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STUDIES OF THE RELATIONSHIP BETWEEN CHEMICAL STRUCTURE AND PORPHYRIA-INDUCING ACTIVITY—V

HYDROLYSIS OF AMIDES AND ESTERS BY CHICK EMBRYO LIVER AMIDASES AND ESTERASES*

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Abstract—The hydrolysis of a series of aliphatic di-esters by a chick embryo liver esterase was studied. The degree of steric hindrance to enzymic hydrolysis in the series paralleled the porphyria-inducing activity and the degree of steric hindrance to chemical hydrolysis as assessed by the magnitude of the six-number. The hydrolysis of a series of amides by a chick embryo liver amidase was studied. The degree of steric hindrance to enzymic hydrolysis in a series of aliphatic amides appeared to parallel the degree of steric hindrance to chemical hydrolysis as assessed by the six-number.

STUDIES of the relationship between chemical structure and porphyria-inducing activity have shown that a series of esters and amides have strong porphyria-inducing activity while the corresponding acids are completely inactive.[1,2] For example the amide, allylisopropylacetamide (AIA; Fig. 1a), is a potent porphyria-inducing drug while the corresponding free acid (Fig. 1b), is inactive.[3] Similarly the ester, diethyl 2,3,5,6-tetramethylterephthalate (Fig. 1c), is a potent porphyria-inducing compound while the corresponding free acid (Fig. 1d) is inactive.[2]

It has recently been demonstrated that for a chemical to induce porphyria it must remain in the liver for a period of at least several hours in order to induce and maintain high levels of δ-aminolevulinic acid (ALA)-synthetase.[5] Consequently, it follows that a porphyrin-inducing drug should possess, in addition to other features, appropriate chemical properties that prevent it from being rapidly metabolized and inactivated by the liver. Thus it is likely that a compound such as AIA will not be readily hydrolyzed by liver amidases to the inactive free acid. Similarly, active esters such as diethyl 2,3,5,6-tetramethylterephthalate should not be readily hydrolyzed by liver esterases to the free acid.

In our previous studies of a series of esters and amides, we have noted that in general those compounds which are sterically hindered from hydrolysis by acid and base are active in inducing porphyria.[1,2] For example, AIA which is branched at the α- and β-carbon atoms, and where there is therefore considerable steric hindrance to hydrolysis by acid or base, is active. On the other hand, the sterically unhindered α-methylbutyramide (Fig. 1e) is inactive. We have thus assumed that the steric features hindering hydrolysis of these compounds by acid and base would also hinder

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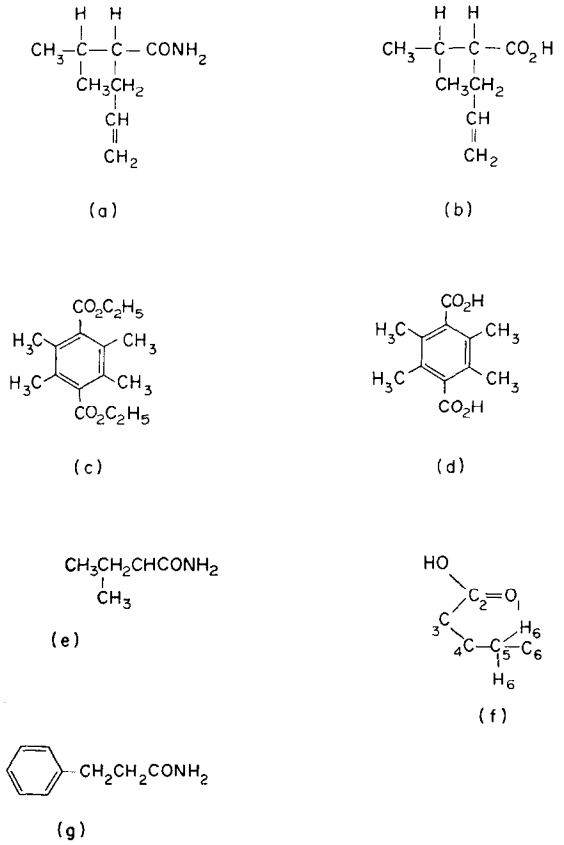

