The Fate of Certain Organic Acids and Amides in the Rabbit

11. FURTHER OBSERVATIONS ON THE HYDROLYSIS OF AMIDES BY TISSUE EXTRACTS

By H. G. BRAY, SYBIL P. JAMES, W. V. THORPE and MARIE R. WASDELL
Department of Physiology, Medical School, University of Birmingham

(*Received* 31 *March* 1950)

Studies of the amidase activity of extracts of various animal tissues have been reported in earlier papers (Bray, James, Ryman & Thorpe, 1948; Bray, James, Raffan, Ryman & Thorpe, 1949*a*; Bray, Thorpe & Wood, 1949*c*; Bray, James, Thorpe, Wasdell & Wood, 1949*b*). The present paper records further observations, including the behaviour of the enzyme towards some saturated long-chain and ω-phenyl-substituted aliphatic amides and the effect of increase in chain length on the degree of hydrolysis. The action of the enzyme upon amides previously examined has been reinvestigated in the absence of chloroform, which was used as a preservative in earlier experiments, since it was found that this substance had some retarding effect on amidase activity (Bray *et al.* 1949*b*). Some other amides have also been examined.

The amides studied can be divided into three groups: unsubstituted aliphatic, ω-phenyl-substituted aliphatic and aromatic amides. Three amides which are readily hydrolysed by tissue extracts and are respectively typical of each group, *n*-valeramide, β-phenylpropionamide and *p*-nitrobenzamide, have been used as substrates in more detailed work. Unless otherwise stated, all the aliphatic amides are derived from the normal fatty acids.

MATERIALS

Amides. Amides used in earlier investigations were obtained as previously described. The other amides or the corresponding acids or esters were purchased from British Drug Houses Ltd., unless stated otherwise. Hexanamide and stearamide were purchased. The following were prepared from the parent acids via the acid chlorides: *o*-, *m*- and *p*-chlorobenzamides, decanamide, tetradecanamide, picolinamide and *iso*nicotinamide (acids of the last two prepared from α- and γ-picolines, Light and Co.). The following were prepared by heating the methyl or ethyl ester of the corresponding acid with NH_3 (sp.gr. 0·880) in sealed tubes: heptanamide (methyl heptanoate from W. J. Bush and Co. Ltd.), octanamide, nonanamide, hendecanamide (ethyl hendecanoate from Prof. T. P. Hilditch), dodecanamide, palmitamide. Amides were prepared from the following acids via the acid chloride.

o-, m- *and* p-*Fluorobenzoic acids.* The appropriate aminobenzoic acid or its methyl ester was converted to the fluorobenzoic acid by a method based upon those of Schiemann & Winkelmüller (1933) and Dippy & Williams (1934).

β-*Phenylpropionic acid.* This was prepared by reduction of cinnamic acid with Na amalgam (Vanino, 1923).

γ-*Phenylbutyric acid.* β-Benzoylpropionic acid was prepared from benzene and succinic anhydride by Friedel-Craft condensation (Somerville & Allen, 1933, 1935) and reduced by Zn amalgam to γ-phenylbutyric acid (Overbaugh, Allen, Martin & Fieser, 1935).

δ-*Phenylvaleric acid.* β-Styrylacrylic acid was prepared from cinnamic aldehyde and acetic anhydride (Perkin, 1877) and reduced with Na amalgam followed by red P and I_2 to δ-phenylvaleric acid (Baeyer & Jackson, 1880).

