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SOLUBILIZATION, PURIFICATION AND PROPERTIES OF ISOCARBOXAZID HYDROLASE FROM GUINEA PIG LIVER

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Abstract—An enzyme preferentially hydrolyzing isocarboxazid was solubilized from guinea pig liver with trypsin. The enzyme was purified by fractionation with ammonium sulfate, followed by chromatography on Sephadex G-200 and DEAE-cellulose. An overall purification of 92-fold was achieved, and molecular weight was estimated to be about 210,000 ± 10,000. Polyacrylamide gel electrophoresis of the final enzyme preparation showed a single band. The purified enzyme is very stable for at least 2 months in the cold (−20°). The K_m and V_{max} of the purified enzyme for isocarboxazid are $2{\cdot}1 \times 10^{-4}$ M and 3·6 nmoles product/mg protein/min respectively. No cofactors are required. The enzyme is significantly inhibited by *p*-chloromercuribenzoate, dibucaine, SKF 525-A and divalent metal ions, i.e. Hg^{2+}, Zn^{2+}, Cu^{2+} and Co^{2+}, but not by EDTA. The pH optimum is 8·0–8·5. The purified preparation catalyzes the enzymatic hydrolysis of β-naphthylamides of three amino acids (leucine, alanine and phenylalanine) and two synthetic penicillins, phenethicillin and propicillin, other than isocarboxazid.

A KEY ENZYME in the metabolism of isocarboxazid (ISOC), a monoamine oxidase inhibitor, is a hepatic amidase-type hydrolase which converts ISOC to benzylhydrazine (BZH).[1–3] In previous papers, the present authors reported the tissue distribution subcellular localization of this enzyme (ISOC hydrolase) in rats,[4] and species differences in the enzyme activity.[5] With regard to arylamidases, several studies have been reported[6–12] and Brecher and Suszkiw[13] summarized and discussed the properties of these enzymes.

The present paper deals with the results of a purification and characterization of ISOC hydrolase of guinea pig liver.

MATERIALS AND METHODS

β-Naphthylamides of L-amino acids (alanine, leucine, phenylalanine, lysine, arginine and glutamic acid) were obtained from Mann Research Laboratories, Inc.; trypsin was obtained from the Boeringer Co.; bovine serum albumin from the Armour Pharmaceutical Co.; Sephadex G-25 and G-200 from Pharmacia Ab; and DEAE-cellulose from Serva. Penicillins were kindly donated by Research Laboratories of Meiji Seika Kaisha Ltd., Kanagawa, Japan.

Assay of ISOC hydrolase activity. ISOC was used as the substrate throughout the purification. The enzyme activity was determined using the colorimetric assay based on conversion of ISOC to BZH. The incubation mixture for enzymatic activity contained phosphate buffer (pH 7·4) 140 μmoles; ISOC, 600 nmoles and the enzyme preparation in a final volume of 1·0 ml. After 30-min incubation at 37° in air, an

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amount of BZH formed was determined as described previously.[14] One unit of ISOC hydrolase was the amount of the enzyme that formed 1 nmole of BZH/min at 37°. Specific activity was defined as units of enzyme activity per milligram of protein. Arylamidase activity was assayed at 37° based on the rate of liberation of β-naphthylamine from 1 mM L-amino acid-β-naphthylamides by the method of Goldbarg and Rutenberg.[15] Penicillins were assayed by determining 6-aminopenicillanic acid liberated from penicillins as described earlier.[14]

Protein determination. Protein determination was carried out according to the method of Lowry *et al.*[16] Protein levels of individual fractions from column chromatography were estimated by measuring the absorbance at 280 nm in the flow system and calculated back to milligram of protein.

Polyacrylamide gel electrophoresis. Disc electrophoresis as described by Davis[17] was used to monitor the purity of enzyme preparations. The gel concentration was 7·5% (pH 9·4).

RESULTS

Purification procedure. Livers obtained from guinea pigs (430–540 g body wt) were used as the enzyme source. The enzyme was purified as described below.

