Biochemical Pharmacology, Vol. 20, pp. 504–507. Pergamon Press, 1971. Printed in Great Britain

Enzymatic hydrolysis of isocarboxazid by rat tissues

(*Received* 17 *February* 1970; *accepted* 26 *June* 1970)

SEVERAL reports have appeared describing the metabolism of isocarboxazid. Schwartz[1] showed that [^{14}C]benzoate was formed *in vivo* by rats after intraperitoneal injection of [^{14}C]isocarboxazid, and Koechlin *et al.*[2] showed the same reaction in man. In addition, Schwartz[3] revealed the hydrolysis *in vitro* of isocarboxazid to benzylhydrazine. These findings demonstrated that isocarboxazid might be hydrolyzed enzymatically to benzylhydrazine and further oxidized to benzoate. The present investigation was undertaken to examine the subcellular localization and tissue distribution of enzyme responsible for hydrolysis of isocarboxazid.

Male rats of the Wistar strain (250–300 g) were used.

Enzyme preparation. Freshly excised tissues were homogenized in a Potter–Elvehjem glass homogenizer with a loosely fitting Teflon pestle in 9 vol. of cold 0·14 M KCl solution containing 0·0025 N NaOH. Subcellular fractions were prepared from whole homogenate by differential centrifugation at 4° using the method of Feigelson and Greengard.[4] Centrifugation for 30 min at 600 *g* sedimented cell debris and nuclei. This supernatant fluid was further centrifuged for 20 min at 12,000 *g* in order to sediment a fraction containing mostly mitochondria. The remaining supernatant fluid was centrifuged for 50 min at 105,000 *g* to give the microsomal fraction and cell sap. Mitochondria and microsomes were washed twice by resuspension in 50 vol. of 0·14 M KCl solution followed by sedimentation at the appropriate gravitational forces. There was no significant difference in the enzyme activities of liver homogenates which were prepared in two kinds of solutions, alkaline KCl solution (pH 10·9) as described above and 0·25 M sucrose solution (data not presented here).

Enzyme assay. The standard assay mixtures of 3·0 ml total volume were incubated for 60 min at 37° in air. Each flask contained 1·0 ml of tissue preparation, 1·0 ml of isocarboxazid solution (3×10^{-3} M) and 1·0 ml of 0·2 M phosphate buffer (pH 7·0) unless otherwise indicated. Protein was assayed by the biuret method[5] and bovine serum albumin (Fraction V) was used as a standard. All experiments were carried out in duplicate. The pH value of the flask contents (pH 7·0) was not affected by the addition of alkaline enzyme source before and after incubation.

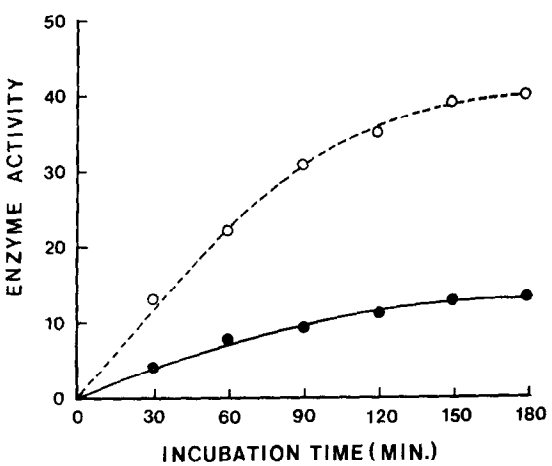

FIG. 1. Relationship between incubation time and benzylhydrazine formation. Duplicate flasks containing 1·0 ml of 10% liver (○——○) or kidney (●——●) homogenate, 1·0 ml isocarboxazid (3×10^{-3}) M and 0·2 M phosphate buffer, pH 7·0, were incubated at 37° for 3 hr. In Figs. 1 and 2, enzyme activity was expressed as mμmoles benzylhydrazine formed/mg of protein/hr. Liver homogenate used here contained about 30 mg protein/ml of the enzyme source.

Colorimetric measurement of benzylhydrazine.[6] The fluorometric assay procedure of benzylhydrazine reported by Roth and Rieder[7] requires a specific reagent, 1,2-napthoquinone-4-sulfonate, and complicated techniques, although the method is excellent in its sensitivity. The quantitative assay method described below was based on the formation of a complex consisting of metaphosphoric acid, Ehrlich's reagent and benzylhydrazine. After deproteinizing the incubation mixtures with 3·0 ml of 15% metaphosphoric acid, 1·5 ml of Ehrlich's reagent solution* was added to aliquots of the centrifuged supernatant (3000 rev/min for 10 min). After standing at least 30 min at room temperature, readings were made at 490 mμ. Residual isocarboxazid in the flask after incubation did not affect the determination of benzylhydrazine. The assay procedure used here could determine a minimum of approximately 2·44 μg (0·02 μmole) benzylhydrazine.

