	10-16	—	23-52	2-42	—	
1.0		Acid	37-61	6-10	70-100	0-17	0	8*
		Amide	6-10	—	25-66	5-43	5-7	
0.25	Para	Acid	20-45	8-24	62-87	4-21	0	15
		Amide	0-29	0-8	12-25	46-81	2	
0.5		Acid	24-42	13-30	62-87	4-21	0	20
		Amide	6-28	1-10	28-38	41-85	1-2	
1.0		Acid	24-49	14-21	44-86	0-16	0	21
		Amide	10-19	3-7	34-42	24-38	1-5	

* These values are probably low, since no values are available for ester glucuronide.

p-Aminobenzoylglucuronide. The solution remaining after the removal of PbS was evaporated to small bulk at 50-60° under reduced pressure. On standing crystallization occurred. The red-brown solid was filtered off, dissolved in water, decolorized with charcoal and recrystallized from aqueous ethanol. The product formed white crystalline flakes (m.p. 193° with decomp.; $[\alpha]_D^{24}$ -5.5° in water). It strongly reduced Benedict's and Fehling's solutions, and appeared to be a complex of equimolecular proportions of *p*-aminobenzoic acid and *p*-aminobenzoylglucuronide. (Found: C, 52.7; H, 4.41; N, 5.82%; equiv. (by titration) 225; NH₂ (diazotizable), 6.45%; glucuronic acid, 40.5%. C₂₀H₂₂O₁₀N₂ requires C, 53.3; H, 4.88; N, 6.23%; equiv., 225; NH₂, 7.12%; glucuronic acid, 43.2%). The yield was 10% of the dose.

Low values for glucuronic acid content were consistently obtained by the Shaffer-Hartmann method. This is probably due to the great lability of these compounds to heat. Pure preparations on warming even slightly, whether dry or in solution, rapidly developed a bright-red colour.

By continuous ether extraction of an aqueous solution of the complex, *p*-aminobenzoic acid was isolated and characterized. By careful evaporation of the remaining solution at a low temperature under reduced pressure, a substance crystallizing as fine white needles was obtained which, after recrystallization from aqueous ethanol, melted (with decomp.) at 208-210°. It was strongly reducing and showed $[\alpha]_D^{24}$ -12° (in water). It appeared to be *p*-aminobenzoylglucuronide. (Found: C, 49.6; H, 4.34; N, 4.78%; equiv. (by titration), 321; NH₂ (diazotizable), 5.05%; glucuronic acid, 54.2% (see above). C₁₃H₁₅O₈N requires C, 49.8; H, 4.8; N, 4.47%; equiv., 313; NH₂, 5.12%; glucuronic acid, 62.1%.)

Further evidence that the compound first isolated was a complex of *p*-aminobenzoylglucuronide and *p*-aminobenzoic acid is provided by the fact that, when products were isolated which were syrups and could not be induced to crystallize directly, in most cases addition of *p*-aminobenzoic acid resulted in the crystallization of the complex.

o-Aminobenzoylglucuronide. The method described above gave a readily soluble syrup which could not be crystallized. It did not form a crystalline complex with anthranilic acid or with *p*-aminobenzoic acid nor has any crystalline derivative yet been obtained.

We were unable to isolate a glucuronide from *m*-aminobenzoic acid. Table 3 shows that this isomer is conjugated with glucuronic acid to a very much smaller extent than the others.

Isolation of oxidation products from *o*-aminobenzamide

This compound (30 g.) was administered to 20 rabbits and 24 hr. urine (8 l.) collected. This was hydrolyzed by boiling with conc. HCl (1/5 vol.) for 1 hr., made slightly acid to litmus and extracted continuously with ether for 48 hr., until the blue fluorescence was no longer visible in the ether layer. Two methods were attempted for the isolation of oxidation products from the ether-soluble material.

