

An Improved Method for Determination of Serum Adenosine Deaminase Activity and its Clinical Applications^{*}

Takahiko SHIOSAKA¹⁾, Hiroji OHMINAMI²⁾, Yuzuru KOBAYASHI¹⁾
and Hiromichi OKUDA²⁾

1) *Department of 1st Internal Medicine, Ehime University, School of Medicine, 791-02 Japan*

2) *Department of 2nd Medical Biochemistry, Ehime University, School of Medicine*

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ABSTRACT

An improved method was devised for the estimation of serum adenosine deaminase activity. The principle of the method consists in measurement of a liberated ammonia by the action of the deaminase with a direct colorimetric determination of ammonia concentration [Okuda et al.]. The technique is relatively easy to estimate the serum enzyme activity and can be applied for routine clinical use. The serum adenosine deaminase activity assayed by this method was elevated in patients with hepatic disease and the others, such as auto-immune diseases, rheumatoid arthritis, malignant lymphoma, leukemia and multiple myeloma.

The presented experiments were designed to clarify the origin of the elevated adenosine deaminase in serum. The heat stability and pH optimum were found to be essentially identical in sera from hepatic disease and the others. However, Michaelis constant (K_m) for adenosine and V_{max} were higher in the sera from patients with hepatic diseases than those of the others. The isoelectrofocusing pattern of the serum enzyme was shown quite different between hepatic disease and the others. The results in the present investigation indicated that the enhanced serum adenosine deaminase activity and its isoenzyme pattern were very useful for clinical diagnosis.

INTRODUCTION

Adenosine deaminase (Adenosine aminohydrolase, E. C. 3, 5, 4, 4) catalyzes the irreversible hydrolytic deamination of adenosine to inosine and ammonia¹⁾. This enzyme is known to be widely distributed in animal tissues and is present in serum.²⁾ Adenosine deaminase activity of serum has been extensively studied to explore its clinical applicability by many investigators and the results suggested greater usefulness for the diagnosis of cancer¹⁾. However, it was rather difficult to estimate the activity of serum adenosine deaminase in ordinary clinical examination. The procedures for adenosine deaminase assay include spectrophotometric method at 265 nm¹⁰⁾ and at 293 nm⁹⁾ neither of which

is sufficiently robust to serve as the basis of a routine clinical examination. The pH stat³⁾, manometric⁷⁾, radioisotopic⁴⁾ and micro diffusion techniques⁵⁾ used for assay of this enzyme are likewise unsuitable for clinical use. In 1962, Konitzner et al.¹²⁾ reported a direct colorimetric determination of blood ammonia. In his procedure, trichloroacetic acid was used as a deproteinizing agent and indophenol formation was followed after neutralization of the clear supernatant mixture with 1 N NaOH. By this method, however, the color development of uric acid and amino acids could not be inhibited. Besides, ammonia might be lost during neutralization. In procedure reported by Okuda et al.¹⁴⁾ using sodium tungstate and sulfuric acid as deproteinizing agent, neutralization is not

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necessary and color development of uric acid and amino acids is specifically inhibited. Then, an improved method which was adopted for the estimation of serum adenosine deaminase was made to use of this colorimetric determination of ammonia. The principle of the assay method consists in measurement of ammonia liberated by the actions of the deaminase using a direct colorimetric determination of ammonia.

Multiple forms of adenosine deaminase may be found even within a single tissue, and the patterns of inheritance of the isoenzymes have been studied^{1, 2, 6, 13, 16, 21}. The enzyme participates in the catabolism of adenosine nucleotides, resulting in the production of hypoxanthine for use in purine salvage pathways. In the heart, this enzyme may play as an important regulatory role to disrupt adenosine, a potent vasodilator⁹. A deficiency of adenosine deaminase has been associated with severe combined immunodeficiency disease in infants¹⁵ but a well defined causal relationship has not been established¹⁹.

The present study was undertaken to determine the adenosine deaminase levels in serum from patients with various disease by the simple colorimetric method and to separate the isoenzymes in the same specimen by preparative isoelectrofocusing technique.

MATERIALS AND METHODS

[8-¹⁴C] adenosine (specific activity 40 mCi/mmoles) was purchased from the Radiochemical Center Amersham. Adenosine was obtained from Sigma. Ampholine carrier ampholytes were purchased from LKB.

