enzyme action and this inhibition is compared with that brought about by a constituent of normal serum.

3. The extent of the inhibition by these agents is dependent on the salt concentration.

4. The serum inhibitor, unlike the inhibitors made by esterifying hyaluronic acid, is destroyed by the testicular hyaluronidase used.

This investigation was aided by a grant from G. D. Searle and Co.

REFERENCES

- Carson, J. F. & Maclay, W. D. (1946). *J. Amer. chem. Soc.* **68**, 1015.
 Chamberlain, N. H., Cunningham, G. E. & Speakman, J. B. (1946). *Nature, Lond.*, **158**, 553.
 Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.
 Haas, E. (1946). *J. biol. Chem.* **163**, 63.
 Hadidian, Z. & Pirie, N. W. (1948). *Biochem. J.* **42**, 260.
 Hahn, L. (1945). *Ark. Kemi Min. Geol.* **19** A, no. 33.
 Hobby, G. L., Dawson, M. H., Meyer, K. & Chaffee, E. (1941). *J. exp. Med.* **73**, 109.
 Humphrey, J. H. (1943). *Biochem. J.* **37**, 460.
 Lineweaver, H. & Ballou, G. A. (1945). *Arch. Biochem.* **6**, 273.
 McClean, D. (1942). *J. Path. Bact.* **54**, 284.
 McClean, D. (1943). *Biochem. J.* **37**, 169.
 McClean, D. & Hale, C. W. (1941). *Biochem. J.* **35**, 159.
 Madinaveitia, J. & Quibell, T. H. H. (1941). *Biochem. J.* **35**, 456.
 Meyer, K., Smyth, E. M. & Dawson, M. H. (1939). *J. biol. Chem.* **128**, 319.
 Robertson, W. B., Ropes, M. W. & Bauer, W. (1940). *J. biol. Chem.* **133**, 261.
 Rogers, H. J. (1946*a*). *Biochem. J.* **40**, 583.
 Rogers, H. J. (1946*b*). *Biochem. J.* **40**, 782.
 Skanse, B. & Sandblad, L. (1943). *Acta physiol. Scand.* **6**, 37.
 Speiser, R. & Eddy, C. R. (1946). *J. Amer. chem. Soc.* **68**, 287.

The Fate of Certain Organic Acids and Amides in the Rabbit

3. HYDROLYSIS OF AMIDES BY ENZYMES *IN VITRO*

By H. G. BRAY, SYBIL P. JAMES, BRENDA E. RYMAN AND W. V. THORPE
Department of Physiology, Medical School, University of Birmingham

(Received 4 July 1947)

In the first two papers of this series it was shown that the rabbit is able to hydrolyze the carbamyl group in benzamide and phenylacetamide (Bray, Neale & Thorpe, 1946), but that the introduction of a hydroxyl group in the position *para* to the carbamyl renders the latter stable, less than 7% hydrolysis occurring *in vivo* (Bray, Ryman & Thorpe, 1947). Further investigations are in progress of the effect of hydroxyl groups in other positions and also of amino and nitro groups. This paper reports a parallel study in which we have examined the activity of various enzyme preparations, especially those from rabbit liver, in hydrolyzing the carbamyl group.

Similar investigations are those of Gonnermann (1902, 1903), in which the action of several enzyme preparations, including pepsin, trypsin and sheep's liver and kidney, on a number of amides and anilides was studied, of Geddes & Hunter (1928) and of Grassmann & Mayr (1933) who examined the action of yeast asparaginase on several amides. The criterion used by Gonnermann as an indication of hydrolysis was the isolation of the unchanged amide or of products of its hydrolysis, whilst Geddes & Hunter

estimated the ammonia liberated. Relevant results from these investigations are summarized in Table 1. Geddes & Hunter claim that asparaginase is present in calf liver and also that yeast asparaginase will hydrolyze glutamine. Glutaminases hydrolyzing this amide but not asparagine have been shown to be present in various tissues, including liver and kidney (Krebs, 1935).

In the present work we have determined quantitatively the degree of hydrolysis by a formol titration method based on that used by Balls & Lineweaver (1939) for assessing the activity of papain with hippurylamide as substrate. We have also compared the stability of the amides used to acid and alkaline hydrolysis.