Chromatography. A solution of the ether-soluble material in ethanol and benzene was poured through an alumina column and anthranilic acid eluted with the same solvent mixture. Anthranilic acid could be readily distinguished from other metabolites in the column owing to its very strong fluorescence in ultraviolet light. It was, however, impossible to elute either with boiling water or organic solvents all the phenolic material which could be shown, by means of the FeCl₃ reaction, to remain on the column.

Fractional crystallization. The ether-soluble material was isolated by evaporation of the extract to dryness *in vacuo* and dissolved in 50% (v/v) aqueous ethanol. By progressive concentration under reduced pressure two main solid fractions were isolated and recrystallized from 60% (v/v) aqueous ethanol. The compounds isolated in all these experiments showed a great tendency to darken in air, especially in solution. The addition of a few drops of SO₂ water prevented this and enabled almost colourless products to be obtained.

The fractions obtained were as follows:

(1) Pale brown needles (yield 150 mg. from solid material extracted from 3 l. urine), soluble in ethanol only with difficulty. Purification was effected by continuous extraction with ether from an aqueous suspension. Ethereal solutions showed an intense blue-violet fluorescence in ordinary light. This compound darkened on heating and decomposed at 248°. It gave a brown coloration with FeCl_3 , a positive indophenol reaction and reduced aqueous AgNO_3 solution in the cold. It gave no yellow coloration with HNO_3 and diazotized solutions did not couple normally with dimethyl- α -naphthylamine or with *N*-(1-naphthyl)-ethylenediamine dihydrochloride, but in concentrated solutions (see p. 435) a violet colour, pale in the case of the first coupling agent and intense with the second, developed slowly, reaching maximum intensity in about 1 hr. With Br_2 water it gave a red-brown coloration. (Found: C, 54.8; H, 4.74; N, 9.2; calc. for $\text{C}_7\text{H}_7\text{O}_3\text{N}$; C, 54.8; H, 4.58; N, 9.14%.) The compound was identical in all respects with synthetic 5-hydroxyanthranilic acid prepared by reductive fission of 4-hydroxy-2-carboxyazobenzene in alkaline solution by means of $\text{Na}_2\text{S}_2\text{O}_4$ (cf. Limpriht, 1891). (Found: C, 54.6; H, 4.67; N, 9.09%; m.p. 248° (decomp.); Limpriht gives 235°; Bamberger & Pyman (1909) give 246–252° depending upon rate of heating.)

The method of preparation of the azo compound fixes only the relative position of the methyl and hydroxyl groups. The position of the amino group was established by conversion of the hydrochloride of the acid to *p*-aminophenol by sublimation *in vacuo*. This was characterized both as such and as its *N*-acetyl derivative. Both the synthetic and biosynthetic specimens of 5-hydroxyanthranilic acid were acetylated by treatment with acetic anhydride at ordinary temperature and in both cases crystalline products were obtained which melted, either alone or mixed together, at 238–239°. The compounds were shown to be monoacetyl derivatives inasmuch as they gave slight brown colorations with FeCl_3 and contained 6.62 and 6.95% N, respectively. Calc. for $\text{C}_9\text{H}_9\text{O}_4\text{N}$, 7.18%. Friedländer (1916) gives m.p. 227° and Kalle and Co. (1911), 224–226° (decomp.).

(2) This fraction on recrystallization yielded two compounds (a) and (b):

(a) Pale brown needles (yield 50–60 mg.), darkening at 230–240° and decomposing at 260°. This compound gave a dark brown coloration with FeCl_3 , no indophenol reaction, no diazo reaction but an intense yellow colour with HNO_3 . It was slightly reducing to aqueous AgNO_3 and gave no coloration with bromine water. In ethereal solution it showed a mauve fluorescence in ordinary light. The properties of this compound were identical with those of synthetic 3-hydroxyanthranilic acid. The only previous record of a compound to which this structure is attributed is due to Keller (1908), who prepared it by the action of red P and HI on damascenine, an alkaloid from *Nigella damascena*. The melting point is stated to be 163–164°, while our synthetic compound decomposed at 260°. Keller did not confirm the structure of his compound by synthesis, but the properties of our compound and its method of preparation suggest that it is, in fact, 3-hydroxyanthranilic acid. *m*-Acetamidobenzoic acid was nitrated as described by Kaiser (1885) to give 2-nitro-3-acetamidobenzoic acid, isolated in 20% yield as pale yellow needles, melting at 245° (Kaiser gives 240–241°). This compound was deacetylated

