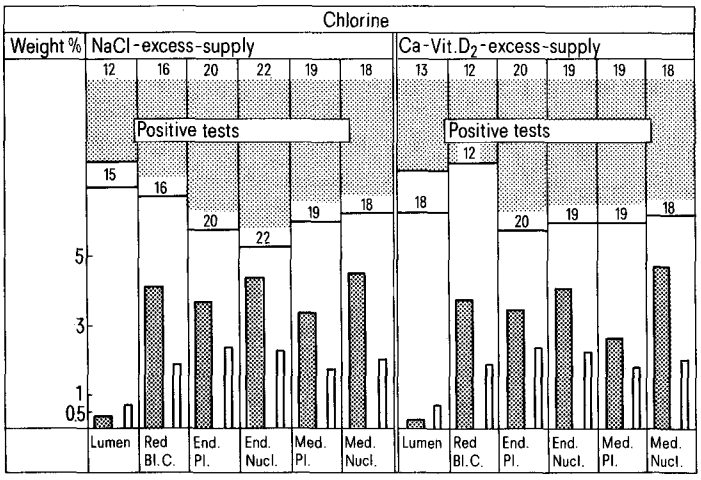


The hatched columns show the number of positive results of total measurements. Black and white columns at the bottom show semiquantitative values in weight percent. Treated rats: black, controls: white. End. Pl., endothelial plasma; Nucl., nucleus, Med., mediocyte, Bl.C., blood cells.

chloride and calcium-vitamin D_2, the latter showed 2-fold higher Cl-values in the vessel wall and the red blood cells (figure). It is of interest that the abundance of Cl in the lumen, which holds a significantly lower level than in the wall of the same vessel, in controls, conversely, were 2-fold higher than in the treated rats.

These results caused us to believe in the real existence of Cl in the tissues even after conventional fixation (glutaraldehyde), washing (distilled water or several puffer solutions), dehydration (ethanol), and embedding (epon 812). The often stressed statement that Cl in epon-impregnated biological tissues has its origin in the epon itself, while the tissue-chlorine has been washed out quantitatively or is removed by the medium, cannot be accepted, mainly for 2 reasons.

Firstly, the amount of Cl in the measured area is usually 4 times higher in tissues than in the tissue-free epon environment. And secondly, we observed differences as already mentioned between controls and rats with metabolic disorder.

Thus it seems that the very labile Cl-ion, though not fixed at its intracellular sites and floating during the preparational procedure, will remain in remarkable amounts in the competent tissue region, i.e. in our case in the vascular wall. Thus for rough comparisons between vessels with similar parameters, it should be possible to find out true differences after experimentally changing a factor like blood pressure. In this connection, the observation of Läuchli et al.[2] must be mentioned, who after osmium fixation, washing, dehydration and Spurr-embedding with radiochemical measurement missed only 4% of the Cl that they had found before fixation.

These results should be verified with the generally accepted freezing methods. They are encouraging in so far as it seems that, even with conventional preparative work, regional differences in the rough element distribution can be estimated.

1 C.H. Becker and A.G.S. Jánossy, Acta biol. med. germ. *36*, 1413 (1977).
2 A. Läuchli, R. Stelzer, R. Guggenheim and L. Henning, in: Microprobe as applied to cells and tissues, p. 107. Ed. T. Hall, P. Echlin and R. Kaufmann. Acad. Press, London-New York 1974.

The localization of mutarotase in rat kidney*

S. Baba, Y. Arimoto, D. Yoshikawa, Y. Toyoda[1], I. Miwa[1] and J. Okuda[1]

Department of Internal Medicine, School of Medicine, Kobe University, Ikuta-ku, Kobe 650, and Department of Clinical Biochemistry, Faculty of Pharmacy, Meijo University, Tempaku-ku, Nagoya 468 (Japan), 7 November 1978

Summary. The localization of mutarotase in rat kidney was investigated by fluorescein-labelled and peroxidase-labelled antibody techniques, and by method of isolation of the nuclei and cytoplasm in non-aqueous solvents. In these immunohistochemical studies, mutarotase was almost exclusively recognized in the nuclei of epithelial cells of renal tubules and glomeruli in rat. The specific activity of mutarotase was found to be 1.5 times higher in the nuclei (122 units/g dry wt) than that in the cytoplasm (80 units/g dry wt) isolated with non-aqueous solvents. These results suggest that mutarotase may be involved in the metabolism of D-glucose in nuclei.