Enzyme assay

Adenosine deaminase was assayed by two different methods. The first method was carried out by direct colorimetric determination of the liberated ammonia described by Okuda et al.¹⁴ It was mainly used for estimation of serum enzyme activity. The reaction mixture consisted of 4 mM adenosine, 10 mM phosphate buffer (pH 7.5) and enzyme solution in a final volume of 0.5 ml. The mixture was incubated at 37°C for 60 min. Then 2 ml of sodium tungstate (Na₂WO₄ · 2H₂O 37.5g, 0.02 N PO₄ 50 ml and 1 N H₂SO₄ 200 ml were made up to final volume of 1,000 ml with H₂O) were added to terminate the reaction and the mixture was centrifuged at 3,000 rpm for 5 min. To 1 ml of the super-

natant, 1.5 ml of reagent A and 1.5 ml of reagent B were added, where reagent A was prepared by dissolving 5g of phenol and 25 mg of sodium nitroprusside in H₂O in a final volume of 375 ml, and reagent B was made up by dissolving 4.2 g of NaOH, 44.6 g of Na₂HPO₄ · 12 H₂O and 5 ml of antiformine (containing 10% Cl⁻) in H₂O in a final volume of 375 ml. The mixture was allowed to stand at 37°C for 30 min, and then the optical absorbance was read at 625 nm.

The second method was carried out by the conversion rate of [8-¹⁴C] adenosine to [8-¹⁴C] inosine¹⁹. The standard reaction mixture for the adenosine deaminase contained 0.4 mM [8-¹⁴C] adenosine (1 μCi/nmol), 50 mM Tris-HCl (pH 7.4) and enzyme in a final volume of 100 μl. After the incubation at 37°C for 30 min, the reaction was terminated by the addition of 50 μl of ethanol (99.5%). The resulted precipitate was removed by centrifugation at 3,000 rpm for 5 min and a 20 μl aliquot of the supernatant was spotted on Whatman 3MM chromatography paper with appropriate carrier. The separation of the substrate from the product was achieved by paper chromatography in n-Butanol : H₂O (43 : 7 containing 5 volume % of ammonia) at room temperature. Inosine was identified under ultraviolet light (254 nm), the spot was cut off and counted in a Packard Tri-cab liquid scintillation spectrometer with 63% efficiency. The formation of inosine was linear with time and protein concentration under these conditions.

Isoelectric focusing

Preparative isoelectric focusing was performed with on LKB model 7,900 unipolar column electrophoresis system according to the method described by Vesterberg and Svensson¹⁸. Blood was collected from patients and erythrocytes were removed by centrifugation. Triton X-100 was added to the serum in a final concentration of 1% and was sonicated at 200 W (Tomy Seiko model UR-200 P) for 30 seconds before isoelectrofocusing. Tissues were homogenized with approximately 10 volumes of 0.25 M sucrose containing 1% Triton X-100 and sonicated at 200 W for 30 seconds. The homogenates were centrifuged at 12,000 g for 20 min and resultant supernatant were subjected to the isoelectric focusing.

RESULTS

Adenosine deaminase activity of human serum was studied to explore its clinical applicability. By the colorimetric determination of ammonia, the assay procedure was found to be relatively simple. Using this method, Fig. 1 was shown the effect of substrate concentration on the rate of an enzyme catalyzed reaction,

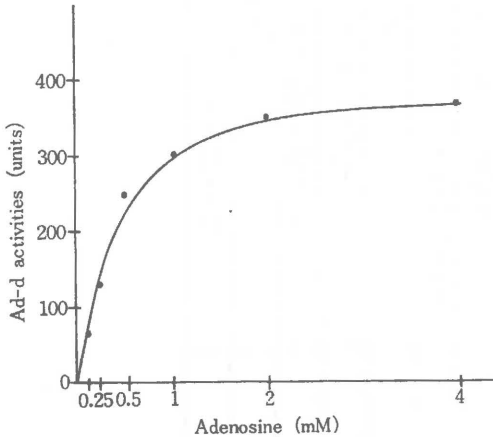


Fig. 1. The effect of substrate concentration of serum adenosine deaminase on the rate of an enzyme catalyzed reaction. Adenosine deaminase was assayed by colorimetric determination of ammonia. 1 unit of this enzyme activity was expressed increment in 1 μ mole of ammonia per hour at 37°C.

