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The Fate of Certain Organic Acids and Amides in the Rabbit

9. LOWER ALIPHATIC AMIDES

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In an earlier investigation, reported in Part 3 of this series (Bray, James, Ryman & Thorpe, 1948), it was shown that rabbit-liver extracts were only slightly active in hydrolysing formamide, acetamide, propionamide and phenylacetamide. It had previously been shown (Bray, Neale & Thorpe, 1946) that phenylacetamide was apparently completely hydrolysed in the intact rabbit, since its excretion products were qualitatively and quantitatively virtually the same as those of phenylacetic acid. In the case of the aromatic amides for which information is available (Bray, Thorpe & Wood, 1949*a*) there is reasonable agreement between results *in vivo* and *in vitro*, so that it seemed desirable to investigate the metabolism of aliphatic amides in greater detail.

These compounds have, in general, attracted little attention, probably because of the difficulty in determining their fate. Schultzen & Nencki (1872) concluded that acetamide was not hydrolysed in the dog. This was supported by Steinhausen's (1914–20) finding that in the phlorrhizinized dog 72% of a dose of the amide was excreted unchanged over a period of 6 days. In the rabbit, however, acetamide appeared to be largely hydrolysed, since Salkowski (1877–8) could detect only a small proportion of unchanged amide in the urine, and Rudzski (1876) claimed that a large proportion of a dose was

excreted as acetic acid. Pommerrenig (1902) reported that small doses were completely catabolized. Indirect evidence that acetamide is slowly and only partially hydrolysed in the rat has been obtained recently by Anker & Raper (1948) in feeding experiments with acetamide labelled with ¹³C. Halsey (1898) found that formamide gave rise to as much urinary formate in the dog as did formic acid itself, and was therefore presumably completely hydrolysed, while Gonnermann (1902) found that both formamide and acetamide were hydrolysed by sheep liver and formamide by sheep kidney. The other amides studied here do not appear to have been investigated previously, except by Fiske (1923), who carried out a study of their hydrolysis by fasting cats, using urinary amide nitrogen estimation as the criterion of splitting. Acetamide was excreted over about 4 days, a total of 70% of the dose appearing unmodified. With the straight-chain amides up to valeramide, the lag in excretion diminished and the extent of hydrolysis increased progressively with increasing molecular weight, *n*-valeramide being almost completely hydrolysed (95–96%) within 24 hr. The extent of hydrolysis of propionamide was 55–57% and of *n*-butyramide 74–80%. The order of stability *in vivo* was the reverse of that towards alkali, with which reagent the degree of hydrolysis

decreases as the molecular weight increases. In our earlier study (Bray *et al.* 1948) we found that shorter chain aliphatic amides in general were much more readily hydrolysed by alkali than by liver extracts.

This paper reports the results of an investigation upon the amides of straight-chain saturated fatty acids from formic to valeric, in which the excretion of unchanged amides was estimated in terms of the increased ether-soluble acid formed as a result of hydrolysis; the hydrolytic effect of rabbit-liver extracts and slices was also observed.

EXPERIMENTAL

A. *IN VIVO* EXPERIMENTS

Methods and materials

Diet and dosage. The rabbits and diet were as previously described (Bray, Ryman & Thorpe, 1947). The amides were administered by stomach tube as solutions or suspensions in water and the acids as solutions of their sodium salts. At the dose levels used none of the compounds showed toxic properties.

Estimation of amides. Owing to the lack of suitable characteristic properties, the amides could only be estimated by a non-specific method. A procedure based on the liberation of NH_3 by alkaline hydrolysis was unsatisfactory and the method finally adopted involved estimation of the increase in the ether-soluble acid after hydrolysis.

Urine (50 ml.) was hydrolysed by addition of 10N-NaOH (5 ml.) and heating for 2 hr. in a boiling water bath; the pH was adjusted to 8, using thymol blue as indicator, the urine cooled and the volume adjusted to 100 ml. The ether-soluble acid content was then estimated as below. By this method 95–102% of added amides were recovered from normal rabbit urine.

