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Abstract

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**MACROMOLECULAR LACTATE DEHYDROGENASE
LINKED TO SERUM IgG OF A PATIENT
WITH LIVER CIRRHOSIS**

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Abstract. An anomalous zymogram of lactate dehydrogenase (LDH) in the serum from a patient with liver cirrhosis was reported. Agar-gel electrophoresis of serum showed an extra LDH band close to the anodic side of LDH5 and a wide band of LDH5. Gel filtration of patient's serum in Sephadex G-200 demonstrated an abnormal LDH fraction eluted between immunoglobulin G (IgG) and macroglobulin in addition to a normal LDH component. Chromatographically abnormal LDH was demonstrated on agar gel as extra and wide LDH5 bands and resembled closely human hepatic LDH in various physico-chemical properties such as inhibition by urea or substrate, stability against heat, and Michaelis-Menten's constant. Immunological analyses demonstrated that abnormal LDH could be in the state combined with IgG. Molecular weight of the complex estimated by gel filtration was approximately 300,000. Mixtures of the heated patient's serum with normal or patient's hepatic LDH showed abnormal LDH fraction by gel filtration, whereas abnormal fraction was not demonstrated when heated normal serum was mixed with normal or the patient's hepatic LDH. These results strongly suggest that the occurrence of anomalous LDH zymogram in patient's serum is due to a formation of LDH-IgG complex, which is based on the binding of essentially normal hepatic LDH and abnormal IgG.

Since the concept of isoenzyme was proposed by Markert and Møller (1) in 1959, a number of papers on LDH isoenzyme have been reported. For the past several years, the presence of anomalous LDH isoenzymes with abnormal electrophoretic mobility has been noticed by some authors (2-13). They presumed that these abnormalities were due to a linkage of LDH isoenzyme to a serum component.

This communication is concerned with the anomalous serum LDH isoenzyme pattern of a patient with liver cirrhosis and with possible cause of the abnormality in his serum.

MATERIALS AND METHODS

Case: The patient examined was a 37-year-old man with edema of the legs and an enlargement of the liver. He had repeatedly eruption on the upper half of his body and was diagnosed autosensitization dermatitis. His serum protein was 5.2 g/dl. Electrophoretic analysis showed albumin 55%, α_1 -globulin 3%, α_2 -globulin 7%, β -globulin 10%, and γ -globulin 25%. Serum immunoglobulins were IgG 1,800 mg/dl, IgA 440 mg/dl, and IgM 185 mg/dl. The total serum bilirubin was 1.61 mg/dl. Thymol turbidity, and zinc turbidity tests were 6 units and 10 Kunkel units, respectively. RA-test for rheumatoid factor was always positive. Serum LDH was 450 units. Serum cholinesterase and alkaline phosphatase were 0.6 Δ pH and 3.5 Bessey units, respectively. Subsequent serial determination of serum GOT, GPT, and LDH activities gave slightly increased values. The patient went downhill gradually, and he expired due to gastrointestinal bleeding. Histologically, the liver obtained at autopsy showed cirrhosis of the postnecrotic type.

Blood samples in the fasting state were obtained from the patient and from healthy subjects as a control. The liver removed from the patient immediately after death was minced and washed with cold saline several times to eliminate the blood. The tissue was then homogenized using a glass homogenizer with a teflon pestle in 0.1 M Tris-HCl buffer solution (pH 8.0) and centrifuged at 20,000 g for 40 min. The supernatant fluid was used for further studies. The normal human liver was also treated as described above.

LDH activity was measured by means of a modified technique of Wróblewski (14). The reaction mixture in a cuvette contained 2.5 ml 1/15 M Sørensen's phosphate buffer (pH 8.0), 1.0 ml enzyme solution, 0.2 ml 2.8 mM NADH (Sigma Chem. Co., U.S.A.), and 0.3 ml 36.4 mM sodium pyruvate (Ishizu Pharmaceutical Co., Japan). The enzymatic reaction was performed at 37°C. A unit of the enzyme activity was defined as a decrease in optical density at 340 m μ of 0.001 per min per ml of the specimen.