and deaminated by boiling for about 1 hr. with aqueous KOH (25%) until the evolution of NH_3 ceased (cf. Griess, 1878), the 2-nitro-3-hydroxybenzoic acid formed being isolated in 70–80% yield, m.p. 162–163° (Griess found 178°). This compound was reduced with SnCl_2 and HCl by the method of Zahn (1900) and 3-hydroxyanthranilic acid obtained as a slightly pink crystalline powder, darkening on heating to 240° and decomposing at 260°. It was a derivative of *o*-aminophenol inasmuch as its hydrochloride on sublimation *in vacuo* yielded this compound and since it gave no diazo reaction, but gave the typical yellow diazo-oxide colour with HNO_3 . Further evidence of the configuration is provided by the fact that it, or its amide, is a metabolite of *o*-aminobenzamide. It contained 8.99% N; $\text{C}_7\text{H}_7\text{O}_3\text{N}$ requires 9.14%. The identity of the compound isolated from urine with this synthetic compound was confirmed by preparing an acetyl derivative by the action of acetic anhydride at ordinary temperature, and comparing it with the acetyl derivative of the synthetic compound, prepared in the same way. This compound does not appear to have been prepared before. Both the synthetic acetate and the acetate of the biosynthetic acid melted at 143–145°, either alone or mixed together. Both gave a slight dark brown coloration with FeCl_3 , which suggests that the compound was the monoacetyl derivative. This was confirmed by the analysis of the synthetic specimen. It contained 7.14% N; $\text{C}_9\text{H}_9\text{O}_4\text{N}$ requires 7.18%.

(b) Pale brown needles (yield 20 mg.), decomposing at 230°. This compound was fairly soluble in aqueous ethanol, from which it was recrystallized, and gave no coloration with FeCl_3 , negative indophenol and diazo reactions, a slight yellow coloration with HNO_3 . Analysis gave C, 52.3; H, 4.62; N, 14.2%. The nature of this compound has not been determined. The analysis suggests that it might be an impure specimen of an aminohydroxyhippuric acid ($\text{C}_9\text{H}_9\text{O}_4\text{N}_2$ requires C, 51.4; H, 4.76; N, 13.34%), but it has not been possible to investigate it further.

The mother liquors from which these compounds were obtained were combined, evaporated to dryness *in vacuo* and chromatographed on alumina (see above). After elution of anthranilic acid, the presence of phenolic compounds on the column could be shown. Elution with boiling water enabled a further small amount of 5-hydroxyanthranilic acid to be isolated.

Isolation of oxidation products from *m*-aminobenzamide

To each of 19 rabbits 2 g. *m*-aminobenzamide were given, and the urine passed in the following 24 hr. was collected and hydrolyzed by boiling for 1 hr. with 1/5 vol. conc. HCl. The hydrolyzed urine was continuously extracted with ether for 48 hr., first at pH 6.5 and then at pH 5.5. The extracts obtained in this way had very similar properties and were recombined (extract A). The urine was then evaporated *in vacuo* to one-quarter bulk and again extracted with ether for 48 hr. (extract B). It was finally evaporated to dryness *in vacuo* at 60–70° and the residue extracted with 96% ethanol (extract C).

