taken up by the liver cells. The cytosolic calcium movement in the liver cells remains to be elucidated.

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References and Notes

1) M. Yamaguchi, Y. Takei, and T. Yamamoto, *Endocrinology*, **96**, 1004 (1975).
2) M. Yamaguchi and T. Yamamoto, *Chem. Pharm. Bull.*, **26**, 1981 (1978).
3) M. Yamaguchi, *Endocrinol. Jpn.*, **27**, 381 (1980).
4) G.H. Hogeboom, "Methods in Enzymology," Vol. 1, ed. by S.P. Colowick and N.O. Kaplan, Academic Press, New York, 1955, p. 16.
5) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
6) M. Yamaguchi and T. Yamamoto, *Chem. Pharm. Bull.*, **23**, 2418 (1975).
7) M. Yamaguchi and T. Yamamoto, *Chem. Pharm. Bull.*, **26**, 1915 (1978).
8) M. Yamaguchi and K. Sugii, *Chem. Pharm. Bull.*, **29**, 567 (1981).

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Comparative Immunological Studies of Mutarotases from Mammals

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The antibodies against purified rat and hog kidney mutarotases (type II) were prepared by injecting each of the purified mutarotases into rabbits and were found to quantitatively precipitate the enzyme activities of rat and hog kidney mutarotases, respectively. Immunological cross-reactivity among four forms of both rat and hog kidney mutarotases and among mutarotases from the kidney, liver, and small intestine of 5 species of animals, rat, hog, mouse, beef, and rabbit, was examined by using these antibodies. It was shown that the four forms of mutarotase in rat kidney as well as those in hog kidney were immunologically identical and that the kidney, liver, and small intestine of each species contain very similar enzymes, though there were clear differences between the enzymes from different species.

Keywords——mammalian mutarotases; multiple forms; anti-mutarotase antibody; immunological identity; double immunodiffusion test; immunoelectrophoresis; quantitative precipitin analysis

Introduction

Mutarotase (aldose 1-epimerase, EC 5.1.3.3) catalyzes the interconversion of the α- and β-anomers of D-glucose and other related sugars. It has been identified in a variety of species including mammals. Relatively high activity of the enzyme is contained in the kidney, liver and small intestine of mammals.[1)]

Mulhern *et al.*[2)] studied the physical characteristics of mutarotases from the kidneys of several animals and described that all of the mutarotases examined seemed to have similar molecular weights and similar shapes. Bailey *et al.*[3)] reported a failnre to produce the antibody against mutarotase from mammals. They attributed their failure to the close structural similarity of the enzymes in various mammals. Furthermore, despite extensive studies on

mutarotases from various species no one has reported the presence of multiple forms of the enzyme until recently.

We first demonstrated the presence of four forms of rat kidney mutarotase[4] as well as of hog kidney mutarotase[5] and also first produced the antibody against the main form (type II) of rat kidney mutarotase.[6] The present studies were directed toward elucidating immunological relationships among the multiple forms of rat and hog mutarotases and among mutarotases of different mammals.

Experimental

Materials——Agarose (A-37) was purchased from Nakarai Chemicals Ltd. Freund's complete adjuvant was from Difco Laboratories. Bovine serum albumin (fraction V) was from Sigma Chemical Co. All other chemicals used were of reagent grade.

Assay of Mutarotase Activity——Mutarotase activity was measured by our method[7] using an oxygen electrode and glucose oxidase. One unit of the enzyme was defined as the amount which converts 1.0 μmol of the α-anomer of D-glucose to the β-anomer per min under the assay conditions used.

Purification of Mutarotases from the Kidneys of Rat and Hog——Rat and hog kidney mutarotases were each separated into four forms (types I—IV), and each of the most abundant forms (type II) was purified to homogeneity as described previously.[4,5] The specific activities of purified rat and hog kidney mutarotases (type II) were 291 and 950 U/mg protein, respectively.