Materials

Amides. The amides which could not be purchased were prepared by the action of aqueous ammonia upon the corresponding acid chloride or upon the methyl ester of the parent acid. Aminobenzamides were prepared by the reduction of the nitrobenzamides with ferrous sulphate (Jacobs & Heidelberg, 1917).

METHODS

Table 1. *Summary of results of some previous investigations of enzymic hydrolysis of amides*

(Results marked * due to Geddes & Hunter (1928). Results marked † due to Grassmann & Mayr (1933). Unmarked results due to Gonnermann (1902).

Action of	Amide						
	Formamide	Acetamide	Propionamide	Benzamide	Salicylamide	Asparagine	Glutamine
Pepsin	—	—	.	—	—	.	.
Trypsin	—	+	.	—	—	.	.
Ptyalin	—	—	.	—	—	.	.
Invertase	—	—	.	—	—	.	.
Maltase	—	—	.	—	—	.	.
Emulsin	—	+	.	—	—	.	.
Sheep's liver	+	+	.	+	.	.	.
Calf's liver	.	.	.	.	.	+	.
Sheep's kidney	+	—	.	+	+	+	.
Yeast asparaginase	—*	—*†	—*†	.	—*	++†	+* —†

Sand. British Drug Houses Ltd. acid-washed sand was found to give more satisfactory results than other preparations tried.

Buffers. The majority of the experiments were carried out 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.). Where other buffers were used details are given in the appropriate section.

Preparation of extracts

The rabbit was killed by a sharp blow on the back of the neck and immediately bled. The liver and kidneys were removed and weighed. The organs were then separately ground with sand and mixed with water (1.5 times the original weight). The resulting *brei* was centrifuged and the supernatant liquid used in the experiments, either directly or after dialysis for 24 hr. The extracts were preserved with chloroform.

Method of estimation of amide splitting

Liver or kidney extract (10 ml.) was added to 50 ml. of a 0.0174M-solution of the amide in the appropriate buffer. The mixture was incubated at 37° in a stoppered flask. At suitable intervals 4 ml. samples were withdrawn, treated with 0.5 ml. neutralized 40% formaldehyde and 1 ml. 0.1N-NaOH and centrifuged. Five ml. of the supernatant liquid were titrated against 0.02N-NaOH using thymol blue, taking the first appearance of blue (pH 9) as end point. Thymol blue was more convenient than phenolphthalein owing to the reddish colour of the digests. With the nitrobenzamides, which are sparingly soluble, 0.00687M-solutions were used. Complete hydrolysis of 4 ml. digest corresponds to a titre of 2.9 ml. 0.02N-NaOH. The corresponding titre for nitrobenzamides is 1.14 ml.

Controls used were (i) the mixture as above, but with boiled extract, and (ii) the mixture without the substrate. The titration values of such controls remained steady throughout the experiments except in a very few cases when the titre of the control increased slightly (up to 0.3 ml. 0.02N-NaOH). The indistinctness of the end point makes it doubtful whether titration differences of less than 0.1 ml. are of any real significance.

Calculation of results

Digests prepared as above using a 0.0174M-solution of the ammonium salt of the acid instead of the corresponding

amide gave results in close agreement with the theoretical values for complete hydrolysis. This theoretical increase in titre was used as the basis for calculation of the degree of hydrolysis.

RESULTS

Experiments with papain

The hydrolysis of benzamide, *p*-hydroxybenzamide and *p*-nitrobenzamide was compared with that of hippurylamide (Balls & Lineweaver, 1939). Digests were made up from 20 ml. of papain solution (3% papain (British Drug Houses Ltd.) in water) and 50 ml. of the amide (0.0174M in acetate buffer pH 4.9). A control for each amide consisted of 20 ml. boiled papain solution with 50 ml. of the amide solution. A control of 20 ml. papain solution with 50 ml. buffer pH 4.9 was also included. Typical results are shown in Table 2 and Fig. 1. Complete hydrolysis in this experiment corresponds to an increase in titre of 2.47 ml. 0.02N-NaOH (0.98 ml. in the case of *p*-nitrobenzamide). *p*-Hydroxybenzamide did not appear to be hydrolyzed at all; the increase in titre of *p*-nitrobenzamide was so small as to be of doubtful significance.