Extract A was evaporated to dryness, dissolved in water, treated with charcoal and progressively concentrated to yield various crystalline fractions and a syrupy residue (D). The crystalline fractions had indefinite melting points ranging from 165 to 195° and all gave positive diazo reactions and a brownish red colour with FeCl_3 . They were

therefore recombined and recrystallized from 96% ethanol. Part of the material (500 mg.) did not dissolve easily. It contained some ash and was purified by extraction with ether from an acidified aqueous solution, followed by recrystallization from water. Yield 20 mg. of mauve needles, decomposing at 253°, which gave a purple coloration with FeCl_3 , a negative diazo reaction, no coloration with HNO_2 , a reddish brown coloration and precipitate with Br_2 water, a positive indophenol reaction, and reduced ammoniacal AgNO_3 . These properties are those of 5-aminosalicylic acid. Recrystallization of the material which was soluble in 96% ethanol (above) yielded as a first fraction 30 mg. of white crystals whose properties, apart from colour, were the same as those of the compound just described. The identity of these two specimens with 5-aminosalicylic acid was confirmed by conversion into the *NO*-diacetyl derivative, m.p. 192°, which did not depress the melting point (194°) of an authentic specimen of 5-acetamidosalicylic acid. Meldola, Foster & Brightman (1917) give m.p. 283° for the acid and 197° for the acetate (actually the diacetate; present authors unpublished). The remainder of the ethanol-soluble material (6 g.) consisted of *m*-aminobenzoic acid. The syrupy residue *D* was treated with acetic anhydride, dissolved in water and fractionated. Three fractions were obtained: (1) *m*-acetamidobenzoic acid (1 g.); (2) a crystalline fraction of lower melting point (1 g.) which on deacetylation and fractionation with ethanol yielded mainly *m*-aminobenzoic acid; (3) a syrupy residue (2 g.) which was chromatographed on alumina, using ethanol-benzene as solvent. The fractions thus obtained after deacetylation gave a purple-brown coloration with FeCl_3 and a slight yellow colour with HNO_2 , but contained relatively large amounts of *m*-aminobenzoic acid which prevented the isolation of the phenolic compound present.

Extract *B*, treated in the same way as extract *A*, yielded three main fractions: (1) white crystals (40 mg.) giving a purple coloration with FeCl_3 , a positive diazo reaction, developing slowly, no yellow colour with HNO_2 and a bright-green colour and precipitate with Br_2 water. Analysis gave: C, 49.2; H, 5.1; N, 17.7%. This compound was neither an acid nor an ammonium salt. It did not melt, but partially sublimed with decomposition at 310°. Its identity has not yet been established, but it is suggested that it might be a hydrated amide of an aminohydroxybenzoic acid. $\text{C}_7\text{H}_8\text{N}_2\text{O}_2 \cdot \text{H}_2\text{O}$ requires C, 49.4; H, 5.9; N, 16.5%; (2) white needles (200 mg.) which did not melt below 300° and contained an appreciable amount of ash; 150 mg. were acetylated and gave a compound which was proved to be 5-acetamidosalicylic acid. Sublimation *in vacuo* of the remainder yielded crystalline *p*-aminophenol, presumably derived from 5-aminosalicylic acid by decarboxylation; (3) a syrup, which on chromatography (as above) yielded 10 mg. crystals and a syrupy fraction. After recrystallization from water the crystals melted at 215° (decomp.) and gave a yellow colour with HNO_2 , but a negative diazo reaction. With FeCl_3 they gave a bluish purple coloration. It is probable that this compound was 3-amino-4-hydroxybenzoic acid, but, owing to the small amount isolated, it was not possible to characterize it finally. The syrup gave a purple-brown coloration with FeCl_3 , but contained also an appreciable amount of *m*-aminobenzoic acid which prevented isolation of the substance responsible.

Extract *C* gave a purple colour with FeCl_3 , but no metabolite of *m*-aminobenzamide could be isolated.

