Deacetylation of Acetamido Compounds by Tissue Extracts

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Several workers have provided evidence that deacetylation occurs both *in vivo* and *in vitro*. Abbott (1942) found that, when *N*-acetyl-glycine and sodium benzoate were administered orally to rabbits, the increase in hippuric acid excretion was only slightly less than when the corresponding amount of glycine was given with the benzoate. Elson, Goulden & Warren (1946) found that the rat deacetylated *N*-acetanilide almost completely. Krebs, Sykes & Bartley (1947) showed that injected *N*⁴-acetylsulphamezathine was deacetylated to a considerable extent by the pigeon, dog and cat. Some aromatic acetamido compounds appear to undergo slight deacetylation in the rabbit. This is probably largely due to spontaneous hydrolysis (cf. Krebs *et al.* 1947; Bray, Lake & Thorpe, 1949).

Deacetylation by tissues *in vitro* has also been recorded. Kimura (1929) observed the deacetylation of certain aliphatic acetamido acids at pH 7.0–7.2. Dog liver hydrolysed *N*-acetyl-DL-leucine more readily than it did *N*-acetyl-glycine, although rabbit and pig liver hydrolysed both compounds to approximately the same extent. Michel, Bernheim & Bernheim (1937) showed deacetylase activity in dog, cat, rabbit, rat and ox liver and in rat kidney. Their preparations hydrolysed *N*-acetyl-L-tyrosine and *N*-acetyl-L-leucine more readily than they did acetanilide. Kohl & Flynn (1940) showed that rat liver preparations hydrolysed *N*⁴-acetylsulphanilamide at pH 7.5, and Shaffer (1942) studied deacetylation of acetamidodisulphonamides by chicken kidney. Krebs *et al.* (1947) showed that sheep kidney, liver and intestinal mucosa and pigeon liver were active in hydrolysing acetamidodisulphonamides at pH 7.3. Bray, James, Raffan, Ryman & Thorpe (1949) and Bray, James & Thorpe (1949) noted differences in the action of liver and kidney preparations of different species towards *N*-acetyl-glycine and *N*-acetanilide which led them to believe that different enzymes were concerned. The present paper presents further evidence for this conclusion and observations on the action of various tissue extracts upon a range of acetamido compounds. It is convenient in this paper to refer to an enzyme which hydrolyses *N*-acetyl-glycine as AG deacetylase and to an enzyme which hydrolyses acetanilide as AA deacetylase.

METHODS

Materials. Aromatic acetamido compounds which could not be purchased were prepared from the corresponding amino compounds by treatment with acetic anhydride.

N-Acetyl-glycine was purchased (Light and Co.). *N*-Acetyl-L-leucine and *N*-acetyl-DL-alanine were prepared by the method of Carter, Handler & Stevens (1941) from L-leucine and DL-alanine. *N*-Methylacetamide was purchased (British Drug Houses Ltd.).

Tissue extracts. These were prepared as described previously (Bray, James, Ryman & Thorpe, 1948; Bray, James, Raffan, Ryman & Thorpe, 1949). Liver was extracted with 1.5 times its weight of water and kidney with 3 times its weight.

Substrates. 0.0137 M solutions of the acetamido compounds in phosphate buffer pH 7.4 were used.

Digests. Substrate solution (50 ml.) was incubated at 37° with tissue extract (10 ml.). Two drops of CHCl₃ were added as preservative. Many experiments with both *N*-acetyl-glycine and acetanilide showed that this amount of CHCl₃ caused no significant inhibition of the enzyme (see Table 4).

Estimation of degree of hydrolysis of aliphatic acetamido compounds. This was determined by measurement of the increase of acidity using a formol titration method similar to that described by Bray *et al.* (1948).