6-*Phenylhexanoic acid.* δ-Benzoylvaleric acid was prepared from adipyl chloride and benzene by Friedel-Craft condensation (Borsche & Wollemann, 1912; Borsche, 1919). The yield from this condensation is very small (10%) since the main product of the reaction is $\alpha\delta$-dibenzoylbutane. δ-Benzoylvaleric acid was reduced by Zn amalgam to 6-phenylhexanoic acid (Borsche & Wollemann, 1912; Borsche, 1919) which was converted without purification (it is very difficult to obtain this acid pure, cf. Borsche, 1919) to the *amide.* Although recrystallized to constant melting point (88–90°) the product was not quite pure. (Found: C, 74·6; H, 8·5; N, 6·6; amide N (by estimation of the NH_3 liberated by acid hydrolysis) 6·8. $C_{12}H_{17}ON$ requires C, 75·4; H, 8·9; N, 7·3; amide N, 7·3%.) The amide N value corresponds to 92·5% of the theoretical value.

Tridecanamide, pentadecanamide, heptadecanamide. These were prepared from the primary alcohols containing one less C atom by the following stages:

$$R.CH_2OH \rightarrow R.CH_2I \rightarrow R.CH_2CN \rightarrow R.CH_2CONH_2.$$

(Dodecan-1-ol and 1-iodohexadecane from British Drug Houses Ltd., tetradecan-1-ol from Prof. T. P. Hilditch.)

iso-*Hexanamide* (β-methylvaleramide) was prepared by the action of conc. H_2SO_4 at ordinary temperature on 2-methyl-*n*-butyl cyanide (British Drug Houses Ltd.).

METHODS

Preparation of digests. The digests were prepared from 0·01204 M solutions (or partial suspensions in the case of the higher unsubstituted aliphatic amides with more than 8 carbon atoms) of the amides in buffer solution (pH 7·4 unless otherwise stated) and tissue extract as previously described (Bray *et al.* 1949*b*). $CHCl_3$ was not added as preservative since experiments were not carried on longer than 6 hr. Where amides were soluble with difficulty the buffer solution was warmed. At pH 7·4 this caused no hydrolysis of the

amides, but in buffers at higher pH some amides were slightly hydrolysed. In such cases the extent of hydrolysis was determined and the appropriate correction made in assessing the extent of enzymic hydrolysis.

Estimation of amide hydrolysis. The NH_3 liberated was determined by the method described by Bray *et al.* (1949*a*).

Estimation of arginase activity. The method was based upon that of Hunter & Downs (1944). Arginine (0·01204 M in phosphate buffer pH 7·4 containing 30 mg. $Co(NO_3)_2$/100 ml.) was incubated with the liver extract. Samples (4 ml.) were withdrawn at intervals and the reaction stopped by heating with 2 N-HCl (2 ml.) at 100° for 10 min. After cooling, 2 N-NaOH (2 ml.) was added and the mixture incubated with urease (one tablet 'urease B.D.H.', British Drug Houses Ltd.) for 40 min. The NH_3 formed was estimated as above.

RESULTS

Hydrolysis of amides by rabbit-liver extracts

The amides were incubated with rabbit-liver extract for 5 hr. at 37°. Experiments of this duration gave values very close to equilibrium values obtained by incubation for a further 15 hr. after addition of chloroform (1·0 ml.) as a preservative. Several determinations were made of the percentage of each amide hydrolysed. The results were sufficiently close to the average values to justify recording the latter as typical values in Figs. 1 and 2.

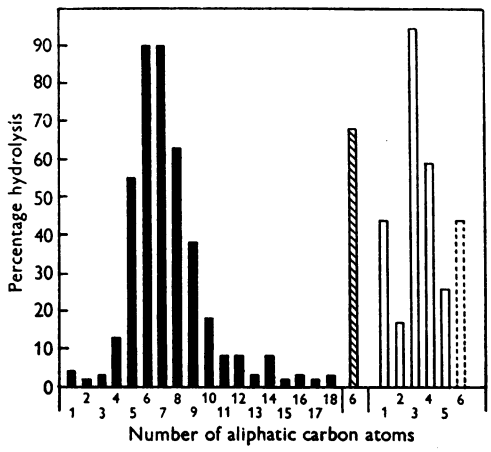

Fig. 1. Percentages of aliphatic amides hydrolysed in 5 hr. at pH 7·4 by rabbit-liver extract. Black bars: amides of normal saturated fatty acids; hatched bar: *iso*hexanamide; unshaded bars: amides of ω-phenyl-substituted normal saturated fatty acids. (The dotted bar represents an amide which was only 92% pure.)