FIG. 1. Chemical structure of: (a) allylisopropylacetamide (AIA); (b) allylisopropylacetic acid; (c) diethyl 2,3,5,6-tetramethylterephthalate; (d) 2,3,5,6-tetramethylterephthalic acid; (e) α-methylbutyramide; (f) numbering system of Newman;[4] (g) 3-phenylpropanamide.

hydrolysis of these compounds to inactive free acids by liver esterases and amidases. In the present paper, we have carried out experiments to determine whether esters and amides which are sterically hindered from hydrolysis by acid and base will also be sterically hindered from hydrolysis by liver esterases and amidases and thus in part explain their activity.

EXPERIMENTAL

Hydrolysis of diethyl glutarate, diethyl β-methylglutarate and diethyl β,β-dimethylglutarate by chick embryo liver esterase

Livers from 17 to 19-day-old chick embryos were cooled on ice and homogenized in ice-cold 1·15% KCl (5 ml/g liver). The homogenate constituted the esterase preparation. Esters were dissolved in a mixture of distilled water and ethanol (9:1; v/v). Solutions of esters (1 ml) were added to test tubes containing esterase (0·5 ml) and 0·1 M potassium phosphate buffer, pH 7·2 (0·5 ml). The colorimetric procedure of Hestrin[6] modified by Lee and Levitt[7] was used for the assay of esterase activity. To several of these test tubes, cooled in ice, alkaline hydroxylamine reagent (4 ml) was

immediately added, and the mixture kept at room temperature for 30 min to allow completion of hydroxamate formation. Two ml of 4 N HCl was added to each tube and the mixture centrifuged. Ferric chloride reagent (2 ml) was added to the clear supernatant and the absorbence of the brown-colored complex determined at 540 nm. This procedure is referred to in Table 1 as the standard treatment and measures the absorbence of ester at the beginning of incubation. The remaining test tubes were incubated for 45 min at 37° and the amount of ester remaining was determined. This procedure is referred to in Table 1 as the enzymic treatment.

TABLE 1. HYDROLYSIS OF ALIPHATIC DI-ESTERS BY A CHICK LIVER ESTERASE

Substrate	Amount added (μmoles)	Net absorbency following:* Standard treatment	Net absorbency following:* Enzymic treatment	Ester hydrolyzed per 100 mg liver in 45 min (μmoles)
Diethyl glutarate†	4	0·545 (0·503–0·584)	0·237 (0·217–0·262)	2·7 (2·51–3·01)
	2	0·281 (0·242–0·306)	0·133 (0·113–0·149)	1·25 (1·03–1·48)
	1	0·158 (0·124–0·180)	0·067 (0·035–0·10)	0·57 (0·37–0·84)
Diethyl β-methylglutarate‡	4	0·556	0·299	2·22
	2	0·303	0·184	0·95
	1	0·138	0·125	0·31
Diethyl β,β-dimethyl-glutarate§	4	0·523 (0·493–0·554)	0·427 (0·383–0·472)	0·81 (0·55–1·07)
	2	0·249 (0·225–0·273)	0·239 (0·224–0·254)	0·30 (0·29–0·30)
	1	0·154 (0·128–0·180)	0·111 (0·093–0·129)	0·33 (0·32–0·34)

* Net absorbency was determined by subtracting the absorbency of a blank solution prepared as follows: to the esterase preparation (1 ml) was added potassium phosphate buffer, pH 7·2 (1 ml) and the resulting mixture allowed to incubate 45 min at 37°. Alkaline hydroxylamine solution (4 ml) was added, and after 20 min 4 N HCl (2 ml) was added, and the protein precipitate was centrifuged. To the clear supernatant solution $FeCl_3$ (2 ml) was added, and the absorbency read at 540 nm.

† Results show the mean and range of three separate experiments. In each experiment the results shown are the mean of duplicates.

‡ Only one experiment was carried out with this compound.

§ Results show the mean and range of two separate experiments.