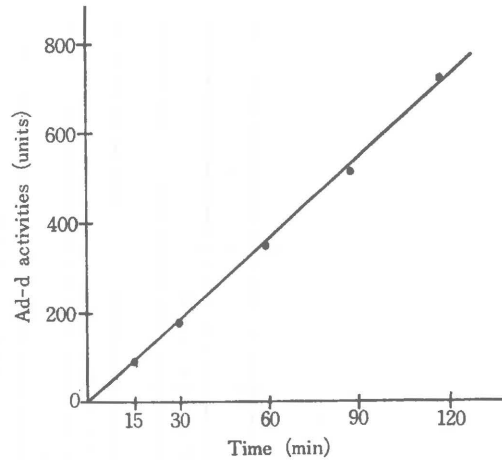


Fig. 2. Time course of the serum adenosine deaminase. Adenosine deaminase was assayed by colorimetric determination of ammonia.

and 4 mM adenosine was finally used on assay as being saturated with the substrate. Fig. 2 was shown time course of the enzyme and was performed at 37°C for 60 min as standardized conditions.

One hundred and twenty normal individuals who were healthy with annual physical examination of hospital, were used as control. Fig. 3 presents the activity of adenosine deaminase obtained for the different diseases. The normal control group showed a relative narrow range with a mean adenosine deaminase activity of

Table 1. Comparison of the serum adenosine deaminase activity from patients with various diseases

	No.	Adenosine deaminase activity (Unit/ml)	P
Control	120	250±64	0.001
Diabetes mellitus	15	514±237	0.001
Hypertension	17	416±133	0.001
Rheumatoid arthritis	8	632±248	0.001
Auto-immune diseases	10	742±261	0.001
Ischemic heart disease	6	353±116	0.001
Renal disease	11	423±206	0.001
Hepatic diseases			
Acute hepatitis	7	1,018±378	0.001
Chronic hepatitis	18	514±129	9.001
Liver cirrhosis	13	649±255	0.001
Ulcus ventriculi	12	255±99	N. S.
Malignant diseases			
of hematopoietic system	11	1,044±555	0.001
the other	8	390±74	0.001

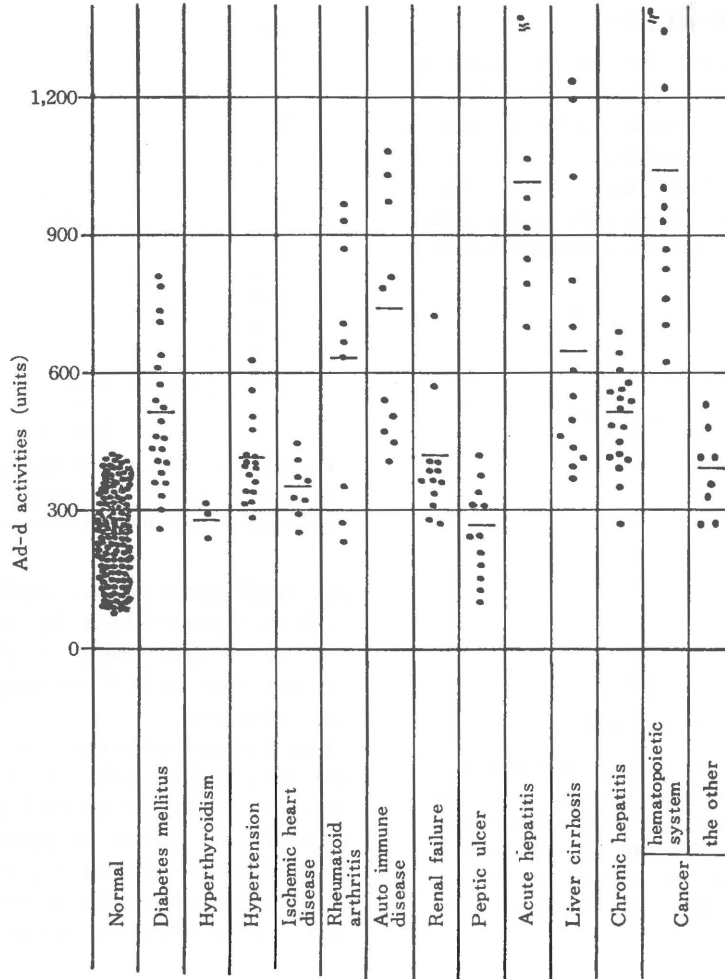


Fig. 3. Serum adenosine deaminase values in subjects with various diseases. Adenosine deaminase was assayed by colorimetric determination of ammonia.

