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# COMPARISON BETWEEN PROCAINE AND ISOCARBOXAZID METABOLISM *IN VITRO* BY A LIVER MICROSOMAL AMIDASE-ESTERASE

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**Abstract**—Characteristics of a liver microsomal amidase with isocarboxazid (ISOC) as substrate were compared to characteristics of a liver microsomal esterase with procaine (PROC) as substrate. Both amidase and esterase activities were mainly localized in the microsomal fraction with low or null activities in other fractions. Higher specific activities were found in smooth endoplasmic reticulum (s-ER) compared with rough endoplasmic reticulum (r-ER). The microsomal amidase-esterase has a pH optimum of 8·5 to 9·0 and a $K_m$ for ISOC of 0·19 mM and for PROC of 0·53 mM. On the basis of sensitivity to certain esterase inhibitors, this amidase-esterase was considered to be a carboxylesterase type; however, no divalent cations were found to activate the amidase-esterase. Since there were no significant differences between ISOC and PROC in their enzymic properties, i.e. subcellular localization, stability, pH optimum and susceptibility to inhibition, it is possible to speculate that this amidase-esterase is responsible for the metabolism of various drugs possessing amido or ester linkage.

Since Kisch *et al.* [1] reported the hydrolysis of procaine (PROC) in 1943, a number of papers have appeared on the metabolism of this drug. However, serum or plasma was in most cases used as an enzyme source; much less attention has been devoted to this phenomenon by microsomal enzymes. Hydrolysis of PROC by the plasma of humans and other mammals appears to be very low [2]. Lee and Livett [3] reported that liver esterases seem to be more active in the metabolism of PROC than the plasma enzymes.

Our previous papers [4, 5] demonstrated that the enzyme which we have studied seems to be a non-specific esterase possessing both amidase and peptidase activities, and isocarboxazid (ISOC), a monoamine oxidase inhibitor, has been used as a standard substrate. An arylamidase purified from guinea pig liver microsomes is known to be responsible for the hydrolysis of compounds, other than ISOC, possessing an amido bond or ester linkage.

The present studies were designed to expand on these findings in comparison with those using PROC as substrate.

## MATERIALS AND METHODS

*Chemicals.* Glucose 6-phosphate was obtained from the Boehringer Co., and bovine serum albumin from the Armour Pharmaceutical Co. PROC and acetanilide were commercially available. All other chemicals used were of analytical grade and all solutions were prepared in redistilled water.

*Enzyme assay.* Amidase and esterase activities are referred to according to substrate name, i.e. ISOCase and PROCase. ISOCase activity was determined using the colorimetric assay based on conversion of ISOC to benzylhydrazine (BZH). The incubation mixture consisting of 0·5 ml ISOC ($2 \times 10^{-3}$ M) in 0·2 M Tris–HCl buffer, pH 8·0, 0·1–0·5 ml enzyme solution and enough redistilled water to make a final volume of 1·0 ml was incubated at 37° for 30 min in air. Product formation of liver amidase-esterase toward ISOC and PROC as substrates was linear up to at least a 1-hr incubation period. The amount of BZH produced was measured according to the method previously described [4, 6]. Enzyme activity and specific activity were expressed as μmoles BZH formed/g of liver wet wt/min and nmoles BZH formed/mg of protein/min respectively.

Acetanilide-hydrolyzing activity (acetanilidase) was determined by measuring the formation of aniline by the method of Krisch [7] with the minor modification of Akao and Omura [8]. PROCase activity was assayed at 37° for 30 min, incubating with 1 mM PROC as substrate, and the product formed was determined by the method of Ting *et al.* [9]. Succinic dehydrogenase activity was measured by the method of Pennington [10] and incubation was carried out for 15 min. The activities of acid phosphatase and glucose 6-phosphatase (G-6-Pase) were assayed by the procedures of Andersch and Szcypinski [11] and Hübscher and West [12].

Protein was determined by the method of Lowry *et al.* [13], using bovine serum albumin as the standard. Phospholipid was extracted with a mixture of ethanol and ether (3:1) from microsomes precipitated with trichloroacetic acid by the method of Volkin *et al.* [14].

Phospholipid phosphorus was determined by the method of Allen [15], and the value obtained was multiplied by 25 to obtain the amount of phospholipid.