Agar-gel electrophoresis was carried out at 150 volts for 30 min in an agar gel according to a modified technique which was originally described by Wieme (15). The gel was prepared by dissolving 0.75 g of agar (Special Agar Noble, Difco Laboratories, U.S.A.) into 100 ml veronal buffer of pH 8.6 and of ionic strength 0.05. After the electrophoretic separation the gel was stained for LDH by placing it in a solution containing 1 mg p-nitroblue tetrazolium (Sigma Chem. Co.), 2 mg phenazine methosulfate (Sigma Chem. Co.) and 4 mg β -NAD (Sigma Chem. Co.) which were dissolved in a mixture of 10 ml 0.05 M pyrophosphate buffer and 2 ml 0.5 M sodium lactate (Sigma Chem. Co.), and then incubated at 37°C for 1-2 hr.

Gel filtration was performed on a column (2.5 \times 35 cm) of Sephadex G-200 (Pharmacia Fine Chem., Sweden) to separate serum constituents according to molecular size and shape. Serum applied to the Sephadex G-200 was eluted with 0.1 M Tris-HCl buffer solution (pH 8.0) at the flow rate of about 8 ml/hr. The eluate was collected continuously in 4 ml aliquots. Gel filtration on Biogel P-300 (Bio. Rad Laboratories, U.S.A.) was performed to estimate the molecular

weight of the LDH isoenzymes. The technique used was essentially the same as that described above. Cytochrome C (Sigma Chem. Co.), ovalbumin (Sigma Chem. Co.) and bovine serum albumin (Sigma Chem. Co.) were used as standards with different molecular weights.

Immunoelectrophoresis was carried out at 150 volts for 80 min in an agar gel containing 1.2g agar and 0.1g sodium azide in 100ml 0.1 M Tris-HCl buffer (pH 8.6, $\mu=0.05$). After the electrophoretic separation, monospecific antisera to IgG, IgA and IgM obtained from horse were applied in troughs and allowed to stand at 4°C until precipitation lines appeared.

RESULTS

Electrophoretic distribution of the patient's serum LDH

The zymogram was characterized by a well-defined extra band close to the anodic side of LDH5 and a broad band of LDH5 (Fig. 1).

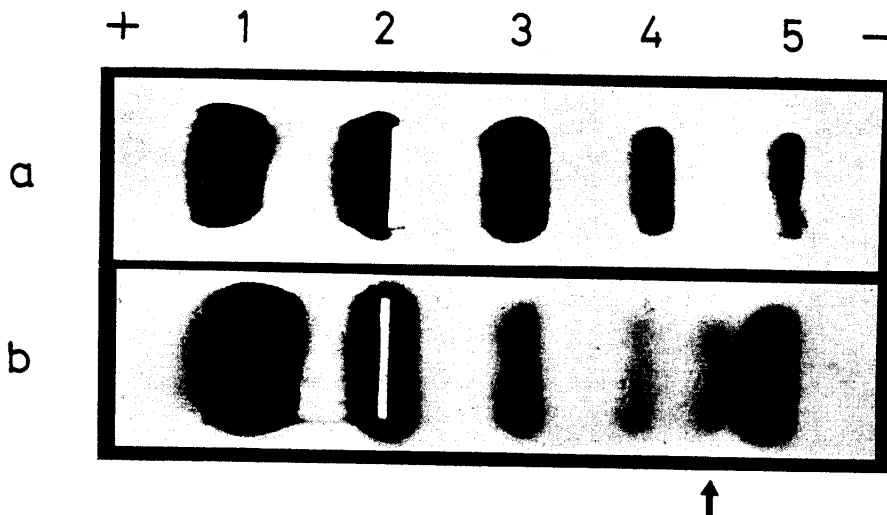


Fig. 1. Electrophoretic patterns of serum LDH in a normal subject (a) and the patient (b). Ten μ l of serum was applied in a trough of agar-gel plate. The total serum LDH activity of the patient was 450 units. The arrow shows an extra LDH band between LDH4 and LDH5.