Experiments with liver extracts

Hydrolysis of benzamide. The degree of hydrolysis of benzamide was determined for each liver extract. Two typical results are shown graphically in Fig. 5. The following figures show the percentage hydrolysis of benzamide effected by 29 different liver extracts in 10 hr. (The figures in parentheses give the percentage hydrolysis at equilibrium.) 38 (47); 20 (58); 11 (44); 35 (44); 20 (32); 19 (44); 19 (46); 9 (34); 27 (77); 15 (53); 14 (25); 4 (40); 17 (52); 38 (60); 16 (37); 35 (38); 44 (47); 32 (44); 39 (62); 32 (45); 33 (38); 27 (35); 44 (55); 25 (52); 26 (49); 15 (51); 14 (40); 15 (33); 22 (32). It can be seen that there is considerable variation. The average values are 24% for 10 hr. and 45% at equilibrium.

Table 2. *Hydrolysis of 0.0174M-solutions of hippurylamide, benzamide, p-hydroxybenzamide and p-nitrobenzamide (0.00687M) by papain at pH 4.9*

Time (hr.)	...	0	0.5	1.0	1.5	2.5	3.75	4.0	5.25	6.75	24	28
Titres in ml. 0.02N-NaOH												
Hippurylamide		6.75	7.05	7.2	7.3	7.55	7.7	—	7.75	7.85	8.05	8.1
Hippurylamide control		7.95	—	—	—	—	—	7.6	—	—	7.6	7.65
Benzamide		6.6	6.75	—	6.8	6.85	—	—	6.9	—	6.9	—
Benzamide control		7.7	—	—	—	—	—	7.7	—	—	7.65	—
p-Hydroxybenzamide		8.05	—	—	—	8.0	—	—	7.95	—	8.05	—
p-Hydroxybenzamide control		8.3	—	—	—	—	—	8.35	—	—	8.45	—
p-Nitrobenzamide		6.6	6.7	6.65	—	6.65	—	—	6.75	—	6.75	—
p-Nitrobenzamide control		7.15	—	—	—	—	—	—	—	—	7.05	—
Enzyme + buffer		6.85	6.8	—	6.7	—	7.0	—	6.8	—	6.85	6.85

Fig. 1. Hydrolysis of 0.0174 M-hippurylamide and benzamide by papain at pH 4.9.

Effect of pH on activity of liver extracts. Several experiments of which the following is typical were carried out in order to determine a suitable pH for the reaction. Benzamide digests were prepared with phosphate buffers of pH ranging from 5.00 to 8.04, the concentration of the amide being 0.210 g./100 ml. To 50 ml. of each were added 10 ml. of liver extract; the usual controls were also prepared. For titrating samples of the digest at pH 5.9 and 6.4 it was convenient to add 2 ml. of 0.1N-NaOH before titration, while to samples of the digests at pH 6.9 and 7.4, 1 ml. 0.1N-NaOH was added. Water (1 ml.) was added to the digest at pH 8.04. The results are shown in Fig. 2. It is evident that the pH (7.4) used throughout the main investigations is a suitable one.

Fig. 2. Effect of pH on hydrolysis of benzamide by rabbit-liver extract.

Concentration of substrate. These experiments were carried out in order to compare the hydrolysis of benzamide and p-nitrobenzamide more closely, as in the main experiments the solutions of the latter were less than half the molarity of those of benzamide. In a typical experiment two digests were prepared, consisting of the buffer (pH 7.4) containing 0.21 g./100 ml. and 0.105 g./100 ml. benzamide respectively, together with the usual controls. The results of two experiments are shown in Fig. 3. Thus the percentage hydrolysis achieved is about 10% greater with the more dilute substrate.

Effect of dialysis. The liver extract was prepared by the method described and its activity towards benzamide and p-nitrobenzamide determined. The remainder of the extract was dialyzed against running tap water for 17 hr. and its activity then determined, allowance being made for the dilution of the extract during dialysis.

The results of two experiments given in Fig. 4 show that there is no great difference in the equi-

Fig. 3. Effect of concentration of substrate upon hydrolysis of benzamide by rabbit-liver extract.

Fig. 4. Effect of dialysis upon activity of rabbit-liver extract.

brum values reached. In almost all experiments in the main section undialyzed extracts were used.

Hydrolysis of various amides by liver extracts. The hydrolytic effect of liver extracts was studied using the three isomeric hydroxy-, amino- and nitrobenz-amides, *o*-bromobenzamide, benzamide, formamide, acetamide, propionamide, phenylacetamide, nicotinamide, asparagine and glutamine. The results of typical experiments are shown in Fig. 5, and those of all experiments are summarized in Table 3.