Step 1. *Separation of microsomal fraction.* A 100-g sample of livers was homogenized in batches of 5 g in 9 vol. of 0·25 M sucrose with a Potter–Elvehjem homogenizer fitted with a Teflon pestle. Microsomal pellets were prepared by centrifugation of a 12,000 *g* (20 min) supernatant at 105,000 *g* for 1 hr followed by washing once with 0·25 M sucrose, and by resuspension in sufficient 0·25 M sucrose to restore the original volume of the 12,000 *g* supernatant. All subsequent steps were carried out at 4°.

Step 2. *Solubilization.* The enzyme preparation from Step 1, having a protein concentration of 47 mg protein/ml, was lyophilized and the lyophilizate was resuspended in 0·25 M sucrose. Solubilization was carried out by adding 1·0 mg trypsin to 10 ml of enzyme suspension (about 5 mg protein/ml) followed by incubation for 1 hr at 37°. After tryptic digestion, the incubation mixture was centrifuged at 105,000 *g* for 1 hr and the supernatant fraction was used as the solubilized enzyme source for further purification. In preliminary experiments, ISOC hydrolase could not be solubilized by freezing and thawing.

Throughout these procedures more than 95 per cent of the enzyme activity could be recovered from the microsomal fraction; moreover, added trypsin could be removed completely by Sephadex G-200 chromatography (0·01 M phosphate buffer, pH 7·4). Proteolysis by trypsin by this standard procedure does not inactivate this enzyme.

Step 3. *Ammonium sulfate precipitation.* Crystalline ammonium sulfate was added up to 70 per cent saturation with continuous agitation. The mixture was allowed to stand for 8 hr and then centrifuged at 16,000 *g* for 20 min at 4°. The active precipitate was resuspended in a small volume of 0·01 M phosphate buffer (pH 7·4) and desalted by Sephadex G-25 chromatography (3 × 70 cm) at 4° on the column previously equilibrated and eluted with 0·01 M phosphate buffer (pH 7·4). The flow rate was 60 ml/hr and the fraction volume was 5 ml each. Eluates from Sephadex G-25 were pooled and assayed for ISOC hydrolase activity.

Step 4. *Fractionation on Sephadex G-200 and DEAE-cellulose.* The enzyme preparation from Step 3 was applied on a Sephadex G-200 column (2·4 × 50 cm) previously

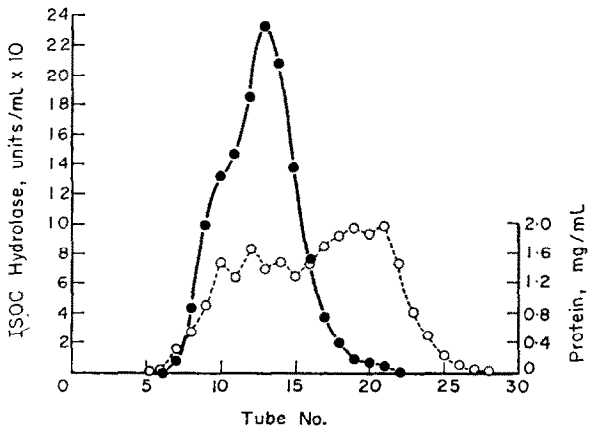

FIG. 1. Gel filtration on Sephadex G-200 (2·4 × 50 cm) of the ISOC hydrolase activity precipitated between 40 and 70 per cent ammonium sulfate saturation. Equilibration of the column and elution were performed with 0·01 M phosphate buffer (pH 7·4). Fraction volume was 5 ml. Proteins, ○- - -○; ISOC hydrolase, ●——●. Enzyme activities in all figures except Fig. 6 are expressed as units per milliliter. Standard assay conditions (see Materials and Methods) were used in this and in the following figures and tables unless otherwise specified.

equilibrated with 0·01 M phosphate buffer (pH 7·4). The same buffer was used for elution. The flow rate was adjusted to 10 ml/hr and 5-ml fractions were collected. A typical chromatographic pattern is shown in Fig. 1. The highest activity is found in fraction number 13 and the active fractions (fraction Nos. 11–15) were pooled and applied on a DEAE–cellulose column (1 × 35 cm) previously equilibrated with 0·01 M Tris–HCl buffer (pH 7·4). The column was eluted by the same buffer, having a linear NaCl gradient from 0 to 0·3 M. The flow rate was 20 ml/hr. The total volume of eluant was about 200 ml. ISOC hydrolase was eluted by 0·12–0·18 M NaCl.