Mutarotase [aldose 1-epimerase, EC 5.1.3.3] catalyzes the interconversion of D-glucose and other sugars, and is known to be present in high activity in the kidney, liver, and small intestine of higher animals[2-5]. Any definite function of this enzyme has not yet been clarified, but it has been postulated that the enzyme may be involved in the transport of sugars[2,6] or in D-glucose metabolism[7,8]. To find a clue to the role of the enzyme, we attempted to determine the localization of the enzyme in rat kidney cells using the fluorescein-labelled and peroxidase-labelled antibody techniques, and the method of isolation of nuclei and cytoplasm in non-aqueous solvents. Since we have recently found that the kidney mutarotase appears in both serum and urine of patients[9] and rats[10] with nephrotic syndrome, it is also of interest to determine the localization of the enzyme.

Materials and methods. Purification of mutarotase from rat kidney. Mutarotase was purified as reported previously[11]. 4 different forms of mutarotase were found to exist in rat kidney, of which a major form (type II) was purified to homogeneity. We used the mutarotase type II for the preparation of anti-mutarotase antibody.

Preparation of anti-mutarotase antibody. 1 mg of 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 then injected into the back of male rabbit once every 2 weeks over 2-3 months. 1 week after the last injection, blood was collected and serum was obtained. γ-Globulin fraction was obtained from the serum by standard procedure of ammonium sulfate fractionation and was thus used as an anti-mutarotase antibody. 1 ml of the solution of γ-globulin fraction neutralized 7.5 units of rat kidney mutarotase type II.

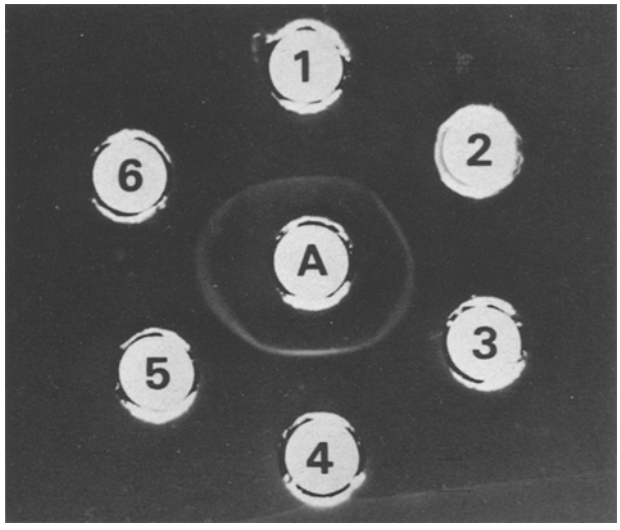


Fig. 1. Double immunodiffusion test of the antibody against rat kidney mutarotase type II with four types of rat kidney mutarotase. Wells contain the antigen or antibody indicated as follows: 1 and 2: rat kidney homogenate; 3: rat kidney mutarotase type I; 4: rat kidney mutarotase type II; 5: rat kidney mutarotase type III; 6: rat kidney mutarotase type IV.

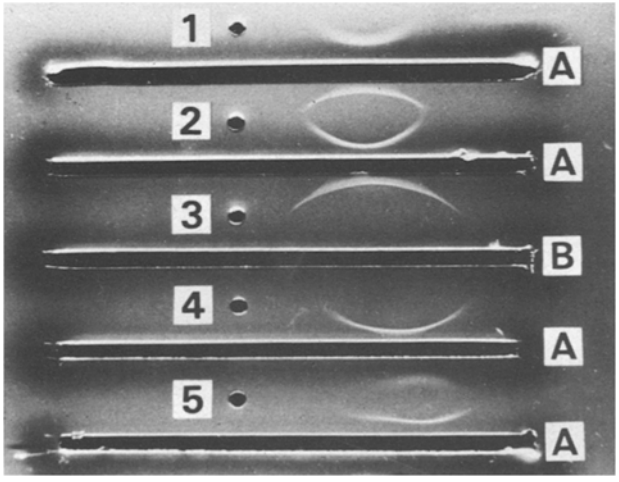


Fig. 2. Immunoelectrophoresis of 4 types of rat kidney mutarotase. Wells and troughs contain the antigen or antibody indicated as follows: A: antibody against rat kidney mutarotase type II; B: normal rabbit serum γ-globulin. 1: Rat kidney mutarotase homogenate; 2: rat kidney mutarotase type I; 3: rat kidney mutarotase type II; 4: rat kidney mutarotase type III; 5: rat kidney mutarotase type IV.