Estimation of ether-soluble acid. Ether-soluble acid was extracted as previously described (Bray *et al.* 1947). Since, however, all the aliphatic acids in this study are volatile, the ether in the 'boiler' was not evaporated as usual but titrated directly after the addition of water (20 ml.), thymol blue being the indicator. 'Fixing' the extracted acid by addition of excess standard NaOH before evaporation was undesirable in estimations on unhydrolysed urines, since it has been shown that an error may be introduced owing to hydrolysis of unchanged amide during evaporation (e.g. phenylacetamide, cf. Bray *et al.* 1946).

Table 1. *Excretion of aliphatic acids and amides by the rabbit**

Compound	Dose (g.)	No. of exps.	Percentage of dose estimated as ether-soluble acid in urine (Ranges in parentheses)		Estimated percentage hydrolysis in rabbit†
			Unhydrolysed	Hydrolysed	
Sodium formate	2–3	5	4 (0–7)	7 (4–12)	—
Formamide	2	3	5 (0–6)	43 (41–45)	60 (55–62)
	3	3	5 (2–8)	48 (47–50)	56 (54–58)
	4	2	2 (1–2)	33 (29–36)	69 (66–72)
		8	Av. 3	43	61
Sodium acetate	2–5	7	1 (0–4)	1 (0–7)	—
Acetamide	1.5	1	0	71	29
	2	4	1 (0–4)	65 (44–78)	37 (22–56)
	2.5	3	2 (0–5)	75 (58–87)	27 (13–47)
	3	4	0	80 (62–94)	20 (6–38)
	5	4	1 (0–3)	72 (62–88)	29 (12–41)
		16	Av. 1	73	28
Sodium propionate	2–3	4	0	0	—
Propionamide	2	4	3 (0–6)	47 (36–59)	56 (50–64)
	3	2	0	52 (48–55)	49 (45–52)
	4	2	0	48 (48)	52 (52)
		8	Av. 1	48	53
Sodium <i>n</i> -butyrate	2–3	4	0	0	—
<i>n</i> -Butyramide	2	3	0	8 (0–16)	92 (84–100)
	3	4	0	17 (12–24)	83 (76–88)
		7	Av. 0	13	87
Sodium <i>n</i> -valerate	2	4	0	0	—
<i>n</i> -Valeramide	1.5	2	0	6 (0–12)	94 (88–100)
	2	2	0	0	100
	3	2	2 (0–3)	5 (0–10)	97 (93–100)
		6	Av. 1	4	97

* The values given for the excretion of amides represent the total excretion, in some cases over periods up to 72 hr. after dosage (cf. Table 2).

† Calculated from individual values.

RESULTS

Ether-soluble acid of unhydrolysed and hydrolysed normal rabbit urine. The average ether-soluble acid excretion by individual rabbits ranged from 661 to 850 mg./24 hr., expressed as hippuric acid. The ether-soluble acid content of hydrolysed urine is conveniently expressed as a percentage of the value for unhydrolysed; it ranged from 148 to 200 %. The average for 107 experiments was 171 %; the average for individual rabbits ranged from 157 to 181 %. The day-to-day variations in output for each rabbit was usually within ± 10 % of the mean values for both unhydrolysed and hydrolysed urines.

Excretion of aliphatic acids and amides by the rabbit. Table 1 summarizes the results obtained, taking the difference between the amount of amide administered and the total amount excreted unchanged as a measure of the extent of hydrolysis. The results with acetamide, propionamide, butyramide and *n*-valeramide are in general agreement with those of Fiske (1923), the degree of hydrolysis increasing with increasing molecular weight. Formamide is an exception, apparently being hydrolysed to a greater extent than acetamide. The possibility of some other type of catabolic process for formamide has not, however, been excluded. Proof of the presence of two of the amides unchanged in the corresponding urine has been obtained by isolation from chloroform extract (acetamide) or the ether extract (propionamide).