*Fractionation of the arylamidase.* Male Wistar rats (230–280 g) were used for all experiments. The liver was removed, weighed and homogenized in 4 vol. of ice-cold 0·25 M sucrose using a Potter–Elvehjem glass

Table 1. Tissue distribution of liver microsomal amidase-esterase toward isocarboxazid and procaine as substrates*

| Tissue | Substrate | |
|---|---|---|
| | Procaine | Isocarboxazid |
| Liver | 151·3 ± 19·0† | 84·3 ± 11·9 |
| Kidney | 22·7 ± 5·6 | 16·0 ± 2·6 |
| Pancreas | ND‡ | 135·0 ± 26·3 |
| Spleen | ND | ND |
| Heart | ND | 10·0 ± 2·6 |
| Brain | ND | ND |
| Stomach | ND | ND |
| Small intestine | 229·0 ± 49·8 | 41·3 ± 7·7 |
| Testis | 56·3 ± 9·0 | 21·0 ± 1·6 |
| Lung | 51·0 ± 6·4 | 18·3 ± 3·1 |
| Plasma | 2·5 ± 0·3 | 2·1 ± 0·3 |

* Incubation was carried out as described in the text.

† Enzyme activity is expressed as nmoles product/g of tissue wet wt/min; mean ± S. E. M. from at least seven rats.

‡ ND: not detectable.

Table 3. Distribution of various hydrolase activities in submicrosomal fractions of rat liver*

| | Submicrosomal fractions | |
|---|---|---|
| | Rough | Smooth |
| Protein (mg) | 7·95 | 4·44 |
| RNA (μg/mg protein) | 233·6 | 17·1 |
| Phospholipid (PL) (μg/mg protein) | 231·1 | 331·3 |
| Glucose 6-phosphatase (μg/mg protein) | 16·3 | 21·3 |
| (μg/PL) | 69·9 | 62·9 |
| ISOC amidase (nmoles/mg protein) | 25·0 | 44·0 |
| (nmoles/PL) | 104·0 | 141·0 |
| PROC esterase (nmoles/mg protein) | 42·0 | 67·5 |
| (nmoles/PL) | 172·0 | 221·0 |
| Acetanilide hydrolase (nmoles/mg protein) | 19·0 | 32·0 |
| (nmoles/PL) | 77·0 | 100·0 |

* The rough and smooth endoplasmic reticulum were isolated by the method of Dallner [18]. For details, see the text.

homogenizer with a loosely fitting Teflon pestle. Subcellular fractionation was carried out by differential centrifugation by the method of Sedgwick and Hübscher [16] based on the method of de Duve *et al.* [17]. Homogenate was centrifuged at 600 *g* for 10 min, then at 5000 *g* for 10 min, 10,000 *g* for 20 min and finally at 100,000 *g* for 60 min, to separate nuclear, mitochondria, lysosomal and microsomal fractions respectively. All subfractions prepared as above were washed once in 1·15% KCl by resuspension and recentrifugation prior to assays of enzyme activity.

When only the isolation of microsomes was necessary, the liver homogenate was first centrifuged at 10,000 *g* for 20 min. After recentrifugation of the supernatant at 100,000 *g* for 60 min, the microsomal pellet was washed once in 1·15% KCl to remove adsorbed protein. The final pellet was then resuspended in 1·15% KCl to contain 1–10 mg protein/ml. Separation of rough (r) and smooth (s) endoplasmic reticulum (ER) was carried out by the method of Dallner [18]. Experiments on the determination of characteristics of the amidase-esterase described here were made using microsomes as an enzyme source.

## RESULTS

*Tissue distribution.* Studies were made to determine the tissue distribution of the amidase-esterase in microsomal fractions of various tissues, i.e. liver, heart, kidney, brain, testis, intestine, stomach, pancreas, spleen, lung and plasma of the rat. Table 1 shows that liver possessed relatively higher ISOCase activity than any other tissue except pancreas; higher PROCase activity was found in liver than in any other tissue tested except intestine. In addition, heart and plasma exhibited a lower enzyme activity, and brain, spleen and stomach had no enzyme activities toward ISOC and PROC.

*Localization of the amidase-esterase in subcellular fractions.* The activities of the amidase-esterase and of known marker enzymes for three subcellular components were studied in the fractions obtained from rat liver. The markers chosen were: succinic dehydrogenase (mitochondria), acid phosphatase (lysosomes) and G-6-Pase (microsomes). Table 2 shows the distribution of the amidase-esterase and of marker enzymes in the various fractions, expressed as percentages of the recovered activities. The distribution patterns of the amidase toward ISOC and esterase toward PROC were roughly the same as that of G-6-Pase.