Isolation of oxidation products from *p*-aminobenzamide

p-Aminobenzamide (30 g.) was fed to 12 rabbits and 24 hr. urine collected. This was hydrolyzed, neutralized and extracted as in the case of *o*-aminobenzamide. The ether-soluble material was recrystallized from aqueous ethanol and a considerable amount of *p*-aminobenzoic acid recovered. The mother liquors gave a bluish coloration with FeCl_3 and ultimately yielded about 15 mg. of a white crystalline material which gave a dark-blue coloration with FeCl_3 , a negative diazo reaction and an intense yellow coloration with HNO_2 . It decomposed at 225–227°. These properties are identical with those of synthetic 4-amino-3-hydroxybenzoic acid, prepared by the nitration of *m*-hydroxybenzoic acid (Griess, 1887) and reduction of the resulting 4-nitro-3-hydroxybenzoic acid with SnCl_2 and HCl (cf. Zahn, 1900). M.p. 226° (Einhorn & Pfyl (1900) give 216°). The identity of the synthetic and biosynthetic acids was confirmed by comparing their *acetyl* derivatives. That of the compound from urine melted (with decomposition) at 238–239°, that of the synthetic compound at 242–244° and a mixture of the two at 240–241°. This acetate does not appear to have been prepared previously.

It should be noted that although these oxidation products were isolated and characterized as acids, it is probable that they are excreted as amides (see p. 434). It is reasonable to conclude that the introduction of the hydroxyl group takes place while the carbamyl group is intact, since the acids themselves give rise to no increased excretion of ethereal sulphate. Whether or not hydrolysis of the carbamyl group takes place more readily in aminohydroxybenzamides than in aminobenzamides is not yet known.

DISCUSSION

The results given in Tables 1–4 and summarized in Table 5 show clearly that there is a considerable difference between the metabolism of the aminobenzoic acids and their amides. Differences in oxidation (as shown by the increase in ethereal sulphate excretion) and in acetylation provide indirect evidence of the comparative stability of the amide grouping in these compounds *in vivo*, while the isolation of unchanged *o*-aminobenzamide, and of *N*-acetyl derivatives of the *m*- and *p*-isomers from the urine of rabbits which had received the amides indicates directly that hydrolysis of the carbamyl group is not complete. Estimations of ether-soluble acid indicate that the group is hydrolyzed to an extent of about 12–33% in the *o*-, 8–12% in the *m*- and 15–21% in the *p*-amide. The corresponding percentages of hydrolysis obtained *in vitro* with liver extracts were 9, 7 and 13 (Bray *et al.* 1948).

The identity of the oxidation products isolated is of particular interest inasmuch as the generalization suggested in a previous paper (Bray *et al.* 1947) can now be more clearly defined. Taking into account the work of other investigators and work in this laboratory, it appears to be a general rule that, in

the hydroxylation *in vivo* of a disubstituted aromatic compound, $R.C_6H_4.X$, where R is $COOH$, $CONH_2$, SO_2NH_2 or CH_3 and X is OH or NH_2 ($NHCOCH_3$ in acetotoluidides), the position of the hydroxyl group introduced is, as might be expected on purely chemical grounds, either para or ortho to X , giving, when R and X are themselves ortho, I or II; when R and X are meta, III or IV (V has not yet been reported) and, when R and X are para, VI.

It has been proved in the present investigation that in the case of *o*-aminobenzamide the two possible oxidation products, I and II, are both formed. Evidence for the formation of the two expected oxidation products of orthanilamide has been put forward by Stubbs & Williams (1947). From *m*-aminobenzamide the main oxidation product isolated corresponded to III. There was, however, evidence of another oxidation product which was probably IV. From *p*-aminobenzamide the only oxidation product isolated was, as would be expected, VI.

The case of the acetotoluidides is of interest since we have shown (unpublished result) that in the rabbit the *o*-isomer gives rise mainly to 2-amino-5-hydroxytoluene (6-amino-*m*-cresol) (I), excreted both as glucuronide and ethereal sulphate, whereas Jaffe & Hilbert (1888) found that the dog excreted a benzoxazolone derivative, presumably derived from 2-amino-3-hydroxytoluene (2-amino-*m*-cresol) (II). We cannot yet say whether this isomer is also formed by the rabbit. In the *m*- and *p*-acetotoluidides we have confirmed Jaffe & Hilbert's (1888) observation that the oxidation of the methyl group takes precedence, aminobenzoic acids appearing as the main excretion products. Phenolic compounds are, however, formed in smaller amounts, particularly with the *m*-isomer, but have not yet been identified. All these compounds are actually excreted as *N*-acetyl derivatives, since only a small amount of deacetylation takes place *in vivo*.