Estimation of degree of hydrolysis of aromatic acetamido compounds. The digests were deproteinized with Zn(OH)₂ (cf. Somogyi, 1930), and the amino compound formed estimated by a procedure similar to that of Bratton & Marshall (1939) for sulphanilamide in blood. The standard used in each case was the amino compound being estimated. Trichloroacetic acid was unsatisfactory as a deproteinizing agent, since it was liable to cause spontaneous hydrolysis of the acetamido compound.

RESULTS

Values for the average percentage hydrolysis of *N*-acetyl-glycine and of acetanilide by extracts of the liver, kidney and brain of various animals are recorded in Table 1. The values from individual experiments were sufficiently near to the average values to justify the latter being taken as typical of the activity of the tissue. The values for mouse liver and kidney and for rat kidney were obtained on the mixed tissues of several animals in each experiment.

It is clear from Table 1 that both the livers and kidneys of the animals examined hydrolyse *N*-acetyl-glycine readily with the conspicuous exception of dog liver which has no significant activity. The liver/kidney activity ratio calculated from 20 hr. values is approximately 1 for all animals except the dog. Consideration of the 3 hr. values, and the fact that the kidney extracts were made up with twice the amount of water used for the liver extracts, suggests that kidney has a greater activity per unit weight of tissue than has liver.

Table 1. *Deacetylase activity of extracts of liver, kidney and brain of various species expressed as average percentage hydrolysis of the substrate*

	<i>N</i> -Acetylglycine						Acetanilide					
	Liver		Kidney		Brain		Liver		Kidney		Brain	
	3 hr.	20 hr.	3 hr.	20 hr.	3 hr.	20 hr.	3 hr.	20 hr.	3 hr.	20 hr.	3 hr.	20 hr.
Rabbit	38	89 (10)	77	88 (6)	8	14 (2)	14	32 (11)	3	13 (6)	0	0 (3)
Guinea pig	72	95 (4)	76	88 (2)	0	5 (2)	32	77 (6)	4	10 (4)	0	0 (2)
Rat	37	84 (6)	58	98 (2)	8	14 (2)	35	67 (10)	9	32 (3)	0	0 (2)
Cat	50	74 (3)	14	72 (2)	—	—	15	35 (3)	24	52 (3)	—	—
Dog	1	4 (6)	76	97 (4)	—	—	15	48 (6)	3	6 (4)	—	—
Horse	40	100 (2)	61	90 (3)	—	—	23	54 (2)	3	9 (2)	—	—
Mouse	10	61 (4)	27	76 (4)	—	—	28	56 (5)	5	14 (4)	—	—

Table 2. *The effect of acetone treatment upon the deacetylase activity of various tissues expressed as percentage of substrate hydrolysed*

Tissue	Exp. no.	<i>N</i> -Acetylglycine				Acetanilide			
		Fresh extract		Acetone-treated extract		Fresh extract		Acetone-treated extract	
		3 hr.	20 hr.	3 hr.	20 hr.	3 hr.	20 hr.	3 hr.	20 hr.
Rabbit liver	1	40	80	79	84	11	33	0	0
	2	44	86	28	100	14	52	0	0
	3*	62	69	84	84	35	42	0	0
	4*	50	98	62	82	8	18	0	0
	5	—	—	44	84	—	—	0	4
Rabbit kidney	1	—	—	92	100	—	—	0	0
	2	—	—	82	100	—	—	0	0
Dog liver		2	15	0	0	22	57	0	0
Dog kidney		—	97	44	76	9	12	0	0
Rat liver	1	48	100	8	30	35	65	8	38
	2	28	81	22	96	39	84	26	74
	3	40	78	24	93	49	80	20	54

* From Bray, James, Raffan, Ryman & Thorpe (1949).

The activity of the extracts towards acetanilide is not like that towards *N*-acetylglycine. The liver/kidney activity ratio only approaches 1 in the case of the cat. For the other animals the kidney appears to have considerably lower activity than has the liver, the liver/kidney activity ratios varying from 2 to 6. Only rat and cat kidney show high activity. The distribution of activity towards acetanilide between liver and kidney in the dog is the reverse of that towards *N*-acetylglycine.