(*a*) *Unsubstituted saturated straight-chain aliphatic amides.* Fig. 1 shows the effect of chain length on the degree of hydrolysis of amides containing 1–18 carbon atoms. Maximum hydrolysis was observed with the amides of hexanoic and heptanoic acids, the degree of hydrolysis falling off progressively on either side. Amides with less than 3 or more than 11 carbon atoms were only hydrolysed to a small extent. The slight hydrolysis observed with the higher aliphatic amides (more than 10 carbon atoms), which were suspended in the digests, does not appear to be due to the limited solubility of these amides, since when the digests were kept for 20 hr. there was no evidence of a slow but continuous hydrolysis. It is of interest that the branched-chain amide with 6 carbon atoms, *iso*hexanamide, was hydrolysed to a greater extent than any of the amides except the corresponding normal amide and heptanamide.

(*b*) *ω-Phenyl-substituted aliphatic amides.* Results for these are shown in Fig. 1. ω-Phenyl substitution appears to facilitate hydrolysis of amides with not more than 4 aliphatic carbon atoms, β-phenylpropionamide being hydrolysed with exceptional ease. δ-Phenylvaleramide and 6-phenylhexanamide are not as readily hydrolysed as the corresponding unsubstituted amides. The value obtained for 6-phenylhexanamide has been shown by a dotted bar in Fig. 1 since the amide was only about 92% pure.

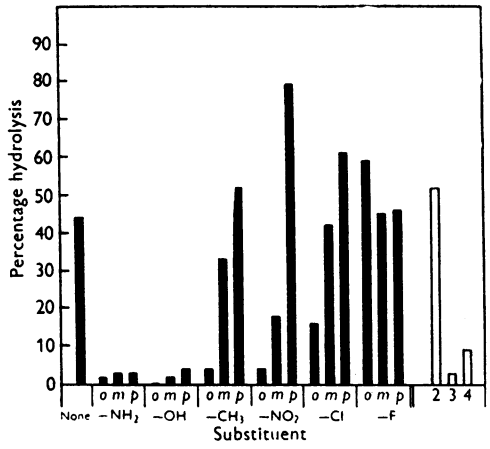

Fig. 2. Percentages of substituted benzamides (black bars) and of picolinamides (unshaded bars) hydrolysed in 5 hr. at pH 7·4 by rabbit-liver extract.

(*c*) *Aromatic amides.* The results for aromatic amides are shown in Fig. 2. Several of these amides have been previously examined using chloroform as preservative (Bray *et al.* 1948). Chloroform has a slight retardant effect (Bray *et al.* 1949*b*), but causes no change in the relative amounts of the different amides hydrolysed. All the values shown in Fig. 2 have been determined in the absence of chloroform. Values for the chloro- and fluoro-benzamides and the 2- and 4-picolinamides have not been previously recorded. It is clear from Fig. 2, that when hydrolysis occurs readily in the benzene series, the position of the substituent has a pronounced influence on the degree of hydrolysis. The effect is less pronounced

in the fluorobenzamides. Substitution with an amino or hydroxyl group practically inhibits hydrolysis since the very low values found are of doubtful significance. Of the pyridine amides only the ortho compound undergoes considerable hydrolysis.

Hydrolysis of amides by extracts of rabbit kidney

The hydrolytic activity of rabbit-kidney extract towards some amides was examined under the same conditions as used with the liver extracts. *n*-Valeramide, β-phenylpropionamide and *p*-nitrobenzamide were hydrolysed (13, 36 and 35% respectively, Table 1). Phenylacetamide and the lower aliphatic amides up to butyramide were not hydrolysed to any significant extent.