Reactivity of the antibody to 4 forms of mutarotase. Reactivity of the antibody to 4 forms of mutarotase was detected using the double immunodiffusion test and immunoelectrophoresis.

Fluorescein-labelled antibody technique. Rat kidney was cut into pieces of about 1 mm in diameter and fixed in 95% ethyl alcohol for 24 h at -20 °C. Dehydration was carried out with ethyl alcohol at -20 °C, following which tissue was embedded in paraffin. Paraffin-embedded sections were prepared and were used for indirect immunofluorescence staining. First, 1 droplet of antibody was placed on the paraffin section for 40 min at 37 °C and was then washed for 15 min in cold phosphate buffered saline (PBS). 2, 1 droplet of fluorescein isothiocyanate (FITC)-labelled anti-rabbit γ-globulin goat serum (Hyland laboratories) was placed and settled for 40 min at 37 °C. After washing with PBS for 15 min, glycerin was mounted and the observation under fluorescent microscope was carried on.

Peroxidase-labelled antibody technique. The procedure of staining with peroxidase-labelled antibody (Miles Laboratories) was the same as in the case of fluorescein-labelled antibody technique described above. After the application of the peroxidase-labelled antibody, the section was stained for 7.5 min with diaminobenzidine tetrahydrochloride (DAB) solution[12], then washed, dehydrated, mounted in balsam and examined by a light microscope.

Isolation of the nuclear and cytoplasmic fractions from rat kidney with non-aqueous solvents. Male Wistar rats weighing about 200 g were killed by decapitation and kidneys were removed, minced with scissors, and frozen in dry ice-acetone. From the powder obtained by lyophilization, the nuclear and cytoplasmic fractions were isolated using non-aqueous solvents (carbon tetrachloride and cyclohexane) by the procedure of Behrens as modified by Siebert[13].

Measurements of activities of mutarotase and NAD pyrophosphorylase. Mutarotase activity was determined by the method of Miwa and Okuda[14]. NAD pyrophosphorylase, a marker enzyme of nuclei, was determined by the method of Kornberg[15].

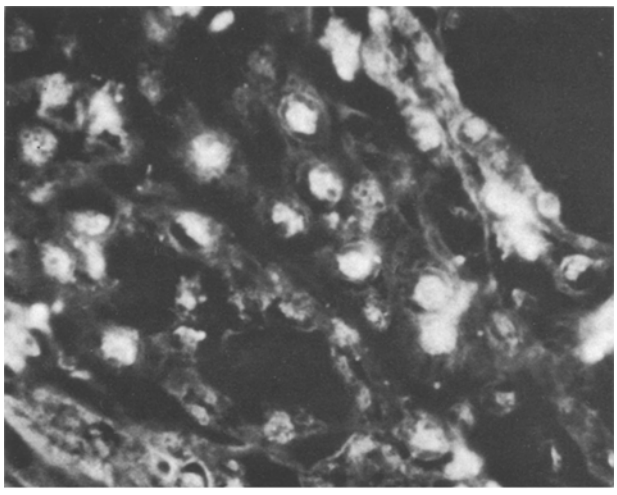
Measurements of RNA and DNA. Measurements of RNA and DNA in the nuclei and cytoplasm from rat kidney was performed by the method of Schmidt and Thannhauser[16].

Results. Specificity of anti-mutarotase antibody[17]. As shown in figure 1, a single precipitin line was observed between 4 types of mutarotase from rat kidney and the antibody against rat kidney mutarotase type II in the double immunodiffusion test, and all single precipitin lines were fused. Moreover, in the method of immunoelectrophoresis, a single line was also observed between each type of mutarotase and the antibody as seen in figure 2. Thus, the antibody was found to be reactive to all 4 forms of mutarotase, and all types of mutarotase were thought to be detected in the fluorescein-labelled and peroxidase-labelled antibody techniques.