N-Acetylglycine is hydrolysed to only a slight extent by rabbit and rat brain and acetanilide not at all.

The suggestion of a differentiation between acetylglycine (AG) deacetylase activity and acetanilide (AA) deacetylase activity was further explored in the following experiments using the two acetamido compounds as substrates.

(1) *Effect of acetone treatment.* Tissues were treated with acetone as previously described (Bray, James, Raffan, Ryman & Thorpe, 1949), and their deacetylase activity compared with that of the fresh

tissue. The results, together with some obtained previously, are given in Table 2 and show that the AG deacetylase activity is not significantly altered by acetone treatment whereas the AA deacetylase activity is destroyed in all the tissues examined except rat liver. Rat liver has a much greater AA deacetylase activity than rabbit or dog liver and, whilst there is a loss of activity on acetone treatment, the acetone preparation still shows considerable activity. The result in Exp. 1 showing considerable loss in both AG and AA deacetylase activity appears to be abnormal.

(2) *Effect of heat.* Extracts of tissues were kept at 50° or 55° for 30 min. before mixing with the substrate. The results in Table 3 suggest that the AA deacetylase activity is impaired to a greater extent by this treatment than AG deacetylase activity. In both guinea pig and rat liver extracts the AG deacetylase is unaffected by heating at 50°, although slightly diminished by heating at 55°. The AA deacetylase is diminished slightly by heating at 50° but greatly at 55°.

Table 3. *Effect of heat upon deacetylase activity of liver extracts*

Percentage of substrate hydrolysed							
Exp. no.	Duration and temperature of heating	<i>N</i> -Acetylglycine			Acetanilide		
		2 hr.	4 hr.	20 hr.	2 hr.	4 hr.	20 hr.
		Guinea pig					
1	Unheated	46	76	—	18	28	100
	0.5 hr. at 50°	49	80	—	11	19	48
	0.5 hr. at 55°	36	60	—	2	2	3
2	Unheated	57	80	—	25	32	70
	0.5 hr. at 50°	45	77	—	10	16	48
	0.5 hr. at 55°	27	49	—	1	2	7
3	Unheated	—	—	—	22	36	93
	0.5 hr. at 50°	—	—	—	8	16	61
Rat							
1	Unheated	14	22	90	—	—	—
	0.5 hr. at 50°	12	22	90	—	—	—
2	Unheated	—	—	—	21	30	—
	0.75 hr. at 50°	—	—	—	4	6	—
3	Unheated	—	—	—	37	46	66
	0.5 hr. at 50°	—	—	—	30	37	60
	0.5 hr. at 55°	—	—	—	14	16	38

Table 4. *Effect of chloroform upon deacetylase activity*

(Values in parentheses have been corrected, see text.)

Experiment ...	CHCl_3 added (ml.)	Percentage of substrate hydrolysed								
		<i>N</i> -Acetylglycine						Acetanilide		
		1		2		3		1	2	3
		3 hr.	20 hr.	3 hr.	20 hr.	3 hr.	20 hr.	3 hr.	5 hr.	20 hr.
Rabbit liver	None	30	—	32	—	15	—	10	13	32
	0.1	—	—	—	—	—	—	10	13	29
	1.0	22	78	34	91	19	95	(9)	(10)	(19)
Guinea pig liver	None	45	—	32	—	—	—	28	41	77
	0.1	—	—	—	—	—	—	29	41	69
	1.0	70	88	46	93	—	—	(30)	(39)	(50)
Rabbit kidney	None	83	—	89	—	—	—	3	4	11
	0.1	—	—	—	—	—	—	3	5	13
	1.0	87	—	81	—	—	—	(3)	(5)	(8)
Rat liver	None	23	—	—	—	—	—	37	46	70
	1.0	24	—	—	—	—	—	(13)	(19)	(33)

(3) *Effect of pH.* We are in agreement with Michel *et al.* (1937) that the optimum action of AA deacetylase occurs at about pH 7.8. The value which we found for AG deacetylase was pH 8.0.