In preliminary experiments, a period of 30 min was shown to be adequate for completion of hydroxamate formation. A linear relationship was demonstrated between the concentration of ester solutions measured by hydroxamate formation and absorbency over the range of 1–5 μmoles. No non-enzymic hydrolysis was observed for any of the substrates under the conditions described. Boiling the esterase preparation for 25 min prior to incubation resulted in complete loss of enzymic activity.

Determination of chick liver amidase activity

Enzymic preparation. Livers from 17 to 19-day-old chick embryos were homogenized in ice-cold 1·15% KCl (4 ml/g liver). In our initial experiments, 1 ml of this homogenate was used, and this is referred to as the "crude amidase" preparation. In our subsequent studies the cold homogenate was centrifuged at 9000 *g* at 4° for 20 min. The supernatant, which retained all the activity of the homogenate, was used, and is referred to as the "amidase" preparation. Amidase activity of the "crude amidase"

or "amidase" preparations was determined by measuring the amount of ammonia released from a series of amides.

Measurement of ammonia. A microdiffusion apparatus developed by Conway[8] and modified by Öbrink[9] was used. Saturated K_2CO_3 (1·5 ml) and a few drops of non-ionic neutral wetting agent (0·025%; NPX Tergitol) were added to the closing chamber. A 1% boric acid solution (1 ml) and a few drops of Tergitol were added to the inner chamber. The solution (1–2 ml) whose ammonia content was to be determined was added to the outer chamber of the microdiffusion apparatus. Saturated K_2CO_3 (1 ml) was added rapidly to the outer chamber and the lid quickly placed in the closing chamber. The ammonia gas diffused into the boric acid solution and the $H_2BO_3^-$ ions formed as a consequence were titrated with 0·02 N HCl using a microsyringe.

Recovery of NH_4^+ added to "crude amidase" preparation. Several solutions of ammonium sulphate of different concentrations were prepared, and aliquots (0·5 ml) containing 40–160 μg of NH_4^+ were added to a mixture of "crude amidase" (1 ml) and 0·5 M potassium phosphate buffer, pH 7·2 (0·5 ml) in the outer chamber of the microdiffusion apparatus. Saturated K_2CO_3 was added to the outer chamber, and the ammonia gas released was determined. The percentage recovery was > 90 per cent.

Hydrolysis of n-*valeramide by "amidase" preparation.* Solutions of *n*-valeramide (0·5 ml) of different concentrations were added to a mixture of "amidase" preparation (1 ml) and 0·5 M potassium phosphate buffer, pH 7·9 (0·5 ml) in test tubes, and the mixtures were incubated at 37° for 6·5 hr. The contents of the test tubes were then transferred to the outer chamber of the microdiffusion apparatus, and the ammonia content of the mixtures was determined (Table 2). No hydrolysis of a solution of *n*-valeramide (1 ml) theoretically capable of liberating 262 μg of NH_4^+ was observed when incubated with 0·5 M potassium phosphate buffer, pH 7·9 (1 ml) for 6·5 hr at 37°. Boiling the "amidase" preparation (1 ml) for 25 min prior to incubation resulted in complete loss of enzymic activity.

TABLE 2. HYDROLYSIS OF *n*-VALERAMIDE BY AMIDASE PREPARATION

Theoretical amount of NH_4^+ releasable from *n*-valeramide solutions (0·5 ml) (μg)	0·02 N HCl used (ml)	Net NH_4^+ recovered* (μg)
262	0·327	63·2
262	0·369	
131	0·271	36·4
131	0·276	
65·5	0·258	31·8
65·5	0·265	
0	0·165	0·0
0	0·180	

* The net NH_4^+ recovered was determined by subtracting the amount of ammonia detected in a mixture of "amidase" preparation (1 ml) and 0·5 M potassium phosphate buffer, pH 7·9 (1 ml) and is expressed as the mean of duplicate samples.