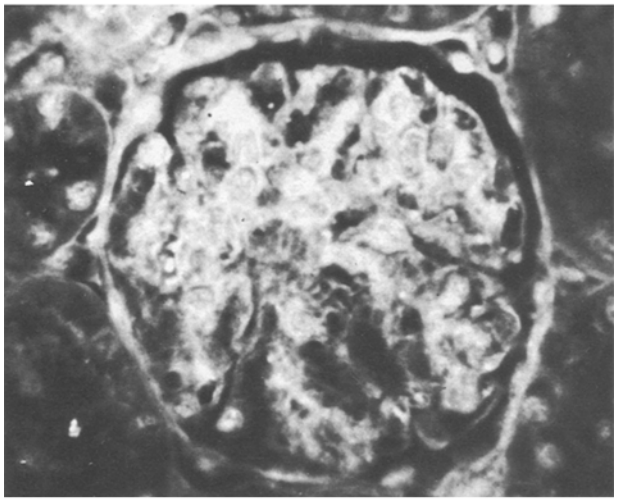
Mutarotase and NAD pyrophosphorylase activities and RNA/DNA ratios in lyophilized powder, nuclei, and cytoplasm of rat kidney

	Lyophilized powder	Nuclei*	Cytoplasm*
Mutarotase**	180	122	80
NAD pyrophosphorylase***	4.4	79.8	Neg.
RNA/DNA	1.1	0.2	10.0

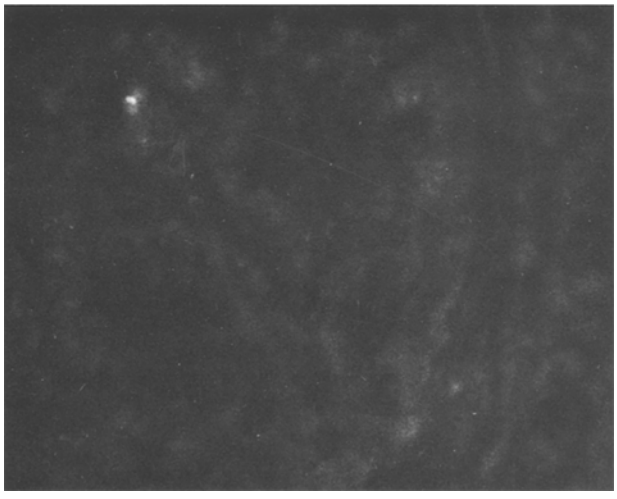
* Prepared by cell fractionation method using non-aqueous solvents; ** units/g dry wt; *** μmoles NAD/h/g dry wt.