Extraction of Mutarotase Activity from Animal Tissues——The whole kidneys, livers, and small intestines of animals were homogenized with a mixture of 0.1 M, pH 7.2 Tris-HCl buffer (1 vol.) and chloroform (0.25 vol.). Homogenates were centrifuged at $10000 \times g$ for 20 min, and the resulting supernatants were dialyzed against 20 mM EDTA buffer (pH 7.0). The crude enzyme preparations thus obtained were used for immunological analyses.

Preparation of Antibodies against Rat and Hog Kidney Mutarotases——One mg of a purified mutarotase (type II) was dissolved in 1 ml of 0.9% NaCl and mixed with the same volume of Freund's complete adjuvant. This mixture was subcutaneously injected into the back and abdominal regions of male rabbits once every two weeks over two months. At one week after the last injection, blood was collected and antisera were obtained by centrifugation. The γ-globulins were prepared by repeated ammonium sulfate precipitation (40% saturation at first and 33% saturation for the succeeding two cycles). The final precipitates were dissolved in 1/15 M phosphate-buffered saline (pH 7.3) and dialyzed against the same buffer. Dialyzed γ-globulin fractions were concentrated by ultrafiltration to a protein concentration of 27 mg/ml and were used for the immunological experiments.

Ouchterlony's Double Immunodiffusion Test——Diffusion plates were prepared by pouring 6 ml of 1% agarose in 1/15 M phosphate-buffered saline (pH 7.3) on each of the glass plates (50 × 100 mm). The agarose gels were about 1.5 mm thick. About 5 μl of an antibody solution was applied to a single inner well and about 3 μl of mutarotase sample to each of the six outer wells. Immunoreactivity was judged after maintaining the gels for 24 h at room temperature.

Immunoelectrophoresis——The plates for immunoelectrophoresis were prepared by pouring 8.4 ml of 1% agarose in veronal buffer (i=0.025, pH 8.6) on each of the glass plates (70 × 100 mm). Wells (2 mm in diameter) and troughs (2 mm in width) were cut in the agar gels (1.2 mm in thickness). Electrophoresis was performed at a constant current of 2.5 mA/cm for 40 min at 4°C after applying samples in the wells. After electrophoresis, antibodies were put in the troughs. The plates were maintained at 4°C for 2 days before checking immunoreaction.

Quantitative Precipitin Analysis——The precipitating effects of various amounts of each of the antibodies on the activity of rat and hog kidney mutarotases (type II) were studied as follows. Reaction mixtures for test samples contained, in a total volume of 1.1 ml, 15 mM Tris-phosphate buffer (pH 7.4), an appropriate amount of rat or hog kidney mutarotase (type II), and various amounts of an antibody (0 to 27 mg of protein). Reaction mixtures for control assays contained normal rabbit serum γ-globulin (0 to 27 mg of protein) instead of an antibody. After incubation for 50 min at 37°C, portions of reaction mixtures were centrifuged at $30000 \times g$ for 20 min to sediment antibody complexes. Mutarotase activity in the supernatants and in the whole reaction mixtures, and protein amounts of precipitates were determined.

Inhibition of Mutarotase Activity——To examine the inhibitory effects of the antibodies against pure rat and hog kidney mutarotases (type II) on mutarotase activity in animal tissues, the crude enzyme preparations were diluted with 20 mM EDTA buffer (pH 7.4) and adjusted to 0.05 U/ml. Each of the samples was divided in half (2 ml each), and an antibody solution (25 μl) was added to one of the halves and a solution (25 μl) of 27 mg/ml normal rabbit serum γ-globulin to the other. After incubation for 3 h at 25°C, remaining mutarotase activity was determined and the percentage inhibition of the activity was calculated.

Protein Determination——Protein concentrations were determined by the method of Lowry *et al.*[8] with bovine serum albumin as a standard.