The highest activities were in fractions consisting of membranous structures, with little or no activity in the cell sap. Furthermore, the amidase-esterase activity was present in both r-ER and s-ER, and the higher specific activity was found in s-ER compared

Table 2. Subcellular distribution of rat liver enzymes*

| | Homogenate† | Distribution (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Nuclear | Mitochondrial | Lysosomal | Microsomal | Supernatant | Recovery |
| Protein | 333·5 ± 12·5 | 22·1 ± 2·3 | 21·2 ± 3·6 | 11·8 ± 1·5 | 14·2 ± 1·5 | 27·2 ± 0·3 | 96·5 ± 3·7 |
| Succinic dehydrogenase | 5·7 ± 0·4 | 24·6 ± 5·7 | 51·9 ± 7·7 | 1·9 ± 0·3 | 0·5 ± 0·1 | 0·4 ± 0·1 | 79·3 ± 3·3 |
| Acid phosphatase | 29·2 ± 3·6 | 20·9 ± 3·3 | 39·0 ± 7·8 | 22·9 ± 2·7 | 14·6 ± 1·2 | 17·7 ± 0·5 | 115·0 ± 12·8 |
| Glucose 6-phosphatase | 5·2 ± 0·9 | 23·1 ± 2·1 | 26·0 ± 4·5 | 15·5 ± 2·2 | 36·5 ± 4·9 | 3·5 ± 1·5 | 106·3 ± 7·6 |
| Isocarboxazid amidase | 0·2 ± 0·03 | 15·5 ± 2·2 | 24·5 ± 3·4 | 18·6 ± 1·0 | 46·1 ± 5·7 | 1·6 ± 0·3 | 106·3 ± 3·9 |
| Procaine esterase | 0·3 ± 0·08 | 17·9 ± 2·0 | 25·6 ± 5·5 | 21·2 ± 2·2 | 34·9 ± 2·9 | 0 | 99·6 ± 8·8 |
| Acetanilide hydrolase | 0·3 ± 0·08 | 14·5 ± 3·6 | 24·7 ± 3·3 | 15·2 ± 0·5 | 31·3 ± 4·3 | 6·7 ± 1·9 | 93·2 ± 5·6 |

* All values are given as mean ± S. E. M. The percentages are based on total recovered activity of more than four experiments.

† Values are expressed as mg protein/g of liver wet wt; enzyme activities were expressed as nmoles product/mg of protein/min. In this table more than five samples were used.

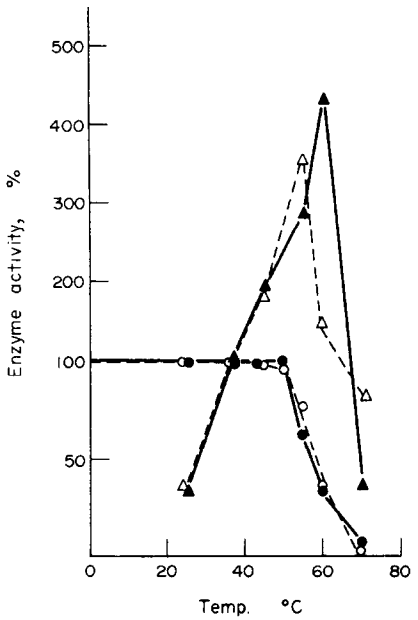


Fig. 1. Temperature optima and temperature sensitivity of a liver microsomal amidase-esterase. Enzyme activities toward ISOC (—▲—) and PROC (—△—) as substrate were measured after 30 min of incubation at 37° at the different temperatures. After a 10-min preincubation without added substrate, incubation was carried out for the next 30 min with added ISOC (—●—) or PROC (—○—).