A

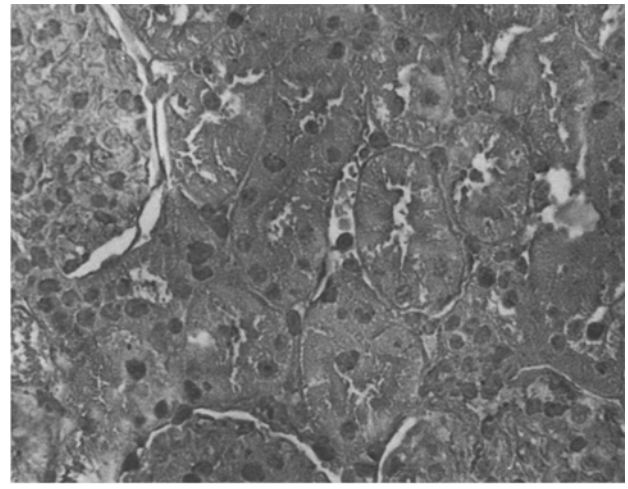


B

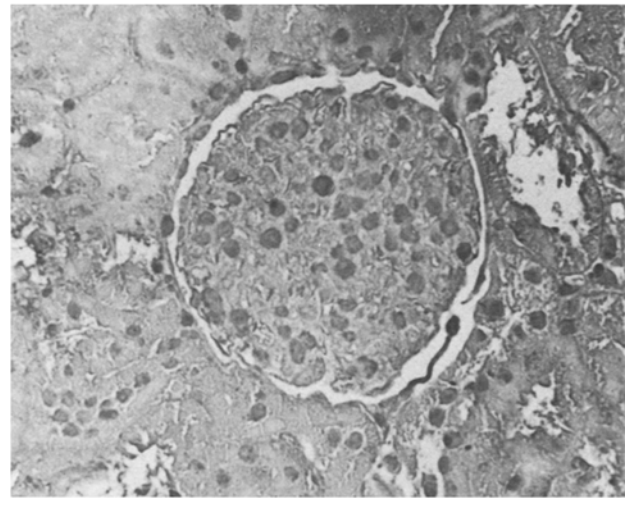


C

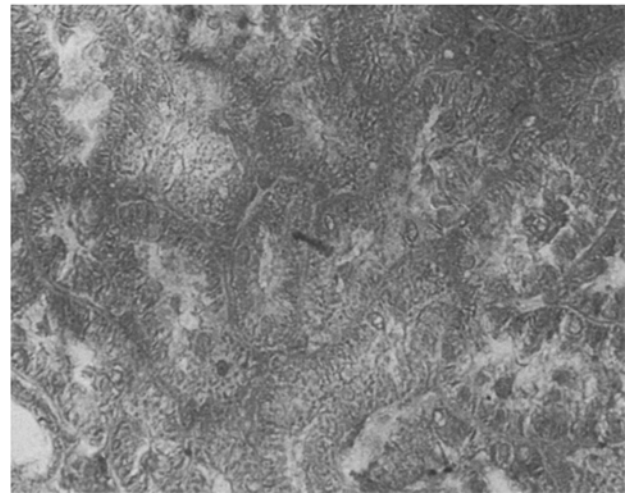
Fig. 3. Immunofluorescent staining of mutarotase in *A* the tubules and *B* the glomeruli of rat kidney, and *C* control staining by blocking test. × 170.



A



B



C

Fig. 4. The localization of mutarotase in *A* the tubules and *B* the glomeruli of rat kidney, *C* control staining by blocking test using the peroxidase-labelled antibody method. × 80.

Staining. In the fluorescein-labelled antibody method, the strong fluorescence was seen in the nuclei of the epithelial cells of any region of renal tubules. The photograph (figure 3A) shows fluorescence localization in a part of the renal tubules. In the glomeruli, fluorescence was observed diffusely (figure 3B). The localization of the enzyme demonstrated by peroxidase-labelled antibody method was almost the same as that shown by fluorescent antibody method. The positive reaction was seen in the nuclei of epithelial cells of renal tubules (figure 4A), and was also clearly recognized in the nuclei of glomeruli (figure 4B). To recognize the specificity of fluorescence and the positive brown color reaction, a blocking test was done as follows: after the treatment with anti-mutarotase antibody, non-labelled anti-rabbit γ-globulin goat serum was placed on the section and then the section was covered with FITC-labelled or peroxidase-labelled anti-rabbit γ-globulin goat serum. No reaction was observed in this test (figures 3C, 4C). No specific staining was seen in another control test as well, in which normal rabbit serum γ-globulin was used instead of the antibody in the staining procedure. Thus, green fluorescence and the positive brown color reaction could be recognized to be specific for the existence of mutarotase.

Mutarotase activity in the nuclear and cytoplasmic fractions isolated from rat kidney in non-aqueous solvents. The nuclear and cytoplasmic fractions from rat kidney were isolated with non-aqueous solvents to eliminate complications introduced by possible diffusion of enzymes across the nuclear membrane during manipulation in aqueous media. As judged by RNA/DNA ratio and NAD pyrophosphorylase activity, reasonably pure nuclei and cytoplasm were obtained. Other fractions were separated as well, but none of them were purer. Therefore, we did not analyze the mutarotase activity of other fractions. As shown in the table, the specific activity of mutarotase was found to be 1.5 times higher in the nuclei (122 units/g dry wt) than in the cytoplasm (80 units/g dry wt). The specific activity (180 units/g dry wt) of mutarotase in lyophilized powder of minced kidney was much higher compared with those in the nuclei and cytoplasm. We think that these contradictory results may be due to the loss of enzyme activity during the preparation with non-aqueous solvents.

Discussion. We have reported[11,18] that 4 forms of mutarotase exist in rat kidney and human kidney and that 4 forms of mutarotase in rat kidney have distinctive isoelectric points, but other properties are almost the same. We also found[17] that 4 forms of the enzyme reacted with the antibody against mutarotase type II from rat kidney and that no spur formation was observed in double immunodiffusion test. This result indicates that all 4 forms of mutarotase have the same antigenic determinants, although the 4 forms of mutarotase have distinct electrophoretic mobility when analyzed by immunoelectrophoresis.

Baily et al.[3] have reported that mutarotase in rat kidney, liver, and small intestine were exclusively found in the supernatant fraction but not in any other fractions isolated by homogenization in 0.2 M sucrose and differential centrifugation using standard procedures. It is thus inferred from their result that mutarotase is an extremely soluble enzyme and leaks easily from the nuclei into an aqueous isolation-media as well as in the case of DNA polymerase[19]. So, we employed non-aqueous solvent method for the isolation of nuclei and cytoplasm. The localization of fluorescence and the positive brown color reaction deposits, specific for mutarotase, in the nuclei of epithelial cells of renal tubules in the histochemical study was compatible with the presence of high mutarotase activity in the nuclear fraction isolated in non-aqueous solvents. However, a discrepancy remained that the nuclei fluorescence and the positive brown color reaction deposits were much stronger, while the nuclear specific activity of mutarotase was only 1.5 times higher than the cytoplasmic. This may be due to the higher concentration of water in cytoplasm than in nuclei[19]. There may be also a possibility that a part of the nuclear mutarotase diffused to cytoplasm, even by the isolation technique using non-aqueous solvents.