Results and Discussion

Immunological Properties of Four Types of Mutarotases in Rat and Hog Kidney

We previously reported that multiple forms of mutarotase exist in rat and hog kidney.[4,5] Those in rat kidney have distinct isoelectric point (pI 5.56, 5.29, 5.24, and 5.04 for types I, II, III, and IV, respectively), but their other properties are quite similar. The distribution patterns of the four types in rat and hog kidney were similar. Keston also reported recently the presence of several forms of mutarotase in hog kidney and intestine.[9]

To elucidate the immunological relationships among the four types of mutarotases in rat and hog kidney, we performed double immunodiffusion, immunoelectrophoresis, and quantitative precipitin analysis. The double immunodiffusion test showed a definite precipitin line between the crude extract of rat kidney and the antibody against purified rat kidney mutarotase type II (Fig. 1). A single precipitin line was also observed between each of the four types of rat kidney mutarotase and the antibody. These precipitin lines formed with the four types of mutarotase and with the crude extract fused completely with each other. These results indicate that the antibody against rat kidney mutarotase type II reacts with each of the four types of rat kidney mutarotase and that the four types are immunologically identical, namely they have the same types of antigenic determinants.

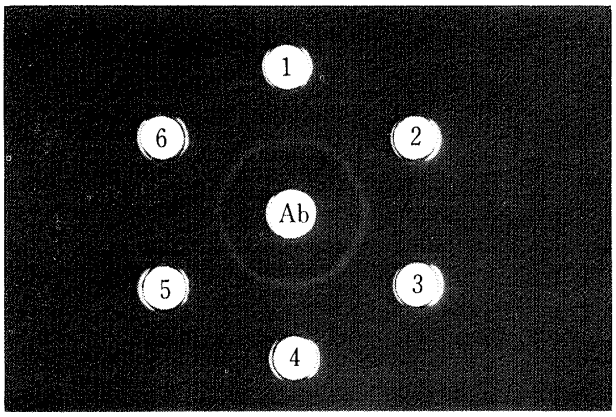

Fig. 1. Double Immunodiffusion Test with the Antibody to Pure Rat Kidney Mutarotase Type II for Identification of Four Types of Rat Kidney Mutarotase

A central well (Ab) was filled with the antibody. Wells 1 and 2 contained the crude extract of rat kidney. Wells 3, 4, 5, and 6 contained types I, II, III, and IV rat kidney mutarotase, respectively.

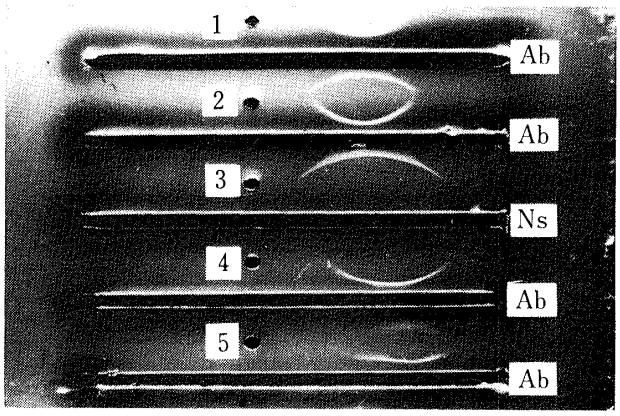

Fig. 2. Immunoelectrophoresis of Four Types of Rat Kidney Mutarotase

Well 1 contained the crude extract of rat kidney. Wells 2, 3, 4, and 5 contained types I, II, III, and IV rat kidney mutarotase, respectively. Four troughs (Ab) were filled with the antibody to pure rat kidney mutarotase type II and one trough (Ns) with normal rabbit serum γ-globulin after electrophoresis.

Immunoelectrophoresis showed that the antibody against rat kidney mutarotase type II produced a single precipitin line with each of the four types of rat kidney mutarotase, while the four types ran with distinct mobilities to the anode in the order types I, II, III, and IV (Fig. 2), indicating that the four types of mutarotase have distinct charges, as described previously[4] on the basis of their isoelectric properties. In immunoelectrophoresis, the precipitin line formed with the crude extract of rat kidney was visualized just as if it were single and was located near type I or II, but this is reasonable because types I, II, III, and IV in the crude extract are known to account for 11, 80, 7, and 2% of the total activity, respectively.[4]

The four types of hog kidney mutarotase were found to react with the antibody against purified hog kidney mutarotase type II in almost the same manner as in the case of rat kidney mutarotase, as judged by double immunodiffusion analysis (data not shown), indicating that these four types are also immunologically identical.