Table 1. *Amidase activity of various tissues*

	Average percentage hydrolysis, with number of experiments in parentheses					
	p-Nitrobenzamide		*n*-Valeramide		β-Phenylpropionamide	
Tissue	3 hr.	5 hr.	3 hr.	5 hr.	3 hr.	5 hr.
Rabbit liver	71 (11)	79 (11)	48 (5)	55 (5)	95 (6)	95 (6)
Guinea pig liver	27 (2)	36 (2)	39 (2)	53 (2)	59 (1)	70 (1)
Rat liver	16 (2)	18 (2)	48 (2)	55 (2)	39 (1)	56 (1)
Dog liver	21 (3)	27 (3)	43 (3)	53 (3)	75 (3)	85 (3)
Horse liver	19 (2)	25 (2)	11 (2)	20 (2)	29 (2)	42 (2)
Rabbit kidney	26 (9)	35 (9)	11 (2)	13 (2)	26 (2)	36 (2)
Rat kidney	4 (1)	5 (1)	6 (1)	9 (1)	12 (1)	17 (1)
Dog kidney	7 (3)	10 (3)	17 (3)	23 (3)	50 (3)	59 (3)
Horse kidney	0 (2)	0 (2)	3 (2)	3 (2)	6 (2)	8 (2)

Table 2. *Effect of acetone treatment on amidase activity of rabbit-liver extract*

		Percentage hydrolysis of					
		p-Nitrobenzamide		*n*-Valeramide		β-Phenylpropionamide	
Exp. no.		3 hr.	5 hr.	3 hr.	5 hr.	3 hr.	5 hr.
1	Fresh extract	79	87	67	71	94	94
1	Acetone-treated extract	22	29	7	11	20	36
2	Acetone-treated extract	7	13	4	7	9	14

Experiments with p-*nitrobenzamide*, n-*valeramide and β-phenylpropionamide*

The following experiments were carried out with a view to showing whether the same amidase was concerned with the hydrolysis of all the amides examined.

(1) *Hydrolysis by extracts of livers of different species.* The hydrolytic activity of extracts of liver and kidney of the rabbit, rat, guinea pig, dog and horse towards the three amides has been determined and the results are summarized in Table 1. The three amides were compared under the same conditions. Except where only one experiment is indicated, the values recorded are averages of a number of experiments. Each individual experiment showed the same pattern in relative activities and the actual results were sufficiently close to the average values to justify presentation of the averages as typical values.

(2) *Effect of acetone treatment.* Rabbit liver was treated with acetone as previously described (Bray *et al.* 1949*a*), and the hydrolytic activity of the product towards the three amides compared with that of fresh liver extract. It can be seen from the examples in Table 2, that the activity towards all three amides is diminished to a similar extent by treatment with acetone.

(3) *Effect of* pH. The three amides dissolved in phosphate solutions of varying pH were incubated with rabbit-liver extract. Highest amidase activity was observed between pH 8·2 and 8·4, whichever amide was used as substrate. Fig. 3 shows a typical curve for each amide. The pH values recorded are those of the digests and remained practically constant throughout the experiment. To obtain the digests of high pH it was necessary to use phosphate solutions of higher pH than that of the final mixture. The phosphate solutions were warmed to facilitate solution of the amide. Under the conditions used no hydrolysis of *n*-valeramide or β-phenylpropionamide was observed, but *p*-nitrobenzamide was hydrolysed to the extent of 3·2% when dissolved in the phosphate solution of pH 10·65 which was used in making the digest of pH 9·47. There was no significant hydrolysis at pH 9·47. The value recorded for hydrolysis of *p*-nitrobenzamide by rabbit-liver extract at pH 9·47 in Fig. 3 has, therefore, been corrected to allow for the initial hydrolysis due to solution in the phosphate solution. The correction was −1·5%. For

the digest pH 8·79 (phosphate solution pH 10·49) it was −0·7%. Solutions of lower pH caused no hydrolysis of *p*-nitrobenzamide.