250±64 units/ml (Table 1). Levels of the serum enzyme activity from patients with rheumatoid arthritis, autoimmune disease, hepatitis acta, liver cirrhosis and malignant disease of hematopoietic system higher than that of the normal subjects, and those patients had a mean adenosine deaminase activity of 632±248, 732±261, 1018±378, 647±255 and 1,044±555, respectively as shown in the table 1. The serum levels of diabetes mellitus, hypertesion, ischemic heart disease, renal diseases, chronic hepatitis and malignant diseases except hematopoietic system were slightly higher and a mean value of 514±234, 416±133, 353±116, 514±129 and 390±74, respectively. The serum enzyme activity was found to be normal level in patients

with ventricular peptic ulcer. Then, enzymatic studies were carried out to characterize the adenosine deaminase enhanced in the serum from various diseases.

Heat stability of serum adenosine deaminase in patients with various diseases

Heat stability of adenosine deaminase was examined in the serum of various patients at 55°C. The enzyme activities of serum from subjects with hepatitis, rheumatoid arthritis and multiple myeloma were strikingly inactivated at 55°C. Any difference was not found in heat denaturation of the serum from patients with those diseases (Fig. 4).

Optimum pH of serum adenosine deaminase in patients with various diseases

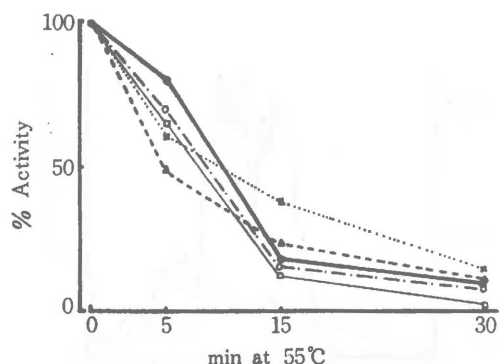


Fig. 4. Heat stability of serum adenosine deaminase in subjects with various diseases. Adenosine deaminase was assayed by colorimetric determination of Ammonia. ●—● Acute hepatitis A, ▲---▲ Acute hepatitis B, ○---○ Rheumatoid arthritis, ×.....× Acute granulocytic leukemia, □—□ Multiple myeloma.

The effect of pH on adenosine deaminase activity of serum from various diseases was shown in Fig. 5. It was found that adenosine deaminase activities in serum from rheumatoid arthritis, malignant lymphoma and hepatitis had the same optimum pH at 6.5.

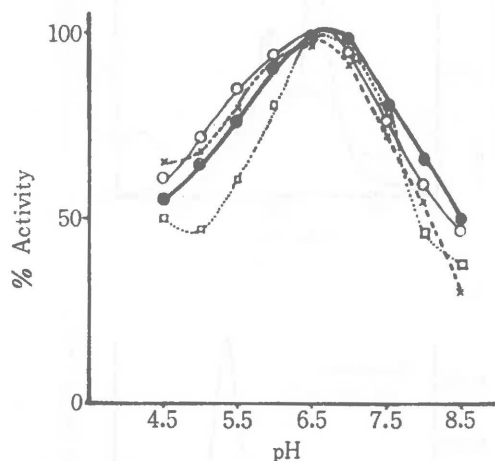


Fig. 5. Optimum pH of serum adenosine deaminase in subjects with various diseases. Adenosine deaminase was assayed by colorimetric determination of ammonia. The phosphate buffer was used in the pH range. ●—● Rheumatoid arthritis, ○—○ Malignant lymphoma, □.....□ Acute hepatitis, × ---- × Chronic hepatitis.

Lineweaver-Burk plot of serum adenosine deaminase in patients with various diseases

Derived kinetic constants of serum adenosine deaminase from hepatitis, liver cirrhosis, leuke-

Table 2. Km and Vmax values of serum adenosine deaminase in patients with various diseases

	Km (mM)	Vmax (units/ml)
Acute granulocytic leukemia	1.75	493
Rheumatoid arthritis	1.75	606
Acute hepatitis	9.10	1,667
Liver cirrhosis	9.10	1,538

mia and rheumatoid arthritis were summarized in Table 2. Apparent Km value of hepatic diseases showed 9.1 mM and Vmax was 1,667 or 1,538 units/ml. On the other hand, apparent km value of leukemia and/or rheumatoid arthritis was 1.75 mM and Vmax was 493 and 606 units/ml, respectively. A distinct difference was found in both Km and Vmax values of the serum enzyme between hepatic diseases and others such as leukemia and rheumatoid arthritis. (Fig. 6)