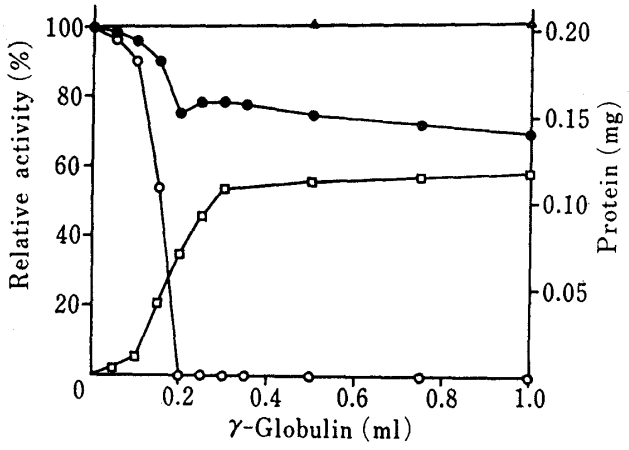

Fig. 3. Immunoprecipitation of Pure Rat Kidney Mutarotase Type II with the Antibody to the Enzyme

The enzyme solutions (0.1 ml, 2.5 units) were incubated with various amounts of the antibody (0 to 1 ml) in a total volume of 1.1 ml. ○: supernatant enzyme activity, ●: total enzyme activity, △: supernatant activity after incubation of the enzyme with normal rabbit serum γ-globulin, □: protein amount of precipitate.

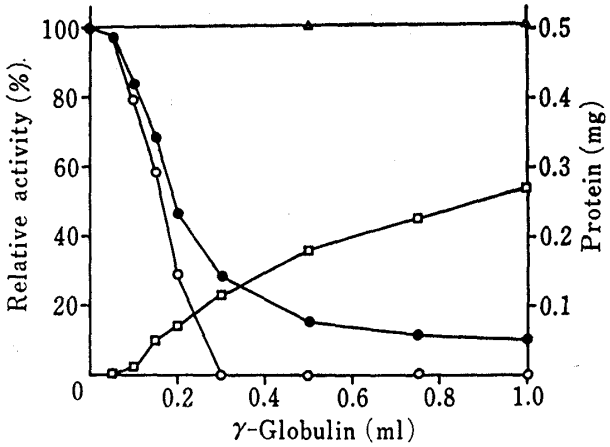

Fig. 4. Immunoprecipitation of Pure Hog Kidney Mutarotase Type II with the Antibody to the Enzyme

The enzyme solutions (0.1 ml, 5.8 units) were incubated with various amounts of the antibody (0 to 1 ml) in a total volume of 1.1 ml. ○: supernatant enzyme activity, ●: total enzyme activity, △: supernatant activity after incubation of the enzyme with normal rabbit serum γ-globulin, □: protein amount of precipitate.

In order to examine whether purified rat kidney mutarotase type II is quantitatively immunoprecipitated by the antibody to the enzyme, the enzyme was incubated in the presence of various amounts of the antibody. After incubation, the enzyme activities in the whole reaction mixture (total enzyme activity) and in the supernatant (supernatant enzyme activity) obtained after centrifugation were assayed. The supernatant enzyme activity was lost progressively as increasing amounts of the antibody were added (Fig. 3). The maximum loss of the total enzyme activity, however, was not great (about 30%). A similar quantitative precipitin pattern was also obtained for the reaction of purified hog kidney mutarotase type II with the antibody to the enzyme except for a more profound loss (about 90%) of the total enzyme activity (Fig. 4). It is possible that some antigenic determinant of hog kidney mutarotase may exist in closer proximity to the catalytic site of the enzyme compared with that of rat kidney mutarotase, and that greater steric interference with the interaction of the substrate and the enzyme therefore occurs in hog kidney mutaratase.

Immunological Identity of Kidney, Liver, and Small Intestine Mutarotases from Various Mammals

Immunological reactivity of the antibodies to pure rat and hog kidney mutarotases (type II) was tested with crude extracts of kidney, liver and small intestine from different species, using a double immunodiffusion technique. The percentage inhibition of mutarotase activity in those tissues caused by the antibodies was also determined. The results obtained are summarized in Table I.