It would be expected that in the compounds under discussion the group X (OH or NH_2) will determine the position of the entering hydroxyl group, group R having no significant directing effect. The hydroxylated compounds, in fact, correspond to those formed when the group R is a hydrogen atom, i.e. in monosubstituted compounds. Phenol is converted in the animal to catechol and hydroquinone (Baumann & Preusse, 1879) and aniline (as acetanilide or form-

anilide) to *o*-aminophenol (as benzoxazolone) in the dog and to *p*-aminophenol in the rabbit (Jaffe & Hilbert, 1888; Kleine, 1896-7). Similar results have been observed if group X is modified, e.g. phenetole is converted to monoethyl hydroquinone in the dog (Kossel, 1880); β -phenylhydroxylamine and cupferron (ammonium nitrosophenylhydroxylamine) to *p*-aminophenol in the rabbit (Sieburg, 1914) and dimethylaniline to *o*-aminophenol in the dog and to

p-monomethylaminophenol in the rabbit (Horn, 1936*a*, *b*). We have also shown (unpublished result) that phenylurea is converted in the rabbit to *p*-hydroxyphenylurea, which is excreted mainly as glucuronide. So far as we are aware, compounds in which the group X and a hydroxyl group occupy positions meta to one another have never been found as products of mammalian metabolism.

SUMMARY

1. The metabolism of *o*-, *m*- and *p*-aminobenzoic acids and their amides in the rabbit has been studied both qualitatively and quantitatively.

2. There is evidence that the carbamyl group is hydrolyzed in the rabbit to an extent of 12-33% in the *o*-, 8-12% in the *m*-, and 15-21% in the *p*-compound.

3. Among the compounds isolated were *m*-acetamidobenzamide and *p*-acetamidobenzamide as metabolites of the amides themselves and *p*-aminobenzoylglucuronide as a metabolite of *p*-aminobenzoic acid. This glucuronide forms a crystalline complex with *p*-aminobenzoic acid.

4. Four aminohydroxybenzoic acids have been isolated from the hydrolyzed urines of rabbits which had received the aminobenzamides under investigation. In this way 3- and 5-hydroxyanthranilic acids were obtained from *o*-aminobenzamide, 3-amino-6-hydroxybenzoic acid from *m*-aminobenzamide and 4-amino-3-hydroxybenzoic acid from *p*-aminobenzamide.

5. This type of biological hydroxylation is discussed from the point of view of the position taken up by the hydroxyl group introduced with respect to the other groups present.

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Separation of Acidic Amino-acids by means of a Synthetic Anion Exchange Resin

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Synthetic ion-exchange resins have recently been widely used for the separation of basic and acidic from neutral compounds. Thus Cannan (1944), by repeated applications of Amberlite IR 4, separated glutamic and aspartic acids quantitatively from protein hydrolysates. Earlier, Freudenberg, Walch & Molter (1942) had used a column of Wolfatite M for the separation of the dicarboxylic amino-acids, and Cleaver, Hardy & Cassidy (1945) investigated the chromatographic separation of amino-acids by organic exchange resins. Recently, Tiselius, Drake & Hagdahl (1947) have employed synthetic ion exchange resins chromatographically for the quantitative separation of amino-acids into groups. The

aim of the present investigation was to extend such methods to cover the separation of the acidic amino-acids from one another (at pH 2.5) after their isolation from the rest of the protein hydrolysate (at pH 3-4). Although completely quantitative recoveries were not obtained, complete separations have been achieved. The separation depends on differences in net charge between the acidic amino-acids at a given pH (Fig. 1). Thus, glutamic acid with pK_1 2.16 and pK_2 4.32 (Neuberger, 1936) has a net charge of -0.31 at pH 4; aspartic acid with pK_1 1.88 and pK_2 3.65 (Miyamoto & Schmidt, 1931) has a net charge of -0.69 at pH 4; whilst cysteic acid with pK_1 1.88 and pK_2 1.30 (Andrews & Schmidt, 1927)