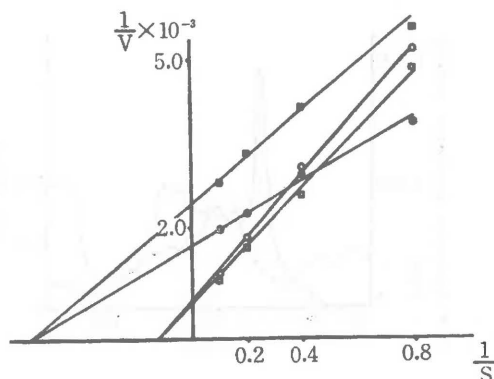


Fig. 6. Lineweaver-Burk plot of serum adenosine deaminase in subjects with various diseases. Adenosine deaminase was assayed by colorimetric determination of ammonia. □—□ Acute hepatitis, ○—○ Liver cirrhosis, ■—■ Acute granulocytic leukemia, ●—● Rheumatoid arthritis.

Preparative isoelectric focusing patterns of serum adenosine deaminase

Isoelectrofocusing over a pH range (3.5 to 10) of serum adenosine deaminase disclosed all the isoenzyme activity between pH 4.0 and 6.0. Thus, isoelectric focusing was carried out using ampholites in the range from pH 4.0 to 6.0.

Eight to nine peaks of adenosine deaminase activity were separated in serum with hepatic disease on isoelectric focusing as shown in Fig. 7. The electrophoretic variants with pI

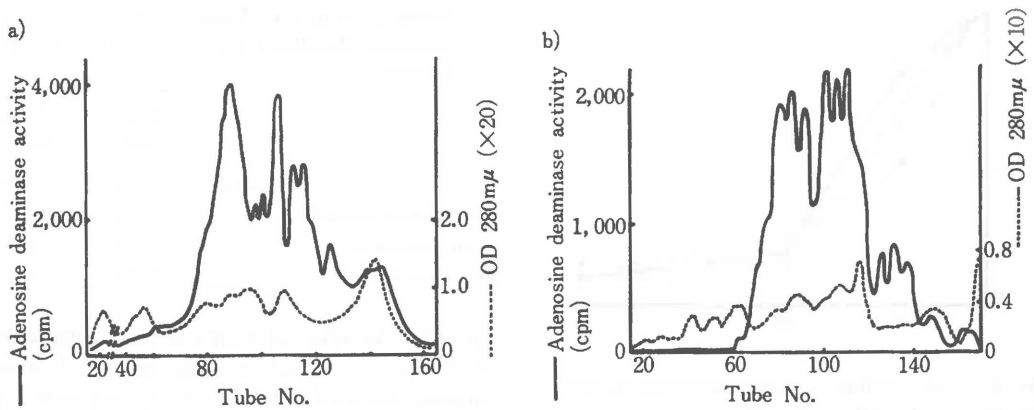


Fig. 7. A preparative isoelectric focusing pattern of serum adenosine deaminase in patients with acute hepatitis and liver cirrhosis. Adenosine deaminase was assayed by the method using $[8-^{14}C]$ labeled adenosine described in "Materials and Methods". Isoelectric focusing over a pH range (4.0 to 6.0) was used. a) Acute hepatitis, b) liver cirrhosis.