No evidence of the excretion of the acids was observed after the first 24 hr., but there was a delayed excretion of some of the amides beyond 48 hr. after administration of the dose. Table 2 shows the percentages of the doses of the amides excreted unchanged in 24 hr. periods after the dose. Here also we are in general agreement with Fiske (1923). The lag period decreases with increasing molecular weight, and hence with increasing degree of hydrolysis.

Table 2. Rate of excretion of aliphatic amides by the rabbit

Amide	Dose (g.)	Average percentage of amide excreted unchanged in 24 hr. periods after dose			
		1st	2nd	3rd	Total
Formamide	2.0-4.0	27	8	4	39
Acetamide	1.5-5.0	62	6	4	72
Propionamide	2.0-4.0	37	10	0	47
<i>n</i> -Butyramide	2.0-3.0	13	0	0	13
<i>n</i> -Valeramide	1.5-3.0	3	0	0	3

B. IN VITRO EXPERIMENTS

Methods

Buffer solution. All the experiments were carried out at 37° at pH 7.4 in a phosphate buffer consisting of 0.2M Na_2HPO_4 (8 ml.) and 0.2M NaH_2PO_4 (2 ml.).

Preparation of digests. (a) Slices were cut from the liver immediately after removal from the animal and placed in the buffer. The required amount (8 g.) was made up to 10 ml. with buffer and added to the substrate in oxygenated buffer to make a tissue concentration equivalent to that of the extracts. The digests were incubated in large flasks filled with O_2 .

(b) Extracts were prepared as previously described (Bray *et al.* 1948). Extract (10 ml.) was added to the substrate (0.01204M) in buffer (50 ml.). In experiments lasting more than 6 hr. it has been our practice to add CHCl_3 as a preservative. It will be seen in Table 3 that this retards the rate of hydrolysis, which was greater in 5 hr. without than in 20 hr. with CHCl_3 . In general, the constancy of controls could not be relied upon for more than 6 hr. in the absence of preservative.

Estimation of amide hydrolysis. The NH_3 formed was estimated by the method previously used (Bray, James, Raffan, Ryman & Thorpe, 1949b). In each experiment a control consisting of tissue or extract and buffer without substrate was included. A digest containing *p*-nitrobenzamide was also always included as a check upon the amidase activity of the liver.

Stability of substrates. All the amides examined were stable in the buffer and no formation of NH_3 was observed when they were incubated with boiled liver extracts.

Table 3. Hydrolysis of aliphatic amides by rabbit-liver extracts

Percentage hydrolysis. (Ranges in parentheses. Superior figures against ranges indicate the number of experiments)

	With CHCl_3			Without CHCl_3	
	3 hr.	5 hr.	20 hr.	3 hr.	5 hr.
Formamide	4 (3-5) ⁷	4 (3-5) ⁴	4 (0-9) ⁶	1 (0-3) ⁴	4 (2-5) ⁴
Acetamide	3 (0-4) ³	—	3 (0-7) ³	—	2 (1-3) ²
Propionamide	1 (0-4) ⁵	2 (0-3) ²	5 (3-8) ⁴	1 (0-2) ²	3 (0-5) ²
<i>n</i> -Butyramide	4 (1-6) ⁴	6 (6) ¹	9 (3-14) ⁴	8 (4-12) ³	13 (6-18) ³
<i>n</i> -Valeramide	11 (4-19) ⁷	13 (4-27) ³	39 (29-45) ⁴	48 (34-83) ⁵	55 (41-85) ⁵
Phenylacetamide	2 (0-5) ⁹	6 (3-8) ²	7 (0-19) ⁸	15 (8-20) ⁴	22 (12-32) ⁴
<i>p</i> -Nitrobenzamide	39* (17-49) ⁷	46* (17-58) ⁷	61* (29-81) ⁷	71 (56-92) ¹¹	79 (67-92) ¹¹

* These values should not be compared with those given in a previous paper (Bray *et al.* 1948) which were obtained with 0.00687M *p*-nitrobenzamide.