Mutarotase is characterized in mammalian tissues by its predominant distribution in kidney, liver, and small intestine, its sensitivity to inhibition by phloridzin, its specificity for sugars which are actively transported, and its kinetic properties that are in concordance with transport process.

From these facts, Bailey et al. have suggested a possible involvement of the enzyme in the absorption of sugars[20]. However, mutarotase was hardly found in the membrane of the rat kidney tubules in our immunohistochemical study. Moreover, we found that no effect of the anti-mutarotase antibody was observed on the active transport of D-glucose in rat small intestine[17]. Thus, we do not think that mutarotase may be involved in the transport of sugars. On the other hand, Gernert and Keston have reported[8] that mutarotase from hog kidney is capable of catalyzing the mutarotation of D-glucose-6-phosphate, and we have found that hexokinase isozymes type I, II, III isolated from rat liver preferentially phosphorylates β-D-glucose[21]. From these data, it could be postulated that mutarotase may be involved in D-glucose metabolism in the nuclei. The true physiological function of mutarotase, however, should be further investigated.

* Acknowledgment. The authors thank Prof. Y. Nishizuka (Department of Biochemistry, School of Medicine, Kobe University) for his valuable advice.

1 Department of Clinical Biochemistry, Faculty of Pharmacy, Meijo University, Tempaku-ku, Nagoya 468, Japan.

2 A.S. Keston, Science *120*, 355 (1954).
3 J.M. Bailey, P.G. Pentchev and J. Woo, Biochim. Biophys. Acta *94*, 124 (1965).
4 J.M. Bailey, P.H. Fishman, S.A. Mulhern and R. Murray, Clin. Biochem. *3*, 11 (1970).
5 I. Miwa, Analyt. Biochem. *45*, 441 (1972).
6 S.A. Mulhern, P.H. Fishman, J.W. Kusiak and J.M. Bailey, J. biol. Chem. *248*, 4163 (1973).
7 A.S. Keston, J. biol. Chem. *239*, 3241 (1964).
8 E. Gernert and A.S. Keston, Archs. Biochem. Biophys. *161*, 420 (1974).
9 I. Miwa, J. Okuda, K. Maeda, S. Umeda, A. Saito and K. Ohta, Chem. pharm. Bull. *25*, 617 (1977).
10 I. Miwa, K. Ikeda, Y. Suzuki, J. Okuda and K. Maeda, Chem. pharm. Bull *22*, 2337 (1974).
11 J. Okuda, I. Miwa and Y. Toyoda, Chem. pharm. Bull. *24*, 2893 (1976).
12 S. Avrameas, in: Meth. Enzymol., vol. 44, p. 713. Ed. S.P. Colowick and N.O. Kaplan. Academic Press, New York 1976.
13 G. Siebert, in: Meth. Cancer Res., vol. 2, p. 287. Ed. H. Busch. Academic Press, New York 1967.
14 I. Miwa and J. Okuda, J. Biochem. *75*, 1177 (1974).
15 A. Kornberg, in: Meth. Enzymol., vol. 2, p. 670. Ed. S.P. Colowick and N.O. Kaplan. Academic Press, New York 1955.
16 G. Schmidt and S.J. Thannhauser, J. biol. Chem. *161*, 83 (1945).
17 J. Okuda, I. Miwa and T. Taguchi, Med. Biol. *96*, 19 (1978), in Japanese.
18 J. Okuda, I. Miwa, Y. Toyoda and S. Baba, Experientia *34*, 563 (1978).
19 G. Siebert and G.B. Humphrey, in: Adv. Enzymol., vol. 27, p. 239. Ed. F.F. Nord. Interscience, New York 1965.
20 J.M. Bailey, P.G. Pentchev, P.H. Fishman, S.A. Mulhern and J.W. Kusiak, Adv. chem. Ser. *117*, 264 (1973).
21 J. Okuda, I. Miwa, K. Inagaki, M. Ueda and K. Taketa, J. Biochem. *84*, 993 (1978).