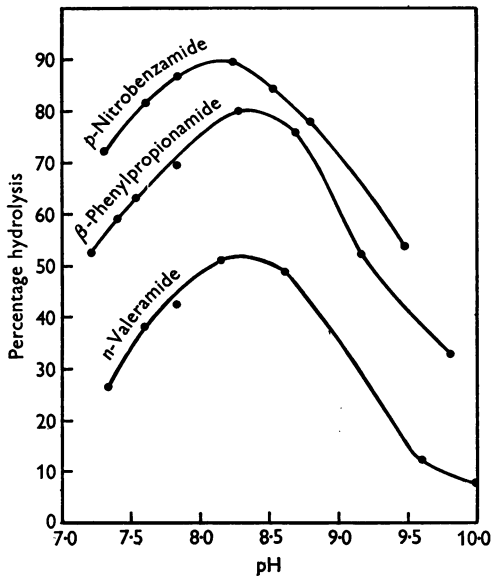

Fig. 3. Effect of pH on the hydrolysis of amides by rabbit-liver extract. Times of digestion: *p*-nitrobenzamide, 3 hr.; *n*-valeramide, 5 hr.; β-phenylpropionamide, 1 hr.

It can be seen from Fig. 3 that the activity at pH 7·4 is sufficiently high to justify routine determinations at this pH. Phosphates have good buffering power at this pH and there is little change in pH on addition of the tissue extract. None of the sparingly soluble amides examined was hydrolysed when warmed with phosphate buffer of pH 7·4.

(4) *Lyophilization of liver extracts.* An extract of rabbit liver was prepared in the usual way. Part was used for determination of amidase activity using *p*-nitrobenzamide as substrate and the remainder lyophilized (75 ml. extract gave about 5 g. dry powder). Part of the powder was suspended in water (25 ml.) to give a solution of concentration corresponding to the original extract and the amidase activity towards the three amides determined. The activity was again determined after the powder had been stored for 3–4 months in a desiccator *in vacuo*. The results of the experiments shown in Table 3, indicate little loss in activity on lyophilization but considerable loss on storage.

(5) *Safranine precipitation.* It was shown in a previous paper (Bray *et al.* 1949*b*) that the addition of an equal volume of an aqueous solution of safranine (0·5%) to rabbit-liver extract according to the method of Geddes & Hunter (1928) gave a precipitate which retained the *p*-nitrobenzamidase activity of the original tissue without appreciable loss. In the experiments recorded in Table 4 the safranine precipitate was suspended in water equal in volume to that of the extract from which it had been obtained. Such suspensions hydrolysed *p*-nitrobenzamide, *n*-valeramide and β-phenylpropionamide to a slightly smaller extent than the original extract. Table 4 also

Table 3. *Effect of lyophilization on amidase activity of rabbit-liver extract*

Exp. no.		Percentage hydrolysis of: *p*-Nitrobenzamide 2 hr.	*p*-Nitrobenzamide 4 hr.	*n*-Valeramide 2 hr.	*n*-Valeramide 4 hr.	β-Phenylpropionamide 2 hr.	β-Phenylpropionamide 4 hr.
1	Fresh extract	52	67	—	—	—	—
1	Lyophilized extract	51	61	25	32	63	80
1	Above after storage 3 months	21	30	9	14	48	60
2	Lyophilized extract	57	74	32	50	72	93
2	Above after storage 4 months	34	50	16	24	52	67

Table 4. *Effect of safranine precipitation on amidase activity of liver extracts*

Exp. no.		Percentage hydrolysis of: *p*-Nitrobenzamide 2 hr.	*p*-Nitrobenzamide 4 hr.	*n*-Valeramide 2 hr.	*n*-Valeramide 4 hr.	β-Phenylpropionamide 2 hr.	β-Phenylpropionamide 4 hr.
				Rabbit liver			
1	Fresh extract	57	77	41	52	80	88
1	Safranine precipitate	43	60	26	46	60	88
2	Safranine precipitate	51	73	30	55	63	93
2	Acetone-dried safranine precipitate	32	49	22	41	44	75
3	Lyophilized precipitate	42	60	22	37	56	80
				Guinea pig liver			
4	Lyophilized safranine precipitate	21	26	21	34	26	44
5	Lyophilized safranine precipitate	14	17	17	21	24	33

shows that considerable activity is retained after drying the safranine precipitate either by acetone or lyophilization.