Fractions containing 5 ml each were collected and active fractions (Nos. 10–16) were pooled and concentrated using a membrane filter to a volume of about 10 ml. The

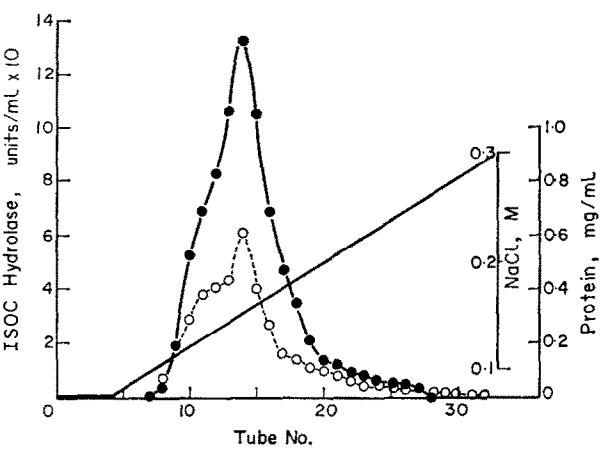

FIG. 2. Chromatography on a DEAE–cellulose column (1 × 35 cm) of ISOC hydrolase activity. Equilibration of the column and elution were performed with 0·01 M Tris–HCl buffer (pH 7·4), containing NaCl. Fraction volume was 5 ml. Proteins, ○- - -○; ISOC hydrolase, ●——●; NaCl gradient, ———.

protein concentration and enzyme activity toward ISOC were determined and the results are shown in Fig. 2. After DEAE-cellulose chromatography, the overall purification is about 92-fold with a recovery of 1·4 per cent of the original enzyme activity present in the liver homogenate.

The purified enzyme is remarkably stable without loss of enzyme activity during storage at −20° for about 2 months. Table 1 summarizes the results of a typical purification procedure.

TABLE 1. PURIFICATION OF ISOCARBOXAZID HYDROLASE FROM GUINEA PIG LIVER

Fraction	Vol. (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Fold purification
Liver homogenate	550	32,500	68,500	2·1	100·0	1·0
Microsomes	200	9400	44,500	4·7	64·9	2·2
Supernatant after trypsin digestion	680	1560	25,000	16·0	36·5	7·6
Ammonium sulfate, 30–60%	100	770	20,000	25·9	29·2	12·8
Sephadex G-25 chromatography	140	685	19,600	28·6	28·6	13·3
Ammonium sulfate, 40–70%	25	360	10,500	29·1	15·3	13·8
Sephadex G-200 chromatography	15	36	3900	108·3	5·7	51·0
DEAE-cellulose chromatography	11	4·9	950	193·9	1·4	92·0

Polyacrylamide gel electrophoresis. The eluates from Sephadex G-200 (0·5 mg) and DEAE-cellulose (0·2 mg) were subjected to polyacrylamide gel electrophoresis (acrylamide concentration, 3·75 and 7·5%; pH 9·4). As shown in Fig. 3, one major and four minor protein bands appear in the Sephadex G-200 eluate (a), whereas the final preparation shows a single band (b). ISOC hydrolase activity after electrophoresis was detected by the following procedure. The gel was incubated for 3 hr at 37° in 0·1 M phosphate buffer (pH 7·4) with 3 mM ISOC as the substrate, then placed in a tube containing both 3 ml of Ehrlich's reagent and 1·5 ml of 5% metaphosphoric acid and the tube was kept for 30 min at room temperature. The enzyme was revealed by an orange single band. Proteins were detected by the Amido-black method followed by destaining in acetic acid–water–methanol, 8:12:1.

Properties of ISOC hydrolase. The reaction velocity was linear with enzyme concentrations up to 25 μg of protein in the presence of 600 nmoles of ISOC, and with time up to 60 min.