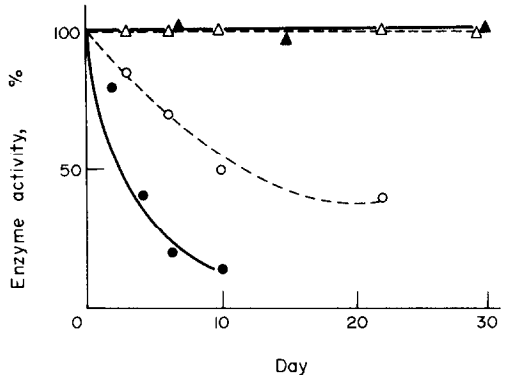


Fig. 2. Stability of a liver microsomal amidase-esterase. The microsomal pellet suspended in 1·15% KCl solution was maintained at room temperature or −20° for 30 days. Enzyme activities with ISOC (—▲—) and PROC (—△—) as substrates were measured after maintenance at −20°. The results for enzyme maintained at room temperature with ISOC (—●—) and PROC (—○—) as substrate are also shown. Protein concentration of the microsomes used in this experiment was 3·6 mg.

with r-ER when expressed per μg of phospholipid (Table 3).

In general, the phospholipid content can be regarded as proportional to the amount of membrane, and it was therefore concluded that the amidase-esterase was bound to the microsomal membranes and not to ribosomes.

*Effects of temperature on the amidase-esterase activity.* As shown in Fig. 1, exposure of the enzyme* without the substrate below 50° caused no inactivation. However, exposure to temperatures higher than 50° resulted in a gradual decrease with almost complete inactivation of enzyme activity at 70°. On the other hand, the activity was gradually increased by temperatures up to 55° and 60° for PROC and ISOC, respectively, and above these temperatures the activity rapidly decreased.

*Stability of the amidase-esterase.* Enzyme activities toward ISOC and PROC gradually decreased within 10–20 days at room temperature, while no loss of enzyme activity was observed at −20° for 30 days (Fig. 2).

*pH optimum.* Microsomal amidase-esterase has a pH optimum at 8·5 to 9·0 for enzymatic hydrolysis of PROC and ISOC (Fig. 3). There was no significant difference in the enzyme activity with the four buffer systems used.

*Effect of substrate concentration on reaction velocity.* The effect of substrate concentration on the rate of the hydrolysis was determined for ISOC and PROC.

* Sp. act.: 1·3 nmoles product/mg of protein/min.

Table 4. Effect of inhibitors on the liver microsomal amidase-esterase toward procaine (P) and isocarboxazid (I) as substrates*

| Inhibitor | Inhibitor concn (M) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $10^{-3}$ | | $10^{-4}$ | | $10^{-5}$ | | $10^{-6}$ | |
| | I | P | I | P | I | P | I | P |
| EPN† | 0 | 0 | 29·5 | 19·4 | 69·8 | 61·4 | 87·5 | 92·5 |
| SKF 525-A | 62·5 | 81·8 | 77·0 | 90·3 | 90·0 | 104·3 | | |
| Sodium fluoride | 84·0 | 44·4 | 103·0 | 71·3 | 104·0 | 98·6 | | |
| *N*-ethylmaleimide | 80·5 | 104·6 | 100·0 | 100·8 | 106·0 | 101·0 | | |
| PCMB‡ | 104·0 | 132·6 | 101·5 | 106·9 | 103·5 | 104·0 | | |
| Eserine | 46·5 | 23·3 | 71·0 | 70·4 | 100·0 | 85·5 | | |
| *p*-Nitrophenyl acetate | 71·7 | 51·3 | 98·6 | 92·3 | 108·7 | 102·9 | | |
| α-Naphthylacetamide | 78·6 | | 96·3 | 72·6 | 105·0 | 98·6 | | |
| Acetylcholine | 107·3 | 103·2 | 100·8 | 102·1 | 109·1 | 99·7 | | |
| Acetanilide | 79·8 | 94·7 | 102·4 | 98·0 | 101·2 | 98·4 | | |
| EDTA | 100·3 | 100·0 | 100·8 | 100·1 | 101·0 | 100·0 | | |

* Numbers in this table show the mean value from three experiments and represent per cent of control.
† Ethyl *p*-nitrophenyl phenylphosphonothioate.
‡ *p*-Chloromercuribenzoate.