The antibody to hog kidney mutarotase type II gave a single precipitin line against each of the crude enzyme preparations from hog kidney, liver, and small intestine, and the three lines fused completely (Fig. 5). The antibody markedly and equally inhibited the enzyme activities of these preparations. A similar result was obtained using a combination of the antibody to rat kidney mutarotase type II and the crude extracts from the three kinds of tissues of rat (not shown in the figure). These results indicate that mutarotases from different tissues of the same species are immunologically identical.

The antibody to rat kidney mutarotase type II produced a clear precipitin line against each of the crude extracts from mouse kidney, liver, and small itnestine, but spur formation was observed between these lines and the ones produced against the crude extracts from rat tissues

TABLE I. Cross-Reactivity on Double Immunodiffusion of Two Kinds of Antibodies with Mutarotases contained in Kidney, Liver, and Small Intestine from Different Animals

Enzyme source	Immunochemical reaction[a]	
	Anti-R-K[b]	Anti-H-K[c]
Rat		
Kidney	+(30.6)[d]	−(3.8)
Liver	+(30.1)	−(2.0)
Small intestine	+(34.1)	−(2.5)
Hog		
Kidney	−(1.9)	+(92.9)
Liver	−(2.0)	+(90.3)
Small intestine	−(2.2)	+(93.0)
Mouse		
Kidney	+(35.4)	−(2.8)
Liver	+(34.0)	−(3.0)
Small intestine	+(19.1)	−(2.4)
Beef		
Kidney	−(7.8)	+(57.2)
Liver	−(5.6)	+(55.6)
Small intestine	−(5.5)	+(55.1)
Rabbit		
Kidney	−(1.0)	−(1.1)
Liver	−(1.2)	−(1.2)
Small intestine	−(1.1)	−(1.1)

a) +: positive cross-reaction, −: negative cross-reaction.
b) Antibody against pure rat kidney mutarotase type II.
c) Antibody against pure hog kidney mutarotase type II.
d) Figures in parentheses are the percentages of inhibition of mutarotase activity in tissues by either anti-R-K or anti-H-K.

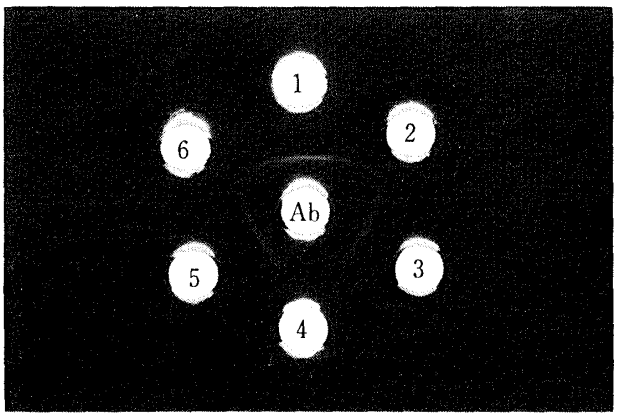

Fig. 5. Double Immunodiffusion Test with the Antibody to Pure Hog Kidney Mutarotase Type II

A central well (Ab) was filled with the antibody. Wells 1, 3, and 5 contained the crude extracts of hog kidney, liver, and small intestine, respectively, and wells 2, 4, and 6 those of rat kidney, liver, and small intestine, respectively.

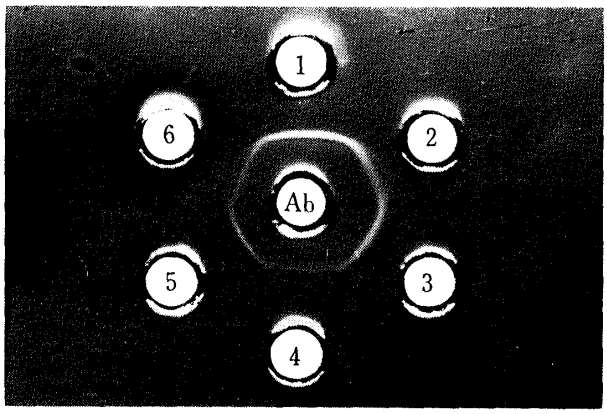

Fig. 6. Double Immunodiffusion Test with the Antibody to Pure Rat Kidney Mutarotase Type II for Examining the Identity between Mutarotase from Rat Tissues and the Enzyme from Mouse Tissues

A central well (Ab) was filled with the antibody. Wells 1, 3, and 5 contained the crude extracts of rat kidney, liver, and small intestine, respectively, and wells 2, 4, and 6 those of mouse kidney, liver, and small intestine, respectively.