Fig. 5. Hydrolysis of amides by rabbit-liver extracts at pH 7.4.

Titration of ammonium salts. Solutions of the ammonium salts (25 ml., 0.0174 M) in buffer pH 7.4 were mixed with 5 ml. liver extract; 4 ml. samples of the mixture were treated with neutralized formaldehyde (0.5 ml. 40%) and 0.1 N-NaOH (1 ml.) and centrifuged. Of the supernatant liquid 5 ml. were titrated against 0.02 N-NaOH. Blank determinations on the corresponding amides were carried out by adding liver extract (5 ml.) to 25 ml. of 0.0174 M-solutions of the amide in buffer pH 7.4; 4 ml. samples were withdrawn, treated with formaldehyde and titrated as above. The difference between the two titrations (corrected for volume) gives the titration due to the ammonia of the ammonium salt, and this represents the ammonia which would be formed by complete hydrolysis of the amide. The

results shown in Table 4 justify the use of the theoretical value as the basis for the calculation of percentage hydrolysis. Incubation of the mixture of ammonium salt and liver extract at 37° for 18 hr. did not affect the titres.

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

Amide	Hydrolysis		Average hydrolysis at equilibrium (%)
	In 10 hr. (%)	At equilibrium (%)	
Benzamide (average values)	24	45	45
<i>o</i> -Hydroxybenzamide	0	0	0
	0	0	
	0	0	
	0	0	
<i>m</i> -Hydroxybenzamide	0	0	1
	0	0	
	2	4	
<i>p</i> -Hydroxybenzamide	0	0	6
	0	0	
	3	10	
	7	13	
<i>o</i> -Aminobenzamide	0	0	9
	0	0	
	3	7	
	8	15	
	16	32	
<i>m</i> -Aminobenzamide	7	13	7
	0	4	
	0	5	
	0	0	
	3	13	
<i>p</i> -Aminobenzamide	2	8	13
	3	11	
	0	0	
	0	26	
	11	20	
<i>o</i> -Nitrobenzamide	12	27	23
	13	28	
	15	15	
<i>m</i> -Nitrobenzamide	12	36	31
	22	45	
	3	17	
	24	24	

Table 3 (cont.)

Amide	Hydrolysis		Average hydrolysis at equilibrium (%)
	In 10 hr. (%)	At equilibrium (%)	
<i>p</i> -Nitrobenzamide	93	100	82
	68	79	
	43	91	
	96	96	
	42	60	
	38	50	
	100	100	
	86	86	
	64	64	
	84	84	
	84	84	
	50	70	
	100	100	
<i>o</i> -Bromobenzamide	8	14	21
	0	28	
	5	22	
Formamide	0	0	0
	0	0	
Acetamide	3	6	2
	0	3	
	0	0	
Propionamide	24	32	25
	5	10	
	9	26	
	6	30	
Phenylacetamide	7	19	10
	6	7	
	0	0	
	8	15	
	4	8	
Nicotinamide	9	9	13
	8	8	
	8	26	
	9	9	
Asparagine	30	37	32
	29	29	
	30	32	
Glutamine	16	16	22
	28	28	

Table 4. *Formol titration of 0.0174M-solutions of ammonium salts, expressed as ml. 0.02N-NaOH*

	Titration	Difference	Difference corrected for volume*	Calculated difference
Ammonium benzoate	6.75	—	—	—
Benzamide blank	4.27	2.48	2.73	2.9
Ammonium <i>p</i> -nitrobenzoate†	5.5	—	—	—
<i>p</i> -Nitrobenzamide blank	4.4	1.1	1.21	1.14
Ammonium <i>p</i> -hydroxybenzoate	7.07	—	—	—
<i>p</i> -Hydroxybenzamide blank	4.75	2.32	2.55	2.9
Ammonium <i>p</i> -aminobenzoate	7.1	—	—	—
<i>p</i> -Aminobenzamide blank	4.2	2.88	3.17	2.9

* I.e. titration due to ammonium salt.

† 0.00687M-solution used owing to small solubility of amide.