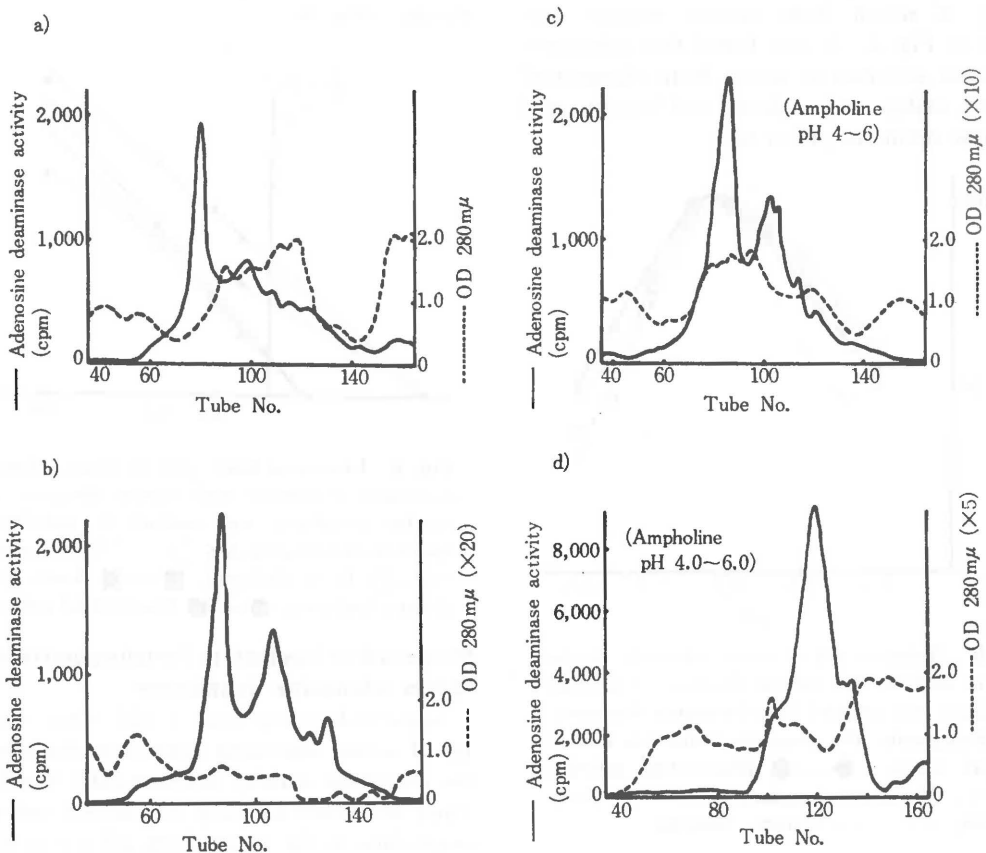


Fig. 8. A preparative isoelectric focusing pattern of serum adenosine deaminase in patients with normal, rheumatoid arthritis, systemic lupus erythematosus and multiple myeloma. The procedures were the same as in Fig. 7. a) normal, b) systemic lupus erythematosus, c) rheumatoid arthritis, d) multiple myeloma.

values were 4.5, 4.7, 4.8, 5.0, 5.1, 5.5, 5.6 and 5.8. On the other hand, two peaks of the activity were found sera from normal, systemic lupus erythematosus, myeloma and rheumatoid arthritis (Fig. 8), and their electrophoretic variants with pI values were 4.9 and 5.1. The isoelectric focusing pattern of the enzyme from human liver tissue demonstrated eight peaks and the pattern was almost identical with that of the serum from hepatic disease (Fig. 9).

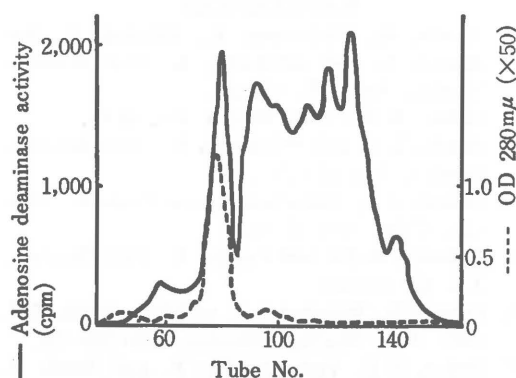


Fig. 9. A preparative isoelectric focusing pattern of adenosine deaminase of human liver tissue from patient of hepatoma. The enzyme solution was prepared from human liver from patient of hepatoma as described in "Materials and Methods". Electrophoresis was carried out as described Fig. 7.

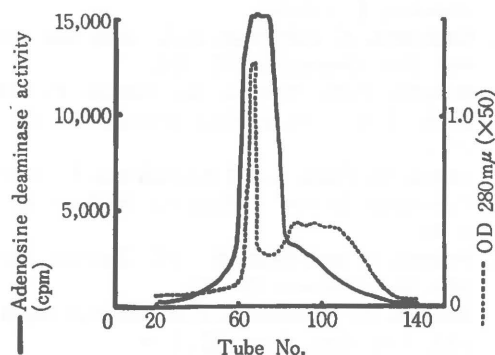


Fig. 10. Isoelectric focusing pattern of adenosine deaminase of human lymphnode from patient of malignant lymphoma. The enzyme solution was prepared from human lymphnode as described in "Materials and Methods". Electrophoresis was carried out as described in Fig. 7.