Hydrolysis of aliphatic amides by rabbit-liver extracts. The degree of hydrolysis is given in Table 3, which also shows the retardation due to use of chloroform as preservative.

Hydrolysis of aliphatic amides by rabbit-liver slices. The degree of hydrolysis is shown in Table 4.

Table 4. *Hydrolysis of amides by rabbit-liver slices*

Amide	No. of exps.	Percentage hydrolysis (ranges in parentheses)		
		2 hr.	5 hr.	6 hr.
Formamide	3	8 (4-13)	10 (6-17)	10 (6-17)
Acetamide	5	0 (0-2)	1 (0-3)	1 (0-3)
Propionamide	4	3 (0-6)	5 (3-7)	5 (3-7)
<i>n</i> -Butyramide	5	18 (15-22)	35 (31-39)	38 (35-43)
<i>n</i> -Valeramide	4	37 (30-43)	65 (49-75)	70 (52-81)
Phenylacetamide	5	12 (9-19)	28 (19-36)	29 (22-40)*
<i>p</i> -Nitrobenzamide	7	37 (30-50)	66 (53-76)	70 (56-82)

* Four experiments only.

Comparison of the hydrolysis of amides by rabbit-liver extract and slices. The percentages of *p*-nitrobenzamide, *n*-butyramide, *n*-valeramide and phenylacetamide hydrolysed by extract and by slices prepared from the same liver are given in Table 5. It will be seen that there are appreciable differences between the degrees of hydrolysis brought about by extracts and by slices. It is difficult to assess the significance of these differences for *p*-nitrobenzamide and *n*-valeramide, but for *n*-butyramide and

phenylacetamide they are considerable. It may also be noted that with *p*-nitrobenzamide there are considerable differences in the initial rate of hydrolysis.

DISCUSSION

Table 6 summarizes the results of both *in vivo* and *in vitro* studies. The results of the former for acetamide, propionamide, *n*-butyramide and *n*-valeramide are in good agreement with those of Fiske (1923) for fasting cats, the degree of hydrolysis

Table 6. *Hydrolysis of amides in the rabbit and by rabbit-liver slices and extract*

Amide	Percentage hydrolysis by		
	Intact animal	Extract (5 hr.)	Slices (5 hr.)
Formamide	61*	4	10
Acetamide	28*	2	1
Propionamide	53*	3	5
<i>n</i> -Butyramide	87*	13	35
<i>n</i> -Valeramide	97*	55	65
Phenylacetamide	100†	22	28
<i>p</i> -Nitrobenzamide	100‡	79	66

* Based on recovery of unchanged amide.

† Only 71% of dose accounted for, but metabolism almost identical with that of phenylacetic acid (Bray *et al.* 1946).

‡ Bray *et al.* (1949a).

increasing with chain length and the lag in excretion decreasing. Formamide is an exception, being hydrolysed to a much greater extent than acetamide, although the rate of excretion is comparable. The lag in excretion (Table 2) is probably not related to delayed absorption of the amides, since Höber & Höber (1937) found that in rat intestine there was little difference between the absorption rates of

Table 5. *Comparison of hydrolysis of amides by rabbit-liver extracts and slices*