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 2, remarkable inhibition was observed with *p*-chloromercuribenzoate (PCMB), SKF 525-A and dibucaine. Inhibition was also noted in the presence of high concentrations of sodium fluoride, procaine, diphenylcarbazide, serotonin, tyramine, norepinephrine, epinephrine, phenelzine and *N*-ethylmaleimide. Little or no inhibition of the enzyme was observed with EDTA, potassium cyanide and iodoacetate even at a concentration of 1 mM.

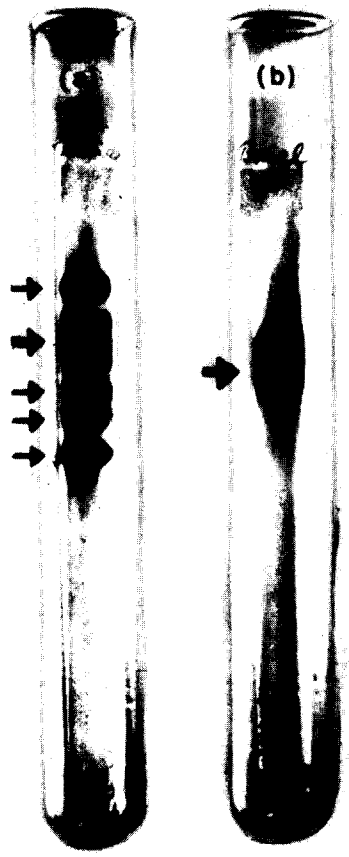

FIG. 3. Disc gel electrophoresis of ISOC hydrolase on polyacrylamide gel. (a) Sephadex G-200 eluant (0·5 mg) and (b) DEAE-cellulose eluant (0·2 mg) were subjected to electrophoresis. A 7·5% polyacrylamide gel was used. Electrophoresis was performed at 4° for 7 hr with 8 mA/tube (0·5 × 10 cm). The proteins were stained with Amido-black.

TABLE 2. EFFECT OF INHIBITORS ON THE ACTIVITY OF ISOCARBOXAZID HYDROLASE*

Inhibitor	Final concn. (μM)				
	1000	100	10	1	0·1
PCMB†	91	49	13	0	
N-ethylmaleimide	43	14	12	0	
Sodium fluoride	56	14	6	0	
SKF 525-A	100	96	82	46	4
Dibucaine	94	86	83	72	48
Procaine	89	74	25	0	
Serotonin	59	14	0		
Tyramine	54	3	0		
Dopamine	18	6	0		
Kynuramine	15	10	0		
Norepinephrine	51	16	0		
Epinephrine	46	18	0		
Diphenylcarbazide	90	29	0		
Phenelzine	49	26	0		

* Numbers in this table show the mean value from three experiments and represent per cent inhibition.

† *p*-Chloromercuribenzoate.

TABLE 3. REVERSAL OF *p*-CHLOROMERCURIBENZOATE-CAUSED INHIBITION OF ISOCARBOXAZID HYDROLASE BY DITHIOTHREITOL*

Addition		Enzyme activity (%)
First	Second	
Control		100
PCMB +	None	43
PCMB +	DTT	73
None +	PCMB and DTT	98
DTT +	PCMB	108
SKF 525-A +	None	16
SKF 525-A +	DTT	17
DTT +	SKF 525-A	16

* A purified enzyme preparation (2·6 units) was first incubated for 10 min at 37° with 0·2 M phosphate buffer (pH 7·4), 0·5 ml plus the addition (first column) listed in the table in a total volume of 1·0 ml. At the end of preincubation, 1·0 ml containing 600 nmoles of ISOC plus the addition (second column) omitted during the first incubation period were added. The mixture was reincubated for a 30-min period at 37°; then enzyme activities were assayed. Controls were treated in the same manner in each case except additions. PCMB: *p*-chloromercuribenzoate (1×10^{-4} M), DTT: dithiothreitol (1×10^{-3} M), SKF 525-A (1×10^{-5} M).