(Fig. 6). With the antibody to hog kidney mutarotase type II, similar spur formation was observed between precipitin lines formed by the crude extracts from hog tissues and those from bovine tissues (not shown in the figure). The spur formation in these case may be due to partially altered antigenic properties of the enzymes from closely related but different species, *i.e.* rat *vs.* mouse and hog *vs.* beef. The antibody to rat or hog kidney mutarotase type II, however, clearly inhibited the enzyme activity of the crude extract from mouse or bovine tissues, respectively.

The antibody against rat kidney mutarotase type II neither reacted with nor inhibited the enzymes extracted from the tissues of hog, beef, and rabbit. A similar situation was also observed with the antibody against hog kidney mutarotase type II and the tissues of rat (Fig. 5), mouse, and rabbit. These results indicate that the enzyme in rat tissues is quite different from those contained in hog, beef, and rabbit, and that the enzyme in hog tissues is different from those in rat, mouse, and rabbit.

References and Notes

1) A.S. Keston, *Science*, **120**, 335 (1954); J.M. Bailey, P.G. Pentchev, and J. Woo, *Biochim. Biophys. Acta*, **94**, 124 (1965); J.M. Bailey, P.H. Fishman, S.A. Mulhern, and R. Murray, *Clin. Biochem.*, **3**, 11 (1970); I. Miwa, *Analyt. Biochem.*, **45**, 441 (1972).
2) S.A. Mulhern, P.H. Fishman, J.W. Kusiak, and J.M. Bailey, *J. Biol. Chem.*, **248**, 4163 (1973).
3) J.M. Bailey, P.G. Pentchev, P.H. Fishman, S.A. Mulhern, and J.W. Kusiak, "Advances in Chemistry Series," Vol. 117, ed. by H.S. Isbell, Am. Chem. Soc., Washington, D.C., 1973, pp. 264—308.
4) J. Okuda, I. Miwa, and Y. Toyoda, *Chem. Pharm. Bull.*, **24**, 2893 (1976).
5) I. Miwa, Y. Toyoda, and J. Okuda, *Chem. Pharm. Bull.*, **29**, 1702 (1981).
6) S. Baba, Y. Arimoto, D. Yoshikawa, Y. Toyoda, I. Miwa, and J. Okuda, *Experientia*, **35**, 1094 (1979).
7) I. Miwa and J. Okuda, *J. Biochem.*, **75**, 1177 (1974).
8) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
9) A.S. Keston, Abstracts of Papers, 11th International Congress of Biochemistry, Toronto, Canada, July, 1979, p. 271.

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Solid-State Nuclear Magnetic Resonance Spectroscopy and Raman Spectroscopy of the Inclusion Compound of Tolbutamide with β-Cyclodextrin[1,2]

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A structural study of the inclusion compound of tolbutamide with β-cyclodextrin (β-CD) in the solid state was attempted by means of carbon-13 high resolution solid-state nuclear magnetic resonance (NMR) experiments. The change in chemical shift of tolbutamide suggested that the phenyl moiety of the drug molecule was included in the cavity of β-CD in the solid state. This view was also supported by the change of the C–H out- of -plane vibration of the phenyl moiety of tolbutamide in the Raman spectrum. The solid-state NMR technique appears to be useful for studies on the nature of inclusion compounds of drug molecules with β-CD.

Keywords——^{13}C high resolution solid-state NMR; magic-angle spinning method; cross-polarization; inclusion compounds; β-cyclodextrin; tolbutamide; chemical shift; Raman spectroscopy