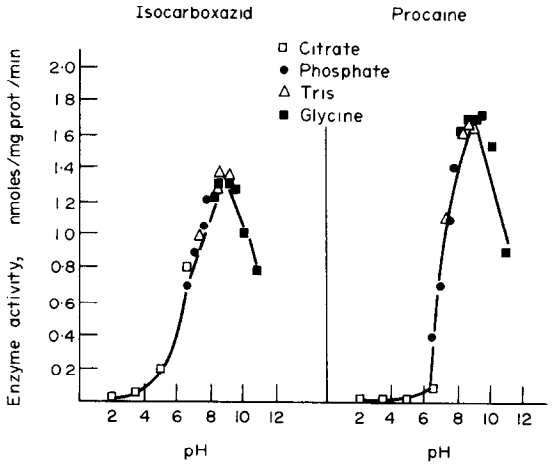


Fig. 3. pH optimum. The following four buffer systems were used: 0·2 M citrate buffer prepared from $Na_2HPO_4$ and citrate (pH 2·3 to 6·5); 0·2 M phosphate buffer prepared from $KH_2PO_4$ and NaOH (pH 6·5 to 8·0); 0·2 M Tris–HCl buffer (pH 7·4 to 9·0); and 0·1 M glycine buffer prepared from glycine and NaOH (pH 8·2 to 11·0). The other conditions were as described in the text.

The apparent $K_m$ value toward ISOC was roughly equal to that of PROC, that is, $K_m$ values were 0·19 mM for ISOC and 0·51 mM for PROC, and $V_{max}$ values were 1·7 nmoles BZH/mg of protein/30 min for ISOC and 67 nmoles *p*-aminobenzoate/mg of protein/30 min for PROC respectively.

*Effects of inhibitors.* A number of substances commonly used in enzyme characterization were tested without preincubation of the enzyme in the presence of the inhibitor. As shown in Table 4, remarkable inhibition was observed with *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonothioate (EPN), an insecticide, and moderate inhibition was also noted in the presence of high concentrations of eserine, SKF 525-A and sodium fluoride. Little or no inhibition of the enzyme was observed with ethylenediaminetetraacetic acid (EDTA), *p*-chloromercuribenzoate (PCMB) and *N*-ethylmaleimide (NEM) even at a concentration of 1 mM.

*Effect of metal ions.* Among divalent metals tested, Hg, Cu, Zn and Co had an inhibitory effect on ISOCase and PROCase. Moreover, none of these metals used were found to activate the amidase-esterase (Table 5).

Table 5. Effect of various divalent cations on the liver microsomal amidase-esterase toward isocarboxazid and procaine as substrates*

| Metal ion ($1 \times 10^{-4}$ M) | Substrate | |
|---|---|---|
| | Isocarboxazid | Procaine |
| None | 100·0 | 100·0 |
| $Cu^{2+}$ | 74·0 | 97·4 |
| $Zn^{2+}$ | 87·0 | 93·8 |
| $Co^{2+}$ | 65·5 | 110·3 |
| $Fe^{2+}$ | 93·0 | 104·8 |

* Numbers represent relative per cent of control. Incubation was carried out as described in the text.

## DISCUSSION

Schöttler and Krisch [19] have demonstrated that many steroid hormone esters are effectively hydrolyzed by pig liver carboxylesterase (EC 3.1.1.1) *in vitro*. Nicotinamide deamidase from rabbit liver microsomes was found to hydrolyze two synthetic aminoacid esters and nitrophenyl acetate at high rates [20]. In addition, formamidase (EC 3.5.1.9) from guinea pig liver hydrolyzed a variety of amides such as acetanilide and several esters of α-naphthyl and *p*-nitrophenyl acetate [21].

The present paper revealed that PROC is found to be more rapidly hydrolyzed by the amidase-esterase than ISOC, and acetanilide was hydrolyzed at a lower rate than either PROC or ISOC. These findings *in vitro* strongly suggest that the enzyme hydrolyzing ISOC is also responsible for the hydrolysis of PROC *in vivo*. Thus, we have observed that ISOC and PROC were hydrolyzed at an equal rate by a highly purified microsomal amidase-esterase from rat and from guinea pig liver.*

On the other hand, the fact that the esterase activity in intestine toward PROC is very much higher than the amidase toward ISOC suggests that there are other hydrolases present in the intestine which are responsible for the hydrolysis of PROC. Recently, Arndt and Krisch [22] and Junge and Krisch [23] have reported that esterase from rat liver and from *Pseudomonas acidovorans* exhibits esterase and acetanilide amidase activity in the same order of magnitude. It is, therefore, possible to speculate that a distinction between "amidase" and "esterase" is not possible.

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* Sp. act.: 1·3 nmoles product/mg of protein/min.

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