(4) *Effect of chloroform.* The results shown in Table 4 indicate that the addition of small amounts (0.1 ml.) of chloroform to the digests (60 ml.) has no significant effect upon either AG or AA deacetylase activity. Larger amounts of chloroform (1.0 ml.), which do not dissolve completely in the digest, also have no effect upon the hydrolysis of *N*-acetylglycine, but there seems to be some evidence of retardation of the hydrolysis of acetanilide. The observed values for the hydrolysis of acetanilide

when 1 ml. of chloroform was added were much lower than when chloroform was absent, but the estimation of the aniline formed under these conditions is complicated by the fact that aniline is readily soluble in chloroform so that the aqueous phase of the digest from which the sample is withdrawn for analysis (it is not practicable to obtain an emulsion from which a representative sample can be taken) does not contain all the aniline. A correction was, therefore, applied to the observed values, taking into consideration the partition of aniline between aqueous and chloroform phases (approx. 1–24), the solubility of chloroform in water and the volume of digest before each sample was withdrawn. The

values in parentheses in Table 4 have been corrected. When values obtained experimentally with known amounts of aniline added to buffer and chloroform were corrected in this way, the corrected values were in close agreement with the amount of aniline added. Whilst the corrected values can be taken as a sufficiently accurate measure of the amount of aniline actually formed it is more difficult to decide whether the observed effect is due to inhibition of the enzyme. No account has been taken of the partition of acetanilide between the two phases, although it is of the same order (approx. 1-11) as that of aniline. The possible accelerating effect of chloroform in diminishing the concentration of aniline formed in the aqueous phase has also been neglected. It seems probable that, since these two factors would tend to neutralize each other, their ultimate effect would be small and thus it may be tentatively concluded, in the absence of more precise information and in view of the fact that the corrected values have never exceeded values obtained without chloroform, that the larger amount of chloroform does retard the hydrolysis of acetanilide.

Hydrolysis of other acetamido compounds

Since all the experiments so far described were carried out using only *N*-acetyl glycine and acetanilide, it seemed desirable to examine the action of tissue extracts upon other substrates. For this purpose two other aliphatic acetamido acids, *N*-methylacetamide and a number of substituted acetanilides were used.

(1) *Aliphatic acetamido acids*. The percentages of *N*-acetyl glycine, *N*-acetyl-DL-alanine and *N*-acetyl-

L-leucine hydrolysed by extracts of rabbit and dog liver and kidney are compared in Table 5. It is clear that acetamido acids other than acetyl glycine are attacked by the deacetylase. The experiments with dog liver are of interest in that only *N*-acetyl-L-leucine is readily hydrolysed. Attention should, perhaps, be drawn to the fact that in these experiments the DL form of acetylalanine was used. This may account for the low values observed (cf. also Kimura, 1929), since if only one optical isomer is hydrolysed complete hydrolysis would be indicated by a value of 50 instead of 100 %.

Table 6 shows that extracts of rabbit liver and kidney which have been treated with acetone to destroy the AA deacetylase activity readily hydrolyse *N*-acetyl-DL-alanine and *N*-acetyl-L-leucine.

(2) *N-Methylacetamide*. No hydrolysis of this acetamido compound by liver extract could be detected. Any methylamine formed would have been measured by aeration into standard acid under conditions similar to those previously employed for determination of ammonia liberated from amides (Bray, James, Raffan, Ryman & Thorpe, 1949).