Effect of pH on the enzymic hydrolysis of n-*valeramide.* Solutions of *n*-valeramide theoretically able to liberate 276 μg NH_4^+ were added to a mixture of "amidase" preparation (1 ml) and 1 M potassium phosphate buffer (1 ml) at varying pH values. Following incubation at 37° for 6·5 hr, the ammonia content of the mixtures was determined. The results are shown in Fig. 2.

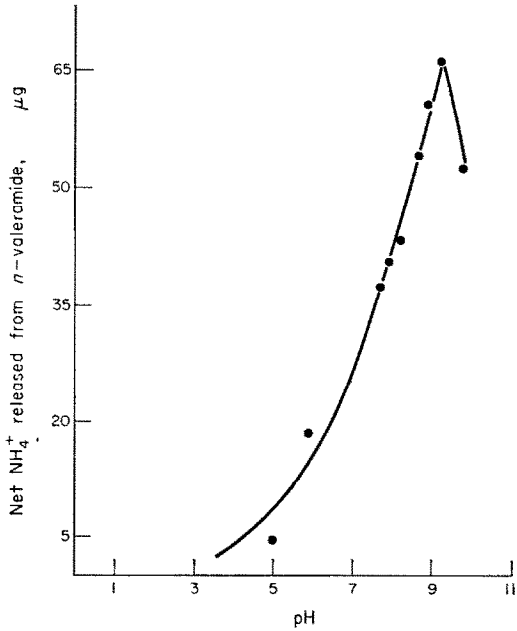

FIG. 2. Effect of pH on the enzymic hydrolysis of *n*-valeramide. The net NH_4^+ released from *n*-valeramide at each pH value was determined by subtracting the ammonia released from a mixture of "amidase" (1 ml) and 1 M potassium phosphate buffer (1 ml) at the same pH. Each point represents the mean of duplicate samples.

Time course of the enzymic hydrolysis of n-*valeramide.* Solutions of *n*-valeramide (0·5 ml) theoretically able to liberate 276 μg NH_4^+ were added to a mixture of amidase (1 ml) and 1 M potassium phosphate buffer, pH 9·2 (0·5 ml) in test tubes and the mixtures were incubated for varying time intervals at 37°. The ammonia content of the mixtures was then determined and the results are shown in Fig. 3.

Hydrolysis of a series of amides by the "amidase". Aliphatic amide solutions containing 17 μmoles equivalent to 306 μg of NH_4^+ in 0·5 ml of water were prepared. Aromatic amide solutions were prepared containing 15 μmoles equivalent to 270 μg of NH_4^+ in 0·5 ml of water.

The amide solutions (0·5 ml) were added to mixtures of "amidase" (1 ml) and 1 M potassium phosphate buffer, pH 9·6 (0·5 ml) in test tubes. Aliphatic amides were incubated for 7 hr and aromatic amides for 5 hr at 37°. The ammonia content of the mixtures was determined and is shown in Table 3.

Determination of porphyria-inducing activity of chemicals. The procedure of Granick[10] using cultures of chick embryo liver cells on Petri dishes and a qualitative scoring technique was employed. The results are shown in Table 4.

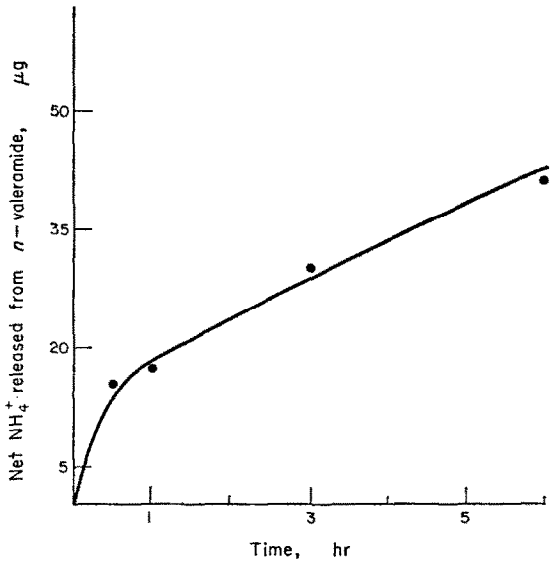

FIG. 3. Time course of the enzymic hydrolysis of *n*-valeramide. The net NH_4^+ released from *n*-valeramide was determined by subtracting the amount of ammonia liberated from a mixture of "amidase" (1 ml) and 1 M potassium phosphate buffer (1 ml). Each point represents the mean of duplicate samples.