Similar results were obtained with guinea pig liver. All attempts to remove the dye from the safranine precipitate by treatment with organic solvents (e.g. *n*-butan-1-ol, *iso*-pentan-1-ol, cf. Forbes, 1927) resulted in complete loss of the amidase activity.

Arginase activity of rabbit-liver extract

It can be seen from the experiments recorded in Table 5 that rabbit liver possesses high arginase activity at pH 7·4. This activity is not diminished by lyophilization or even by storage of the lyophilized material in a desiccator for 11 weeks. Treatment with safranine gave a precipitate in which the original activity was largely retained. It has been shown above (Table 2) that acetone treatment of rabbit-liver extracts caused considerable loss in *p*-nitrobenzamidase activity. In Exps. 1 and 2, Table 5, this loss in activity towards *p*-nitrobenzamide is again clearly shown, but the arginase activity is unimpaired by the acetone treatment. These experiments also show that cobalt nitrate, which was added to the buffer for the arginase determinations, had no significant effect upon the *p*-nitrobenzamidase activity.

Table 5. *Arginase and amidase activity of rabbit-liver extracts and the effect of various treatments*

Exp. no.		Percentage changed: Arginine 0·25 hr.	Arginine 1 hr.	Arginine 3 hr.	Arginine 4 hr.	*p*-Nitrobenzamide 0·25 hr.	*p*-Nitrobenzamide 1 hr.	*p*-Nitrobenzamide 3 hr.	*p*-Nitrobenzamide 4 hr.
1	Untreated extract	79	80	82	83	38	59	74	76
1	Acetone-treated extract (precipitate)	61	80	80	80	2	7	12	13
2	Untreated extract	76	76	76	76	19	56	70	71
2	Above + $Co(NO_3)_2$	—	—	—	—	23	56	70	71
2	Acetone-treated extract (precipitate)	70	78	79	79	—	—	0	4
2	Above + $Co(NO_3)_2$	—	—	—	—	—	—	3	3
3	Lyophilized extract (after storage 11 weeks)	—	82	84	—	6	16	27	30
4	Safranine-treated extract (precipitate)	17	43	66	71	9	29	56	66

Urease activity of liver extracts

No ammonia was liberated from urea (0·01204 M in phosphate buffer 7·4) when incubated with extracts of rabbit or guinea pig liver. The extracts were very active in hydrolysing *p*-nitrobenzamide.

DISCUSSION

The examination of the effect of liver extract upon 47 amides has shown that less than half (20) are hydrolysed to an extent greater than 30 % in 5 hr. About the same number (21) are hydrolysed to the extent of less than 10 %, i.e. barely significant or no significant hydrolysis. In general, it appears that (1) only aliphatic amides with 5–10 carbon atoms are readily hydrolysed, (2) all ω-phenyl-substituted aliphatic amides examined were hydrolysed to a significant extent, (3) nuclear substitution of benzamide with —NH_2 or —OH in any position prevents hydrolysis, (4) with the other substituents examined (—NO_2, —CH_3, —Cl, —F) substitution in the *para* position enhances, and in the *ortho* position diminishes the ease of hydrolysis, the fluorobenzamides being exceptional in that *ortho* substitution enhances and *meta* and *para* substitution has no significant effect. Of the amides of pyridine carboxylic acids only 2-picolinamide is significantly hydrolysed.