Experiments with kidney extract

The scope of these investigations was limited by the small amounts of tissue available. In general it may be said that kidney extracts possess feeble amide-hydrolyzing properties. The rate of hydrolysis in some cases was found to be very slow, e.g. in one experiment *p*-nitrobenzamide was hydrolyzed to an extent of 35 % in 30 hr. while, in another, 60 hr. was required to bring about the same change. It is, perhaps, significant that Waelsch & Busztin (1937) found that aqueous extracts of horse kidney showed no enzymic activity of this type, but that glycerol extracts were able both to hydrolyze and synthesize benzamide. We found that glycerol extracts of rabbit kidney were not more active than aqueous extracts.

Experiments with proteolytic enzymes of animal origin

Trypsin. Solutions of benzamide (0.21 g./100 ml.); *p*-hydroxybenzamide (0.23 g./100 ml.); phenylacetamide (0.23 g./100 ml.); and *p*-nitrobenzamide (0.115 g./100 ml.) in phosphate buffer pH 8.04 were prepared and to each 3 % pancreatin (British Drug Houses Ltd.) (10–50 ml.) was added. Similar solutions treated with boiled enzyme solution served as controls. A further control consisted of buffer and enzyme solution without substrate. The solutions were incubated at 37° and samples (4 ml.) withdrawn at intervals and titrated as before. In no case was any significant hydrolysis observed.

Pepsin. Solutions of benzamide, *p*-hydroxybenzamide, *p*-nitrobenzamide and phenylacetamide were prepared as in the last experiment, but using an acetate buffer pH 1.2. To 50 ml. of each solution was added 3 % pepsin (British Drug Houses Ltd.) solution (10 ml.). These were incubated along with controls as above and samples withdrawn and titrated as before. No significant hydrolysis was observed. Similar experiments at pH 5.9, using a phosphate buffer, also gave negative results.

Inhibition of enzyme action by various substances

The discrepancy observed between the complete hydrolysis of phenylacetamide which occurs *in vivo* (Bray *et al.* 1946) and its relative stability to liver extracts *in vitro* suggested that phenylacetamide or a metabolite, e.g. phenylacetic acid or phenaceturic acid, might have an inhibiting effect upon the enzyme responsible for amide hydrolysis. Benzamide and *p*-nitrobenzamide were used as substrates. It was found that 30 mg. of these substances/60 ml. digest produced significant inhibition. Typical results are given in Table 5.

Table 5. *Inhibiting effect of various compounds upon hydrolysis of benzamide and p-nitrobenzamide by liver extracts*

Amide	Compound added	Hydrolysis at equilibrium (%)
Benzamide	—	61
	Phenaceturic acid	43
	Phenylacetic acid	34
	Phenylacetamide	30
<i>p</i> -Nitrobenzamide	—	85
	Phenaceturic acid	75
	Phenylacetic acid	57
	Phenylacetamide	46

It may be noted that phenaceturic acid, the chief metabolite of phenylacetamide in the rabbit, shows less inhibiting effect than either phenylacetamide or phenylacetic acid.

Hydrolysis of amides by acid and alkali

In order to compare the stability of the amides to acid and alkali with their stability to liver extracts, a series of experiments was carried out in which they were incubated with acid and alkali under standard conditions and the amount of ammonia formed estimated by aeration into standard acid. The conditions chosen in the case of acid were heating 0.001 mol. with *N*-HCl (25 ml.) in a boiling-water bath for 1 hr. Under these conditions benzamide was hydrolyzed to the extent of 33 %.

The alkaline conditions were similar except for using *N*-NaOH for hydrolysis, and resulted in 73 % hydrolysis of benzamide. Table 6 shows the results, together with those obtained from the enzyme experiments. The order of stability to acid and alkali is not greatly different, but stability to enzymic hydrolysis clearly depends upon other factors.

Table 6. *Hydrolysis of amides by liver extracts, acid and alkali*

Amide	Hydrolysis by liver extract at equilibrium (%)	Hydrolysis by <i>N</i> -HCl in 1 hr. (%)	Hydrolysis by <i>N</i> -NaOH in 1 hr. (%)
<i>p</i> -Nitrobenzamide	82	35	79
Benzamide	45	33	73
Asparagine	32	50	—
<i>m</i> -Nitrobenzamide	31	27	81
Propionamide	25	87	78
<i>o</i> -Nitrobenzamide	23	1	21
Glutamine	22	—	—
<i>o</i> -Bromobenzamide	21	—	—
Nicotinamide	13	28	73
<i>p</i> -Aminobenzamide	13	30	54
Phenylacetamide	10	87	79
<i>o</i> -Aminobenzamide	9	8	64
<i>m</i> -Aminobenzamide	7	26	70
<i>p</i> -Hydroxybenzamide	6	19	14
Acetamide	3	88	75
<i>m</i> -Hydroxybenzamide	1	32	55
<i>o</i> -Hydroxybenzamide	0	9	9
Formamide	0	83	81