TABLE 3. HYDROLYSIS OF A SERIES OF AMIDES BY THE "AMIDASE" PREPARATION

Substrate	Micromoles of amide hydrolyzed by 9000 *g* supernatant from 0·21 g liver in 7 hr*
n-Valeramide	4·82 (4·56–5·16)
α-Methylbutyramide	2·47 (2·21–2·60)
β-Methylbutyramide	1·24 (1·12–1·37)
α-Propylvaleramide	2·04 (1·81–2·35)
Allylisopropylacetamide	0·10 (0 –0·30)
3-Phenylpropanamide†	3·97 (2·34–5·76)
Benzamide‡	0·01

* In each experiment the ammonia released was determined by subtracting the amount of ammonia released from a mixture of amidase (1 ml) and 0·5 M potassium phosphate buffer (1 ml). The results show the mean and the range of three separate experiments. In each experiment results shown are the means of duplicate samples.

† Hydrolysis for 5 hr only.

‡ Hydrolysis for 5 hr and only one experiment carried out.

DISCUSSION

In the first part of our study, we have attempted to determine whether esters which are sterically hindered from hydrolysis by acid and base will be sterically hindered from hydrolysis by liver esterase. The idea that the action of hydrolytic enzymes is

hindered by steric factors in substrate molecules receives support from the following considerations. Many similar models have been proposed to explain the mechanism of ester hydrolysis by esterases and that shown in Fig. 4 contains the essential features of current ideas.[11] In this mechanism, imidazole functions as a general base catalytic agent since it attracts the serine hydroxyl hydrogen and thus increases the nucleophilicity of the serine hydroxyl oxygen atom. This oxygen atom attacks the carbonyl carbon of the ester, and the acylated enzyme intermediate III is formed. An acidic group facilitates rupture of the acyl oxygen bond of the ester by functioning as a general acid catalytic species. Deacylation of the enzyme is accomplished by nucleophilic attack of water at the carbonyl carbon of the acylated enzyme intermediate III forming the intermediate IV which subsequently breaks down forming the free enzyme and acid as shown in V. It seems reasonable that such a mechanism should be sensitive

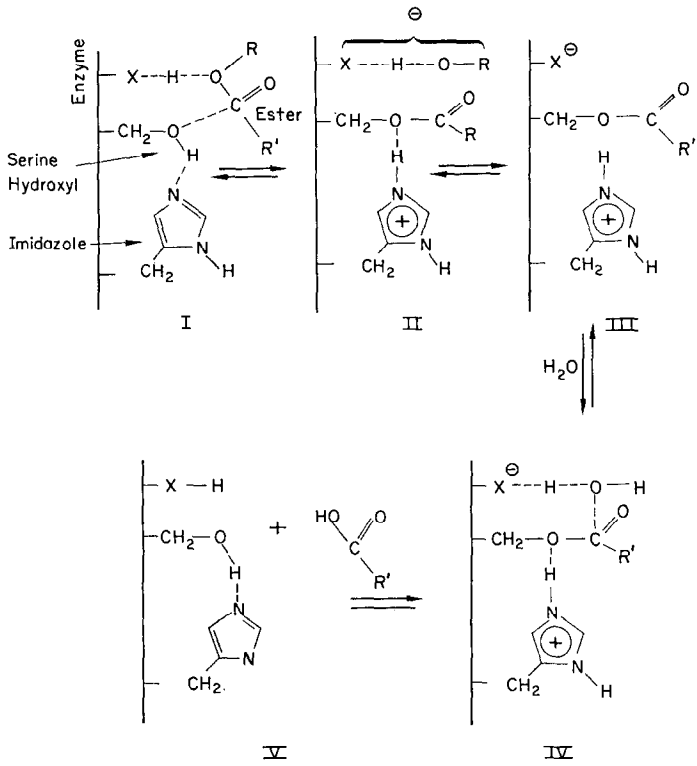

FIG. 4. Proposed mechanism of the hydrolysis of esters by esterase.