Reversal of inhibition of ISOC hydrolase by dithiothreitol. Although addition of reductants, i.e. reduced glutathione. cysteine and dithiothreitol (DTT) did not activate the enzyme (data not presented here), partial reversal of the inhibition by PCMB was observed by the addition of DTT. The data in Table 3 show that 57 per cent inhibition of ISOC hydrolase by PCMB could be reversed partially by 1 mM DTT. However, significant inhibition of the enzyme by SKF 525-A could not be reversed by DTT. As is well known, SKF 525-A has been widely used as an inhibitor of drug-metabolizing enzymes in liver microsomes, and this compound has an ester bond in its chemical structure. The inhibitory effect of SKF 525-A on ISOC hydrolase, when plotted by the method of Lineweaver and Burk,[18] indicates that the mechanism of inhibition was the competitive type for ISOC and its K_i value was $6{\cdot}7 \times 10^{-7}$ M.

Effects of metal ions. Among metals tested, Hg^{2+}, Zn^{2+}, Cu^{2+} and Co^{2+} inhibited ISOC hydrolase. None of the metals tested were found to activate the enzyme (Table 4).

TABLE 4. EFFECT OF VARIOUS DIVALENT CATIONS ON THE ISOCARBOXAZID HYDROLASE*

Metal ions	Final concn. (μM)		
	1000	100	10
Control	100	100	100
Hg^{2+}	0	2	34
Zn^{2+}	4	12	67
Cu^{2+}	3	22	71
Co^{2+}	54	86	98
Fe^{2+}	93	102	100
Mn^{2+}	100	100	102
Mg^{2+}	110	100	101
Ca^{2+}	100	108	105

* Each addition was made 10 min before the beginning of incubation in the reaction mixture containing 0·1 M Tris–HCl buffer (pH 7·4) and 2·6 units of the purified enzyme, except substrate. Numbers represent relative per cent of control.

Optimum pH. The purified preparation of ISOC hydrolase has a pH optimum at 8·0–8·5 for enzymatic hydrolysis of ISOC (Fig. 4). There was no significant difference in the enzyme activity with the four buffer systems used.

Substrate specificity. Twenty-four compounds having amido bond in their molecules were tested, and 6 of the 24 compounds were found to be hydrolyzed by ISOC hydrolase (Table 5). The rates of hydrolysis were determined and are presented in Table 5. ISOC is the compound most susceptible to enzyme-catalyzed hydrolysis.

Effect of substrate concentration on reaction velocity. The effect of substrate concentration on the rate of ISOC hydrolase-catalyzed hydrolysis was determined for ISOC, two synthetic penicillins and leucyl-β-NA. The results are shown in Table 5 and Fig. 5. Penicillins have the highest V_{max} values and the K_m values are the lowest for three L-amino acid-β-naphthylamides.

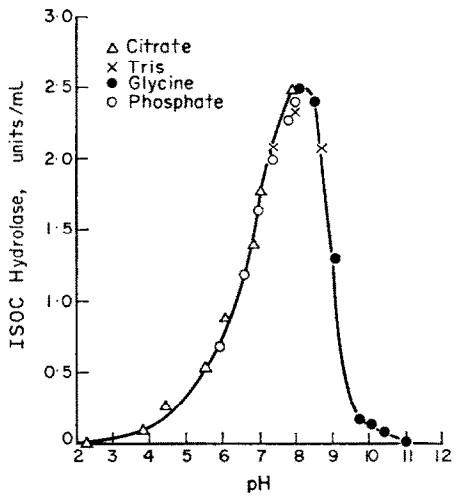

FIG. 4. Optimum pH. The following four buffer systems were used: (△), 0·2 M citrate buffer prepared from Na_2HPO_4 and citrate (pH 2·3–8·0); (○), 0·2 M phosphate buffer prepared from KH_2PO_4 and NaOH (pH 6·0–8·0); (×), 0·2 M Tris–HCl buffer (pH 7·4–8·7); (●), 0·1 M glycine buffer prepared from glycine and NaOH (pH 8·2–11·0). The other conditions were as described for the standard assay procedure. In this experiment a purified ISOC hydrolase having specific activity of 2·5 units was used.

Effect of the temperature on ISOC hydrolase activity. As shown in Fig. 6, the activity is gradually increased up to about 30–40° with increasing temperatures, and above 40° an abrupt increase of the activity is observed. Maximal activity is observed at 53°, and the activity rapidly decreased with further increasing temperature. Figure 6 also shows that the exposure of the enzyme without substrate below 45° caused no inactivation; however, exposure to 55° resulted in complete inactivation of the enzyme activity.