The mechanism of amide hydrolysis by enzymes

When acid and alkali were used for the hydrolysis of amides the ammonia formed was estimated, whereas in the enzyme experiments the formol titration gave a measure of the acid formed. If the mechanism of the enzymic reaction is, like that catalyzed by acid and alkali, a hydrolytic one expressed

then both methods of estimation should yield the same result. To confirm this, the progress of hydrolysis in some enzymic digests was followed by both methods. Fig. 6 shows that the two methods yield essentially the same results. The fact that no increase in acidity of the enzymic digests was apparent until formaldehyde was added suggests that the mechanism is a hydrolytic one.

Fig. 6. Comparison of aeration and formol titration methods for estimation of hydrolysis of amides by rabbit liver extract.

The theoretical possibility of the enzymic conversion of the amide being due to an oxidative mechanism such as

with subsequent conversion of the hydroxylamine to ammonia, has, however, been explored to the extent of testing for the presence of hydroxylamine. The Pucher and Day reaction (Pucher & Day, 1926) applied to various digests gave negative results up to 48 hr. incubation. The amounts of hydroxylamine which could be formed, however, are near the lower limit of sensitivity of the test. Hydroxylamine is converted in the tissues into nitrite and ammonia (Lewin, 1888-9):

The same change is brought about by liver extracts. Amounts of hydroxylamine sufficient to give a good Pucher and Day test were added to liver extracts (140 mg./50 ml.) and incubated for 20 hr. The extracts then failed to give a Pucher and Day reaction, but the presence of nitrite was easily detected by the Griess-Ilosvay diazotization test. This test applied to the various amide digests was, like the Pucher and Day test, always negative.

Further evidence against an oxidative mechanism, at least an aerobic one, was provided by comparing the results obtained with digests incubated under the stated conditions with those from digests incubated in an atmosphere of oxygen or nitrogen. No significantly different results were obtained.

Other properties of liver extracts

The liver extracts used for the experiments described in this paper were capable of reducing the three isomeric nitrobenzamides to yield diazotizable material. They were also able to oxidize hydroxybenzoic acids and amides to dihydroxy compounds (cf. Bray *et al.* 1947), which could be detected by means of the colorations given with ferric chloride. Both these properties of the extracts are being investigated in more detail.

Attempts to obtain active dried liver preparations. When dried by means of acetone and ether, rabbit liver yielded inactive preparations. Dried ox liver, however, showed some enzymic activity. These investigations are being continued.

DISCUSSION

The main object of this research was to compare the hydrolysis of amides *in vivo* with that by enzymes *in vitro*. The results set forth above show that in general the activity of liver extracts in hydrolyzing amides is similar to that of the intact animal, since we have already shown that the metabolism of benzamide is virtually indistinguishable from that of benzoic acid (Bray *et al.* 1946), and that *in vivo* the carbamyl group of *p*-hydroxybenzamide is hydrolyzed to only a very slight extent (Bray *et al.* 1947). Investigations in progress in this laboratory suggest that *in vivo* *o*- and *m*-hydroxybenzamides are incompletely, if at all, hydrolyzed, that the three isomeric aminobenzamides are hydrolyzed only partially (Bray, Lake, Neale, Thorpe & Wood, 1948), and that *m*- and *p*-nitrobenzamides are probably completely hydrolyzed. Nencki (1873) states that acetamide is excreted unchanged by dogs, although his evidence does not preclude the possibility of a slight degree of hydrolysis.

So far, phenylacetamide appears to be the only exception to this general agreement, inasmuch as it

is completely hydrolyzed *in vivo** (Bray *et al.* 1946), but only to an extent of 10 % by liver extracts. This apparent discrepancy may not be as great as it appears at first, since under the dynamic conditions prevailing in the living animal products of a reaction of this type are continuously removed, so that it can proceed further towards completion than under static conditions such as those used in *in vitro* experiments. Thus it is possible that amides which appear to be hydrolyzed with difficulty *in vitro* could be completely hydrolyzed *in vivo*. Phenylacetamide is excreted (as phenaceturic acid) much more slowly than benzamide or benzoic acid (as hippuric acid). Since, however, phenylacetic acid is eliminated as slowly as phenylacetamide, it would seem that it is the conjugation with glycine which is delayed rather than the hydrolysis of the amide.