Exp.	Time (hr.)	Percentage of hydrolysis of							
		<i>p</i> -Nitrobenzamide		<i>n</i> -Butyramide		<i>n</i> -Valeramide		Phenylacetamide	
		Extracts	Slices	Extracts	Slices	Extracts	Slices	Extracts	Slices
1	0.5	19	13	—	—	15	10	1	2
	2	48	39	—	—	36	33	6	9
	4	65	62	—	—	48	56	10	16
	6	71	71	—	—	54	66	12	22
2	0.5	23	7	—	—	7	7	—	—
	2	47	30	—	—	22	30	—	—
	4	63	47	—	—	38	44	—	—
	6	73	56	—	—	43	52	—	—
3	0.5	32	17	2	8	—	—	3	6
	2	62	50	7	22	—	—	11	19
	4	72	70	12	32	—	—	17	31
	6	88	82	16	38	—	—	20	40
4	0.5	37	—	2	6	—	—	—	—
	2	64	—	9	20	—	—	—	—
	4	77	—	15	34	—	—	—	—
	6	81	—	19	43	—	—	—	—

acetamide and *n*-valeramide. The explanation more probably lies in the rate of reabsorption of these compounds in the kidney. The earlier finding (Bray *et al.* 1948) that in the case of phenylacetamide there is less correlation between the degree of hydrolysis *in vivo* and by extracts *in vitro* than with aromatic amides is shown by the present investigation to apply to other aliphatic amides. The amides studied were, with the exception of *n*-valeramide, hydrolysed to a much greater extent *in vivo* than would be expected from their *in vitro* hydrolysis by extracts. It is, however, possible to offer an explanation for this discrepancy, taking into account the observed lag in excretion of formamide, acetamide and propionamide. In these three amides it may be suggested that the extra reaction time allowed by the delay in excretion compensates for the feeble hydrolytic power of the enzyme responsible. This factor does not operate with *n*-butyramide, but there is instead evidence of more efficient enzyme action provided by the experiments with slices. In these experiments (Tables 4 and 5) the conditions used presumably approximate somewhat more closely to those *in vivo*, and the results show that the hydrolytic activity is much greater than in extracts. With phenylacetamide the percentage hydrolysis observed *in vitro* is 22–29 % compared with an *in vivo* hydrolysis of 100 % (Bray *et al.* 1946). In the earlier investigation, however, it was shown that there was a delayed excretion of the metabolites of phenylacetamide in the rabbit. Thus in the case of all five amides there are secondary factors which may be interpreted as accounting to some degree for the discrepancy between the observed *in vivo* and *in vitro* results. It should, however, be remembered that the *in vivo* results for formamide, acetamide, propionamide, *n*-butyramide and *n*-valeramide were obtained by determination of the amount of amide excreted unchanged. In the case of *n*-butyramide and *n*-valeramide the *in vitro* experiments indicate that

hydrolysis of these amides occurs, but there is no significant enzymic hydrolysis of the other three. This leaves open the possibility that these three amides may be metabolized by some process other than simple hydrolysis, or in some organ other than the liver. Whether the enzyme responsible for the hydrolysis of aliphatic amides is the same as that which hydrolyses aromatic amides is not yet certain. The evidence at present available indicates that the hydrolysis of the aliphatic amides is depressed to a somewhat greater extent by chloroform than is that of *p*-nitrobenzamide. The possibility that different enzyme systems may be involved is being explored.

SUMMARY

1. The metabolism of formamide, acetamide, propionamide, *n*-butyramide, *n*-valeramide and their parent acids has been studied in rabbits by estimating the extra ether-soluble acid in unhydrolysed and hydrolysed urines after administration of the compounds.

2. The percentages of the doses of the amides excreted unchanged were 39, 72, 47, 13 and 3, respectively.

3. There is a considerable lag in the excretion of formamide and acetamide and a smaller lag with propionamide.

4. The hydrolysis of the above amides and phenylacetamide by rabbit-liver extracts and slices has been compared. Formamide, acetamide and propionamide were only hydrolysed to a very small extent. The degrees of hydrolysis for *n*-butyramide, *n*-valeramide and phenylacetamide were: for extracts, 13, 55 and 22 %; and for slices, 35, 65 and 28 %, respectively.

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