to hindrance by steric factors in an ester molecule since both acylation and deacylation depend upon nucleophilic attack at a carbonyl carbon atom. Shielding of the carbonyl carbon by bulky groups in close proximity would hinder the approach of a nucleophile and thus reduce the rate of reaction. Fife and Milstein[12] have studied the effect of steric factors in the deacylation of acylchymotrypsins formed from a series of *p*-nitrophenyl esters. These workers found that increasing steric bulk in the acyl group leads to decreased rates of deacylation. Thus propionyl-chymotrypsin is deacylated

much more rapidly than trimethylacetyl- or 3,3-dimethylbutyrylchymotrypsin. Acylation of the enzyme could not be achieved when *p*-nitrophenyl triethylacetate was employed. Thus increased branching at both the alpha- and beta-carbons of the acyl group decreased the rates of acylation and deacylation. Since it has been established that an acyl enzyme intermediate is formed during the hydrolysis of amide substrates, it follows that amide hydrolysis must also be sensitive to steric factors.[13] It is of interest that the hydrolysis of a series of esters has been found to be more susceptible to steric factors when catalyzed by cholinesterase than when catalyzed by hydroxide ion.[14]

TABLE 4. PORPHYRIN ACCUMULATION IN PRIMARY CULTURES OF CHICK EMBRYO LIVER CELLS INDUCED BY A VARIETY OF CHEMICALS AND MEASURED BY FLUORESCENCE MICROSCOPY

Compound	Concentration (μg/ml)	Intensity of fluorescence
Benzamide*	50	0
	5	0
n-Valeramide†	175	0
3-Phenylpropanamide†	250	3
	100	1
	50	trace

* The cover slip technique was used for testing this compound.
† The Petri dish technique was used for evaluating this compound.

Hestrin[6] developed a rapid micromethod for the estimation of short chain carboxylic acid esters based on their ability to react with hydroxylamine quantitatively in an aqueous alkaline solution. The method was shown to be useful for determination of acetylcholine in the presence of a large excess of acetate and choline. This assay has been modified by Lee and Levitt[7] and used for estimating the concentration and investigating the enzymic hydrolysis of local anesthetic esters such as procaine. The principal modification employed by Lee and Levitt[7] was to increase the time allowed for hydroxamate formation. Thus, in the case of procaine, 10 min was required for maximum hydroxamate formation at room temperature, which was considerably greater than that required by acetylcholine. In our experiments maximum hydroxamate formation was found in a period of 2 min with diethyl glutarate and at least 10 min for diethyl β,β-dimethylglutarate. For this reason, in our studies a period of 20 min was used to ensure complete hydroxamate formation.

In the first part of our study, we have attempted to determine whether esters which are sterically hindered from hydrolysis by acid and base will be sterically hindered from hydrolysis by a liver esterase. In order to estimate the degree of steric hindrance to hydrolysis by acid or base we have previously utilized[2] the empirical rule of six which was developed by Newman.[4] Newman numbered the atoms in an acid consecutively, starting with the carbonyl oxygen as one (Fig. 1f) and called the number of atoms in the six position the six-number. "The rule of six states that in reactions involving addition to an unsaturated function containing a double bond, the greater the number of atoms in the six position the greater will be the steric effect."[4] It was

anticipated that the inactive di-ester, diethylglutarate (six-number = 3), would be hydrolyzed relatively easily while the most active compound, diethyl β,β-dimethylglutarate (six-number = 9), would be hydrolyzed with the greatest difficulty. The moderately active diethyl β-methylglutarate (six-number = 6), it was believed, would occupy an intermediate position. These expectations were borne out by the results shown in Table 1 indicating that the degree of steric hindrance to enzymic hydrolysis in this series of di-esters parallels the degree of steric hindrance to hydrolysis by acid and base as assessed by the magnitude of the six-number. However, only a limited number of substrates have been tested and the results must be treated with reserve.