The fact that the liver extracts used in this study were active in hydrolyzing asparagine and glutamine

* These findings have been confirmed during the present investigation.

suggests that it might be profitable to investigate the possible identity of the liver enzyme(s) with asparaginase or glutaminase.

SUMMARY

1. The hydrolysis of 18 amides by rabbit-liver extracts has been studied and the results compared with some obtained on the rabbit *in vivo*.
2. A suitable pH for the reaction was 7.4.
3. Rabbit-kidney extracts behave in a qualitatively similar manner, but are less active.
4. The action of papain, pepsin and trypsin upon some of the amides is recorded.
5. There is considerable difference between the stability of the amides to hydrolysis by HCl or NaOH and by liver extracts.

We are indebted to the Royal Society for a Government Grant which defrayed part of the cost of this work. The results for the percentage hydrolysis of amides by acid and alkali were obtained by Mr P. B. Wood, whom we wish to thank.

REFERENCES

- Balls, A. K. & Lineweaver, H. (1939). *J. biol. Chem.* **130**, 669.
- Bray, H. G., Neale, F. C., Lake, H. J., Thorpe, W. V. & Wood, P. B. (1948). *Biochem. J.* **42**. In the Press.
- Bray, H. G., Neale, F. C. & Thorpe, W. V. (1946). *Biochem. J.* **40**, 406.
- Bray, H. G., Ryman, B. E. & Thorpe, W. V. (1947). *Biochem. J.* **41**, 212.
- Geddes, W. F. & Hunter, A. (1928). *J. biol. Chem.* **77**, 197.
- Gonnermann, M. (1902). *Pflüg. Arch. ges. Physiol.* **89**, 493.
- Gonnermann, M. (1903). *Pflüg. Arch. ges. Physiol.* **95**, 278.
- Grassmann, W. & Mayr, O. (1933). *Hoppe-Seyl. Z.* **214**, 185.
- Jacobs, W. A. & Heidelberger, M. (1917). *J. Amer. chem. Soc.* **39**, 1437.
- Krebs, H. A. (1935). *Biochem. J.* **29**, 1951.
- Lewin, L. (1888-9). *Arch. exp. Path. Pharmacol.* **25**, 306.
- Nencki, L. v. (1873). *Arch. exp. Path. Pharmacol.* **1**, 420.
- Pucher, G. W. & Day, H. A. (1926). *J. Amer. chem. Soc.* **48**, 672.
- Waelsch, H. & Busztin, A. (1937). *Hoppe-Seyl. Z.* **249**, 135.

Leaf Protease of Tobacco and other Plants

By M. V. TRACEY, *Rothamsted Experimental Station, Harpenden, Herts.*

(Received 4 July 1947)

The work described in this paper was undertaken to obtain information on the properties and activities of protein- and peptide-splitting enzymes of the green leaves of tobacco and other plants. These enzymes will be referred to as proteases.

Greenberg & Winnick (1945) list eleven plant proteases that have been obtained in a relatively high state of purity. All, with the exception of bromelin, pinguinain and solanain, are obtained from latex. These three are obtained from fruits, although bromelin also occurs in the green leaves of the pineapple. Other plant proteases which have been studied in detail are those of seeds, both dormant and germinating. Reports on proteases in the green

leaves of plants that do not produce latex are few, and frequently amount to little more than an assertion of their presence, based in much of the early work on evidence that seems open to question.

The presence of proteases in the green leaf of tobacco has been noted by Loew (1900) (action on egg albumin; none detected on casein, fibrin or gelatin); Oosthuizen & Shedd (1913) (possibly present in cured leaves); Fodor & Reifenberg (1925, 1926) (present in green and dried leaves, peptone substrate); Silberschmidt (1934) (stem sap of *Nicotiana glauca* grafts, peptone substrate); and by Nito & Kitamura (1936) ('trypsin' and 'erepsin' in fresh leaves, 'trypsin' lost on drying). Much of the diffi-