In contract, isoelectric focusing pattern of the enzyme from human lymphnode tissue was the same as in the serum enzyme from systemic

lupus erythematosus and rheumatoid arthritis (Fig. 10).

DISCUSSION

The adenosine deaminase has been investigated in human sera from different diseases. Koehler and Benz have demonstrated that the mean levels of serum adenosine deaminase from patients with liver cancer were higher than that of normal control subjects²¹. In 1957, Straub et al.¹⁷ reported that higher correlation existed between the "Carcinogenesis" and an elevated adenosine deaminase activity in blood plasma. However, the procedure of assay with Nessler's reagent as proposed by Straub et al.¹⁷ was found to be relatively robust. Spectrophotometric assays at 293 nm⁹ and at 265 nm¹⁰, assays based on radioactive substrate⁴ and the pH stat measurement technique are not available for routine clinical diagnosis. The method for this was presented in the measurement of ammonia liberated by the actions of deaminase with a direct colorimetric determination of ammonia. After the incubation, the reaction mixture was deproteinized by sodium tungstate and sulfuric acid and the resultant clear supernatant was reacted with indophenol reagents. In this assay procedure, the reaction of indophenol with uric acid and amino acids was specifically reduced under standard conditions. Moreover, the described technique with direct colorimetric determination of ammonia was found to be simple and capable of producing a precise data for routine clinical application as compared with the conventional methods.

The levels of adenosine deaminase activities were examined in serum from various diseases. It was found that the enhanced enzyme activities were revealed in a groups of patients with hepatic disease, auto-immune disease, rheumatoid arthritis and malignant disease of hematopoietic system. The groups of patients showed elevated serum adenosine deaminase activities were separated into two classes. One was a group of patients with liver diseases and the other was lymphnode system. The authors suggested that it was important to clarify the tissue of the elevated serum adenosine deaminase for clinical use. Then, experiments were designed to clarify the organ of the elevated serum enzyme.

The heat stability and the optimum pH were

found to be essentially identical in sera from hepatic disease and the other, such as auto-immune disease, rheumatoid arthritis and cancer of hematopoietic system. While apparent K_m value for adenosine and V_{max} showed the striking difference between sera from patients with hepatic disease and the other non hepatic diseases. Organ specificity is demonstrable for the different electrophoretic pattern from various tissues²⁰. According to the method by Weyden and Kelley²⁰, an isoelectric focusing of the large molecule species of adenosine deaminase obtained by gel filtration of liver tissue extract was revealed five to six different isoenzyme forms. And small molecule species adenosine deaminase showed several electrophoretic variants by an isoelectro focusing.

In the present case, isoelectric focusing of all species of this isoenzyme from liver tissue was revealed eight to nine different electrophoretic forms. However, the isoelectric focusing pattern of the enzyme originated in lymphnode was found at only one peak distinctly differed from liver tissue. Then, to clarify the origin of serum adenosine deaminase elevated in various diseases, some investigators were tried to analyze this serum enzymes on an isoelectric focusing. In serum, isoelectric focusing pattern of adenosine deaminase showed a remarkable difference between hepatic injury and disease of lymphnode system. The isoelectric focusing pattern of serum adenosine deaminase from hepatic injury was found eight to nine peaks and was similar to that observed with liver tissue. On the other hand, the isoelectric focusing pattern of serum enzyme from disease of lymphnode system was found just two peaks. And in case of rheumatoid arthritis, auto-immune disease and normal subjects, 4.9 of pI value was lower than 5.1 in its serum enzyme, however, in case of multiple myeloma was reversed. The electrophoretic pattern of serum adenosine deaminase from rheumatoid arthritis and systemic lupus erythematosus were identical to that observed with lymphnode tissue with 4.9 of pI.

The isoelectrophoretic pattern of serum adenosine deaminase from hepatic injury was similar to that observed with normal tissue, suggesting that serum enzyme was originated from liver tissue. On the other hand, the electrophoretic patterns of the serum enzyme from rheumatoid

arthritis and systemic lupus erythematosus were quite similar to that observed with lymphnode tissue.

It seemed that the serum enzyme in those diseases might be originated from lymphnode tissue. The results in the present investigation indicate that the estimation of serum adenosine deaminase activity and an analyse of the isoenzyme pattern on an isoelectric focusing are very useful for clinical diagnosis.

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