Determination of molecular weight. Molecular weight of ISOC hydrolase was estimated by gel filtration in Sephadex G-200 (2·8 × 40 cm) according to the method of Andrews,[19] using the following proteins as references: horse heart cytochrome *c* (12,400), bovine pancreas trypsin (24,000), bovine serum albumin (68,000) and beef liver catalase (240,000). Cytochrome *c* (1 mg), trypsin (1 mg), albumin (5 mg), catalase

TABLE 5. EFFECT OF SUBSTRATE CONCENTRATION ON REACTION VELOCITY*

Substrate	$K_m \times 10^{-4}$ M	V_{max} (moles/ml/min) $\times 10^{-6}$	Rate of hydrolysis
Isocarboxazid	2·1	3·6	100
Phenethicillin	29·4	6·9	56
Propicillin	43·5	6·7	48
L-Alanyl-β-NA	0·43	1·3	26
L-Leucyl-β-NA	0·73	1·5	26
L-Phenylalanyl-β-NA	0·70	1·5	26

* Each tube contains 2·6 units of the purified enzyme. K_m and V_{max} values were obtained by Lineweaver–Burk analysis. The value, 100, was assigned to that substrate hydrolyzed at the highest rate. The substrate concentration was 1 mM in all cases.

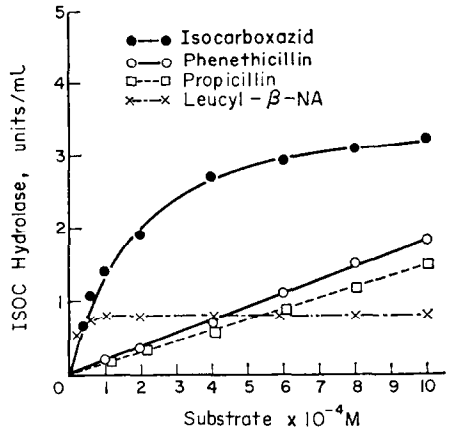

FIG. 5. Comparison of rates of hydrolysis with different substrates by purified ISOC hydrolase. (●), ISOC; (○), phenethicillin; (□), propicillin; (×), leucine-β-naphthylamide. The standard incubation mixture containing appropriate concentrations of the substrates ranging from 1·0 to 10·0 × 10^{-4} M was incubated as described in Materials and Methods.

(0·2 mg) and ISOC hydrolase (0·3 mg) in 1·0 ml of the 0·05 M Tris–HCl buffer (pH 7·4) containing 0·1 M KCl were applied on the column. The elution was carried out with same buffer. The flow rate was 12 ml/hr, and the fraction volume was 5 ml. From Fig. 7, molecular weight of ISOC hydrolase can be estimated to be 210,000 ± 10,000.

Localization of ISOC hydrolase in subcellular particles. Since the possible location of hydrolyzing enzymes in the lysosomes has been reported, the distribution of ISOC hydrolase in the various subcellular fractions was investigated. Four particle fractions, mitochondrial, lysosomal, heavy microsomal and microsomal, were successively

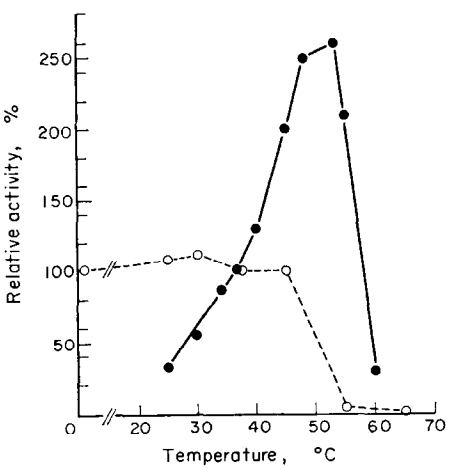

FIG. 6. Temperature optima and temperature sensitivity of ISOC hydrolase. Enzyme activity with ISOC as substrate was measured after 30-min incubation at the different temperatures (●——●); (○- - -○) shows the results for enzyme maintained at appropriate temperatures indicated for 10 min without substrate, then reincubated with ISOC for 30 more min at 37°.