Chemicals. Isocarboxazid was kindly furnished by Takeda Chem. Ind., Osaka, Japan. Benzylhydrazine was synthesized essentially according to the procedure of Grandberg and Kost[8] and Kost and Sagitullin.[9] Benzylhydrazine hydrochloride prepared in this laboratory was recrystallized from ethanol before use (m.p. 209–111°). The other chemicals used were of the highest purity available commercially.

Isocarboxazid hydrolyzing activities in liver and kidney were determined. Figure 1 shows that the reactions were linear for 60 min and markedly greater activity was seen with liver than with kidney. The results concerning the effects of substrate concentrations ranging from 5×10^{-5} to 2×10^{-3} M on conversion of isocarboxazid to benzylhydrazine are illustrated in Fig. 2.

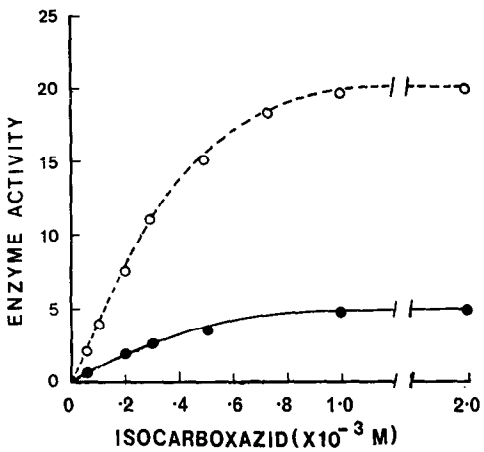

FIG. 2. Relationship between the substrate concentrations and enzyme activity. Duplicate flasks containing 1·0 ml of 10% liver (○——○) or kidney (●——●) homogenate, 1·0 ml of phosphate buffer, pH 7·0, and 1·0 ml of graded amounts of isocarboxazid dissolved in redistilled water were incubated at 37° for 60 min.

The increase in benzylhydrazine showed linearity with respect to the substrate concentration up to approximately $0{\cdot}3 \times 10^{-3}$ M in liver as well as kidney homogenates.

Subcellular localization of the enzyme responsible for hydrolysis of isocarboxazid was studied by means of differential centrifugation of 10% liver homogenate in KCl solution. Incubation of several subcellular fractions with added isocarboxazid (3×10^{-3} M) was carried out according to the standard procedure and aliquots of the incubation media were used to determine the benzylhydrazine formed. The isocarboxazid hydrolyzing enzyme was found to localize mostly in the microsomal fraction of the cell (Table 1).

Studies were made to determine the tissue distribution of the isocarboxazid hydrolyzing enzyme in microsomal fractions of various tissues, i.e. liver, heart, brain, kidney and plasma of the rat. Table 2 showed that liver and kidney possess high enzymatic activity, while heart and plasma exhibited a lower level, and brain was lacking in this property.

* One g of Ehrlich's reagent was dissolved in a mixture of 20 ml ethanol and 10 ml glacial acetic acid. This solution was prepared freshly before use.

TABLE 1. SUBCELLULAR LOCALIZATION OF ISOCARBOXAZID HYDROLYZING ENZYME*

Subcellular fraction	Enzyme activity/g liver wet wt.†	Specific activity‡
Nuclei and debris	1·6	12·0
Mitochondria	1·7	10·0
Microsomes	9·1	79·0
Cell sap	nil	nil
Unfractionated homogenate	6·7	26·0

* Reaction mixture consisting of 1·0 ml of subcellular fractions prepared from liver homogenate, 1·0 ml of 3×10^{-3} M isocarboxazid, and 1·0 ml of 0·2 M phosphate buffer, pH 7·0, was incubated at 37° for 60 min. All experiments were carried out in duplicate and the results are shown as mean values of three experiments.

† Enzyme activity was expressed as micro-moles benzylhydrazine formed per hour.

‡ Specific activity was expressed as millimicro-moles benzylhydrazine formed per milligram of protein per hour.