(3) *Aromatic acetamido compounds*. The substrates for these experiments were the *o*-, *m*- and *p*-isomers of acetanilide substituted with $-\text{COOH}$, $-\text{CONH}_2$, $-\text{CH}_3$, $-\text{OH}$, $-\text{Cl}$ and $-\text{NO}_2$. Rat-liver extract, which has high deacetylase activity, was used, and in each experiment the amount of each of the three isomers hydrolysed was compared with amount of acetanilide hydrolysed. The results recorded in Fig. 1 are the average values from duplicate experiments which were in good agreement in each case. The value (41 %) indicated for acetanilide in this diagram is the average of the values obtained from

Table 5. *Hydrolysis of some acetamido acids by tissue extracts*

	Percentage of substrate hydrolysed					
	<i>N</i> -Acetyl glycine		<i>N</i> -Acetyl-DL-alanine		<i>N</i> -Acetyl-L-leucine	
	3 hr.	5 hr.	3 hr.	5 hr.	3 hr.	5 hr.
Rabbit kidney	78	96	32	36	46	58
	—	—	—	—	58	61
Rabbit liver	38	54	29	34	75	82
Dog kidney	85	94	24	25	52	52
Dog liver	0	0	—	—	16	22
	0	2	4	5	23	30

Table 6. *Deacetylase activity of acetone-treated rabbit liver and kidney expressed as percentage of substrate hydrolysed*

Substrate	Liver		Kidney			
			Exp. 1		Exp. 2	
	3 hr.	20 hr.	3 hr.	20 hr.	3 hr.	20 hr.
<i>N</i> -Acetyl glycine	44	84	92	100	82	100
<i>N</i> -Acetyl-DL-alanine	9	15	18	30	14	32
<i>N</i> -Acetyl-L-leucine	54	56	62	80	60	94
Acetanilide	0	4	0	0	0	0

the twelve experiments (range 33–58 %). Values for *o*-acetotoluidide and *o*-hydroxyacetanilide were not determined, since *o*-toluidine and *o*-aminophenol do not give satisfactory colours with the Bratton & Marshall reagent. *o*-Substituted acetanilides appear to be very readily hydrolysed compared to the *m*- and *p*-isomers.

Fig. 1. Hydrolysis of aromatic acetamido compounds by rat-liver extract.

DISCUSSION

Michel *et al.* (1937), in their investigation of the effect of tissue extracts upon acetanilide, attempted to relate the enzyme responsible to other enzymes and to determine whether it could deacetylate both aliphatic and aromatic acetamido compounds. They concluded that there was only one deacetylase since no preparation was obtained which was active towards acetanilide and not also towards aliphatic acyl derivatives. These workers showed that the pH optima for the hydrolysis of acetanilide and chloroacetyl-L-leucine were at approximately pH 7.8, and further stated that the liver/brain ratio for the hydrolysis of acetanilide and chloroacetyl-L-tyrosine were in close agreement.

It is evident from our results that the enzyme responsible for the hydrolysis of acetanilide is different from that which hydrolyses acetylglutamine. If the two substrates were decomposed by the same enzyme then the degree of hydrolysis of the one would be expected to vary with that of the other. Reference to Table 1 shows that with the exception of dog liver all the tissues examined possess high hydrolytic activity towards acetylglutamine, whereas the activity of kidney extracts in hydrolysing acetanilide is in general low. The difference between the two enzyme systems is most clearly seen in the dog, where the degree of hydrolysis of acetylglutamine brought about by the kidney is high and by the liver

negligible, whereas acetanilide is readily hydrolysed by liver but only to a very small extent by kidney. We have found that treatment of the extracts of rabbit and dog liver and kidney with acetone effectively deprives these extracts of AA deacetylase activity whilst leaving the activity towards *N*-acetylglutamine not significantly impaired (Table 2). The difference between the AG deacetylase and AA deacetylase is further emphasized by the relative resistance to heat treatment of the former (Table 3), and the probable inhibition of the latter by chloroform (Table 4). Rat-liver extracts appeared to be more resistant to acetone treatment, although the response to heat and chloroform was similar to that of rabbit liver and kidney extracts. Michel *et al.* (1937) used the similarity of the liver/brain activity ratios for acetanilide and chloroacetyl-L-tyrosine as an argument that the two compounds were hydrolysed by the same deacetylase. They found, and we have confirmed, that the activity of brain extracts is very feeble, so feeble, in fact, that activity ratios based on these values can hardly be regarded as significant.