In the second part of our study, we have attempted to assess whether amides which are sterically hindered to hydrolysis by acid or base will also be sterically hindered to hydrolysis by a liver amidase. Bray *et al.*[15] demonstrated the presence of an amidase in rabbit liver and showed that *n*-valeramide and 3-phenylpropanamide (Fig. 1g) were excellent substrates. For this reason these substrates were selected as substrates for measuring amidase activity in chick embryo liver. The results shown in Tables 2 and 3 and Fig. 3 show that these substrates are also good substrates for chick embryo liver amidase. Bray *et al.*[15] studied the effect of pH on the rabbit liver amidase and showed that the enzyme has optimal activity at pH 8·4. In our studies (Fig. 2), the activity of the enzyme rose as the pH was increased from pH 5, reaching an apparent maximum at pH 9·2. At higher pH values the activity appeared to drop, although this could not be established with certainty using phosphate buffer. In subsequent experiments a pH of 9·2, at which no significant non-enzymic hydrolysis was noted, was used. It was anticipated that AIA (six-number = 8) would be hydrolyzed with the greatest difficulty. This was borne out (Table 3) by the observation that AIA was not a substrate for the enzyme. It was expected that *n*-valeramide (six-number = 3) and α-methylbutyramide (six-number = 3) would be good substrates of the amidase. This was found to be the case. It was thought that α-propylvaleramide (six-number = 6) and β-methylbutyramide (six-number = 6) would occupy an intermediate position. This was found to be the case.

These results show that in the case of aliphatic amides the degree of steric hindrance to enzymic hydrolysis parallels the degree of steric hindrance to chemical hydrolysis as assessed by the six-number. However, only a limited number of substrates have been tested and these results must be treated with reserve.

There is no steric hindrance to hydrolysis in 3-phenylpropanamide, and it was therefore anticipated that it would be a good substrate for the amidase which is the case (Table 3). On the other hand, there is no steric hindrance to hydrolysis in benzamide which is not a substrate for the enzyme. Thus in the case of aromatic amides we cannot infer that if a substrate is not sterically hindered from hydrolysis by acid or base that it will be a substrate for the amidase.

In order to relate these findings to the porphyria-inducing activity of amides, the activity of benzamide, *n*-valeramide and 3-phenylpropanamide was measured in the chick embryo liver cell system of Granick.[10] The results are shown in Table 4. The activity of the remaining amides used in this study has been previously determined.[1,2] It has been shown that for a chemical to induce porphyria it must remain in contact with liver cells for a period of at least several hours in order to induce and maintain high levels of ALA-synthetase. The potency of AIA as a porphyria-inducing drug may, therefore, be partially explained on the basis of its resistance to hydrolysis to

the inactive free acid by liver amidase. The fact that *n*-valeramide, α-methylbutyramide and β-methylbutyramide are substrates for the liver amidase resulting in their conversion to free acids may partially explain their inactivity. The fact that α-propylvaleramide which has considerable porphyria-inducing activity is as good a substrate for the liver amidase as the inactive β-methylbutyramide shows that activity in this series of compounds is not explained completely on the basis of resistance to hydrolysis by the liver amidase, and that other factors must contribute. 3-Phenylpropanamide has only moderate potency as a porphyria-inducing drug, and it is, therefore, not surprising that it is a good substrate for the amidase. Benzamide has no activity as a porphyria-inducing drug and is not hydrolyzed by the amidase. Either benzamide does not possess any intrinsic activity or it is rapidly inactivated by other liver enzymes such as microsomal oxidases or reductases.

In summary, it appears that although steric factors play a part in the activity of the compounds, other physical and chemical factors must be of importance as well. Hansch[16] has recently had considerable success in relating physico-chemical properties of drugs with their biological activities by taking account of steric, electronic and hydrophobic properties of drugs. Further studies of structure-activity relationships of porphyria-inducing drugs along the lines proposed by Hansch for biologically active compounds should be fruitful.

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