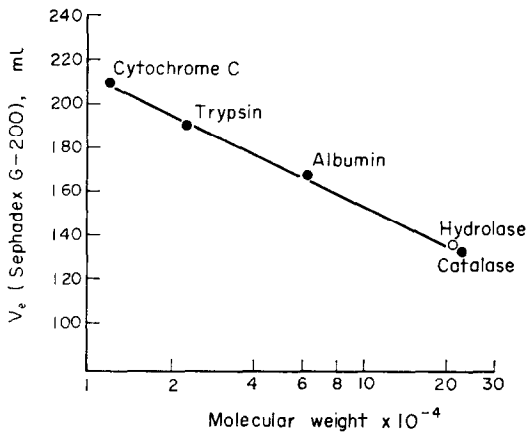

FIG. 7. Determination of the mol. wt of ISOC hydrolase. Plots of elution volume, V_e against log (mol. wt) for proteins on Sephadex G-200 column (2·8 × 40 cm) equilibrated with 0·05 M Tris–HCl buffer (pH 7·4), containing 0·1 M KCl. The following proteins were used as references: cytochrome *c* (12,400), trypsin (24,000), bovine serum albumin (68,000) and catalase (240,000). Mol. wt of each protein used here were obtained from Andrews.[19]

separated by centrifuging at 9,000 *g* (10 min), 15,000 *g* (20 min), 56,000 *g* (20 min) and 105,000 *g* (60 min) respectively. A pellet including most of the microsomes with minimum contamination with lysosomes (heavy microsomal plus microsomal) showed 87 per cent of the total ISOC hydrolase activity present in whole homogenate, and only 12 per cent of the enzyme activity was obtained in lysosome-rich subfraction (15,000 *g* pellet).

DISCUSSION

According to the usual chromatographic and electrophoretical criteria, the hydrolase described here appears to be homogeneous. It catalyzes the hydrolysis of several compounds possessing an amido bond. Among six amino acid-β-naphthylamides tested, leucyl-, alanyl- and phenylalanyl-β-naphthylamides were found to be substrates, while glutamyl-, lysyl- and arginyl-β-naphthylamides were not.

ISOC hydrolase seems to be different from other known arylamidases located in particles, and from penicillinamidase (EC 3.5.1.11) which has been discussed in a review by Hamilton–Miller.[20] Penicillinamidase has a pH optimum of 10 for the hydrolytic reaction, and is capable of hydrolyzing benzylpenicillin (penicillin V) and phenoxymethylpenicillin (penicillin G).

Several known hydrolases which are commercially available, i.e. chymotrypsin (EC 3.4.4.5), arylamidase (EC 3.5.1.4), 4-glucuronidase (EC 3.2.1.31), leucine aminopeptidase (EC 3.4.1.1) and cathepsin (EC 3.4.4.9) failed to hydrolyze ISOC under optimum conditions. ISOC hydrolase can also be distinguished from acetanilide hydrolase[21,22] by substrate specificities, resistance to trypsin digestion, pH optimum and cofactor requirement.

The fact that the sulfhydryl groups of ISOC hydrolase are involved in the enzyme activity is suggested by the recovery experiment with DTT (Table 3). Various arylamidases can be distinguished by their metal ion activation. Felgenhauser and

Glenner[10] found that divalent metal ions activated microsomal enzymes, but not lysosomal ones. The present hydrolase is markedly inhibited by divalent metal ions and is not activated by Mn^{2+}, Mg^{2+}, Ca^{2+} or Fe^{2+}. Furthermore, the present enzyme is stable on storage at least for 2 months in the cold (−20°) without addition of any reductants.

Recently, Järvinen *et al.*[23] reported a partial purification of a hydrolase from guinea pig liver microsomes. Properties of ISOC hydrolase are very similar to those of the former enzyme; however, the lack of activity with acetanilide and 2-acetylamino fluorene appears to distinguish between the two enzymes.

The results obtained for the subcellular distribution of ISOC hydrolase activity suggest that this enzyme seems to be located in microsomal fraction rather than lysosomal. In addition, as seen with several microsomal enzymes, ISOC hydrolase can be induced by phenobarbital treatment but not by 3-methylcholanthrene. Details will be reported in a later paper.

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