The experiments with *n*-valeramide, β-phenylpropionamide and *p*-nitrobenzamide were carried out with a view to showing whether the same amidase was concerned with the hydrolysis of all the amides. The results obtained showing the activities of extracts of livers of different species and the effects of various treatments of rabbit-liver extracts do not warrant the conclusion that there is more than one amidase concerned with the hydrolysis of these amides. It has already been shown (Bray *et al.* 1949*a*) that this amidase is distinct from certain other amidases, e.g. glutaminase, asparaginase which appear to have more specific activity. It has also now been shown that preparations containing the amidase have no urease activity and that the amidase activity of rabbit-liver extract can be differentiated from its arginase activity since the latter is not diminished by treatment of the extract with acetone.

SUMMARY

1. The amidase activity of rabbit-liver extract has been examined using 47 amides as substrates. Nearly half of these amides are readily hydrolysed.

2. The extent of hydrolysis is influenced by the number of carbon atoms in straight-chain aliphatic

amides and by the position of substituents in atomatic amides.

3. Experiments with typical aliphatic, ω-phenyl-substituted aliphatic and aromatic amides suggest that the same amidase is concerned with the hydrolysis of all the amides.

4. The amidase is most effective between pH 8·2–8·4.

We wish to make grateful acknowledgement to Prof. T. P. Hilditch, F.R.S., who provided the ethyl hendecanoate and *n*-tetradecanol for the preparation of hendecanamide and pentadecanamide. We have to thank Mr P. B. Wood for assistance in the preparation of some of the amides. We are indebted to the Royal Society for a Government Grant which defrayed part of the cost of this work. The micro-analyses were carried out by Drs Weiler and Strauss, Oxford.

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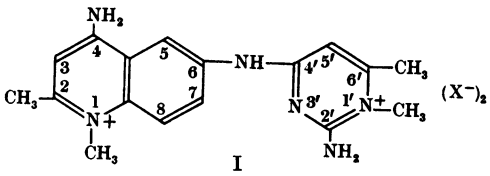
Determination of Antrycide

By A. SPINKS
Imperial Chemical Industries Research Laboratories Ltd., Hexagon House, Manchester 9

(*Received* 21 *March* 1950)

'Antrycide'* (I, 4-amino-6-(2′-amino-1′:6′-dimethylpyridinium-4′-amino)-1:2-dimethylquinolinium dichloride or dimethosulphate) was developed in the laboratories of Imperial Chemical Industries Ltd. by Curd & Davey (1949, 1950) and their collaborators.



I

It has outstanding activity against numerous *Trypanosoma* species, notably *T. congolense*, in mice and cattle. Davey (1947) found that its action varied according to the form in which it was administered subcutaneously. Very soluble salts, for example the dimethosulphate (I, $X^- = CH_3SO_4^-$), were more toxic than sparingly soluble salts, for example the dichloride (I, $X^- = Cl^-$), when these were given in large amounts as dispersions. The prophylactic action of the dichloride, however, persisted longer than that of the dimethosulphate. If the subcutaneous reservoir of drug was removed, prophylactic activity was soon lost. These findings suggest that Antrycide, when given as a 'soluble' salt, may be more rapidly absorbed but less persistent than when given as an 'insoluble' salt, persistence possibly depending on slow seepage of Antrycide into the blood stream from the subcutaneous reservoir. Persistence owing to such an effect was demonstrated by Browning & Gulbransen (1934) for a styrylquinoline derivative.

In order to examine these possibilities a method of determination was needed. It was clear that such a method must be very sensitive, since 1 μg. of Antrycide exerts a slight but definite therapeutic effect when administered subcutaneously to a mouse. Although several methods failed because of inadequate sensitivity, and some for other reasons as well, a short account of them will be given, as they may be applicable to other problems. A brief summary of the preferred method has already been published (Spinks, 1949).

* The name 'Antrycide' is a trade mark of Imperial Chemical (Pharmaceuticals) Ltd.