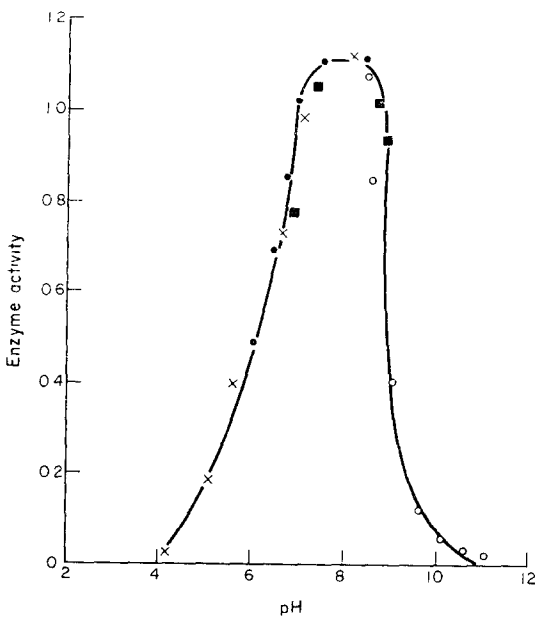

FIG. 3. The pH optimum of isocarboxazid hydrolyzing enzyme. The following four buffer systems were used: (×), 0·2 M citrate buffer prepared from Na_2HPO_4 and citrate (pH 2·5–8·1); (●), 0·2 M phosphate buffer prepared from KH_2PO_4 and NaOH (pH 6·1–8·0); (■), 0·2 M tris–HCl buffer (pH 6·9–8·9); (○), 0·1 M glycine buffer prepared from glycine and NaOH (pH 8·3–11·1). The other conditions were as described for the standard assay procedure. In this experiment a lyophilized preparation of rat liver microsomes, which contained about 0·64 mg protein/ml of the enzyme source, was used. Enzyme activity in this figure is expressed as μmoles benzylhydrazine formed/mg of protein/hr. The higher enzymatic activity in this figure than those shown in Figs. 1 and 2 results from the difference of protein contents per ml of the enzyme sources used.

TABLE 2. HYDROLYSIS OF ISOCARBOXAZID BY THE HOMOGENATES OF VARIOUS TISSUES*

Tissue	Total enzyme activity/ tissue wet wt.	Enzyme activity/g tissue wet wt.	Specific activity
Liver	110·4	9·6	27·0
Kidney	3·6	1·7	8·0
Heart	0·1	0·1	0·6
Plasma		0·06	1·0
Brain	nil	nil	nil

* Incubation conditions are as in Table 1, except that 1·0 ml of 10% tissue homogenates in KCl solution and plasma were added instead of subcellular fractions. Enzyme activity and specific activity are expressed as defined in Table 1.

In order to determine a pH optimum of the enzyme, four buffer systems were used. From the data presented in Fig. 3, the pH optimum of the enzyme was found to be 7·5–8·0 with a steep decrease in activity on both sides of the optimum. Moreover, there was no significant difference in the enzyme activity of these four systems.

Benzylhydrazine is well known as a more potent monoamine oxidase (MAO) inhibitor than isocarboxazid.[10,11] Therefore, various aspects of the pharmacological activities of isocarboxazid may be, at least in part, due to the actions of benzylhydrazine formed *in vivo*. Thus, it has been demonstrated that hydrolysis to benzylhydrazine is required for MAO inhibition by isocarboxazid.[12,13]

In summary, the results presented here indicate that the transformation of isocarboxazid to benzylhydrazine is catalyzed by the hydrolyzing enzyme localized mainly in microsomal fractions of liver and kidney. Although the enzyme proposed here is similar in some respects to the amidase-type hydrolase reported by Mcmonigle and Horita,[14] results concerning the localization of these enzymes in subcellular fractions of the cell are not identical. Further studies on these enzymes are now in progress in this laboratory.

Acknowledgements—The authors are grateful to Professor Komei Miyaki, Director of this Institute, for his continuous encouragement. The authors are also indebted to Dr. Isamu Saikawa for synthesis of benzylhydrazine.

Department of Pharmacology and Toxicology,
Institute of Food Microbiology,
Chiba University,
Chiba, Japan

TETSUO SATOH
KAYOKO MOROI

REFERENCES

1. M. A. SCHWARTZ, *J. Pharmac. exp. Ther.* **130,** 157 (1960).
2. B. A. KOECHLIN, M. A. SCHWARTZ and W. E. OBERHAENSLI, *J. Pharmac. exp. Ther.* **138,** 11 (1962).
3. M. A. SCHWARTZ, *Proc. Soc. exp. Biol. Med.* **107,** 613 (1961).
4. P. FEIGELSON and O. GREENGARD, *J. biol. Chem.* **236,** 153 (1961).
5. A. G. GORNALL, C. J. BARDAWILL and M. M. DAVID, *J. biol. Chem.* **177,** 751 (1949).
6. T. SATOH and K. MOROI, *Analytica chim. Acta* **51,** 449 (1970).
7. M. ROTH and J. RIEDER, *Analytica chim. Acta* **27,** 20 (1962).
8. I. GRANDBERG and A. N. KOST, *Chem. Abstr.* **50,** 11320 (1956).
9. A. N. KOST and R. S. SAGITULLIN, *Chem. Abstr.* **52,** 9071 (1958).
10. J. BARSKY, W. L. PACHA, S. SARKAR and A. ZELLER, *J. biol. Chem.* **234,** 389 (1959).
11. A. L. GREEN, *Biochem. Pharmac.* **13,** 249 (1964).
12. S. HESS, H. WEISSBACH, B. G. REDFIELD and S. UDENFRIEND, *J. Pharmac. exp. Ther.* **124,** 189 (1958).
13. M. A. SCHWARTZ, *J. Pharmac. exp. Ther.* **135,** 1 (1962).
14. J. J. MCMONIGLE and A. HORITA, *Archs int. Pharmacodyn. Thér.* **178,** 53 (1969).