The action of dog liver upon the aliphatic acetamido acids appears to be exceptional. While the other tissues examined hydrolysed *N*-acetylglutamine, *N*-acetyl-DL-alanine and *N*-acetyl-L-leucine readily, dog liver caused appreciable hydrolysis of only *N*-acetyl-L-leucine (Table 5). Kimura (1929) also observed that dog liver hydrolysed *N*-acetyl-L-leucine but not *N*-acetylglutamine. It seems unlikely that the hydrolysis of *N*-acetyl-L-leucine is brought about by AA deacetylase since in both rabbit and dog kidney AA deacetylase activity is low (Table 1). Furthermore, in rabbit kidney and liver acetone treatment destroys AA deacetylase, but has no effect upon the activity towards *N*-acetyl-L-leucine (Table 6). The experiment with rabbit liver (Table 5) indicates that *N*-acetyl-L-leucine is more readily hydrolysed by liver than the other two acetamido acids. *N*-Acetyl-L-leucine is hydrolysed by dog liver to a much smaller extent than by any other tissue examined. It seems reasonable, therefore, to ascribe this hydrolysis to the presence of very small amounts of AG deacetylase in the absence of definite evidence of the existence of a separate enzyme for *N*-acetyl-L-leucine in dog liver.

It is generally agreed that the deacetylation and acetylation processes are closely interrelated, so that our evidence that different enzymes are responsible for the deacetylation of aliphatic and of aromatic acetamido compounds is in accord with the suggestions made by Stekol (1938) and Bloch & Rittenberg (1945, 1946) that the same may hold for the acetylation of aliphatic and aromatic amino compounds. The behaviour of dog-liver extracts in deacetylating aromatic acetamido compounds very much more actively than the aliphatic ones would be

expected, if it be assumed that the liver has the dominant role in acetylation and deacetylation processes, since *in vivo* only aliphatic amino compounds are acetylated to a considerable degree. In the rabbit, which acetylates aromatic amino compounds and deacetylates aliphatic compounds (if its behaviour towards *N*-acetylglycine (cf. Abbott, 1942) is typical), it would be expected that liver extracts would deacetylate aliphatic acetamido compounds more readily than aromatic. This is in accordance with our findings (Table 1).

The influence deacetylation *in vivo* may have upon subsequent metabolic paths has been discussed by Smith & Williams (1948), who suggest that the difference between the configuration of the hydroxylation products of acetanilide in the dog and the rabbit is due to the fact that the former probably deacetylates it prior to hydroxylation while the latter does not. While direct evidence that this is true for the rabbit is available, as far as we are aware no deacetylation studies on acetanilide in the dog have been carried out. The findings of Krebs *et al.* (1947) that acetylsulphamezathine is deacetylated in the dog suggests that acetanilide would undergo similar treatment, and we have recently shown (Bray, James & Thorpe, 1949) that Jaffe & Hilbert's

(1888) isolation of benzoxazolone as a metabolite of acetanilide in the dog may be interpreted as evidence of deacetylation, the compound isolated being an artifact formed by condensation with urea of the unconjugated *o*-aminophenol during the evaporation of the urine in the process of isolation.

SUMMARY

1. The deacetylase activity of extracts of various tissues has been examined.

2. The deacetylase hydrolysing *N*-acetylglycine appears to be distinct from that hydrolysing acetanilide.

3. *N*-Acetyl-DL-alanine and *N*-acetyl-L-leucine, but not *N*-methylacetamide, are hydrolysed by the acetylglycine deacetylase.

4. Many substituted acetanilides are readily hydrolysed, especially those with the substituent in the ortho position.

5. The correlation between deacetylase activity of tissues *in vitro* and the process of deacetylation *in vivo* is discussed.

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