Chromatographic distribution of the patient's serum LDH

In normal human serum the LDH fraction separated by Sephadex G-200 appeared as a single component between albumin and IgG, whereas in the patient's serum an unusual extra component of LDH was found between IgG and macroglobulin in addition to the normal LDH fraction (Fig. 2). This normal component was designated Type-I for the sake of identification. The larger type of LDH, that was eluted between IgG and macroglobulin, was designated Type-II.

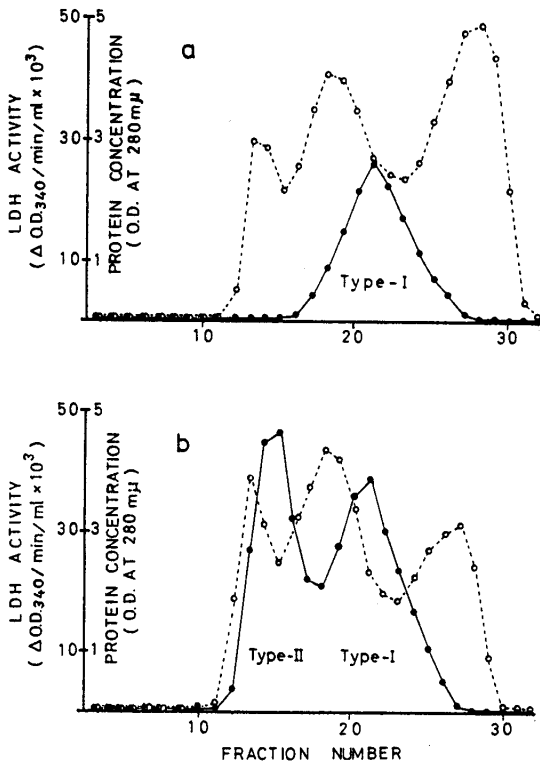


Fig. 2. Chromatographic patterns of serum LDH in a normal subject (a) and the patient (b) in relation to serum proteins. An anomalous chromatographic LDH fraction is demonstrated as Type-II. LDH is indicated by the solid line and proteins by the broken lines.

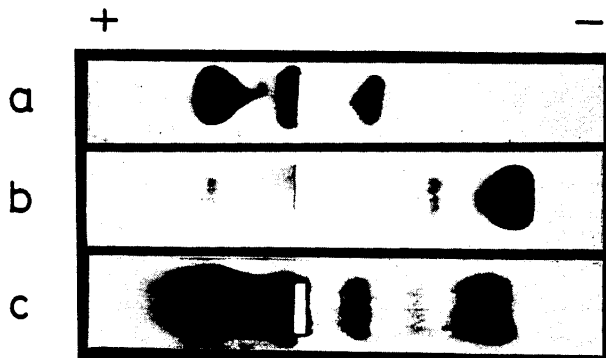


Fig. 3. Electrophoretic patterns of Type-I (a) and Type-II (b) fractions as compared with a zymogram of the patient's serum LDH (c). The abnormal band was resulted from Type-II fraction, but not from Type-I.

By electrophoresis of the Type-I three bands of LDH, i.e., LDH₁, LDH₂, and LDH₃, were disclosed. When the Type-II was separated electrophoretically the major portion of the LDH activity was demonstrated as a wide band of LDH₅ and a small amount of the activity was found in other electrophoretic fractions (Fig. 3).

After heating normal and the patient's sera at 50°C for 155 min, each of sera was fractionated by gel filtration. The large portion of the Type-I activity was retained, whereas the activity of the Type-II was almost completely lost. Addition of the heated normal serum to the LDH, which was isolated from the liver of normal human or the patient and was previously proved to exhibit no activity in the Type-II zone, did not cause virtually any changes in the sense of the chromatographic distribution of LDH. In contrast to this, the gel fractions of the mixtures of the heated patient's serum with the normal or the patient's hepatic LDH showed the occurrence of the LDH activity in the Type-II zone (Fig. 4).

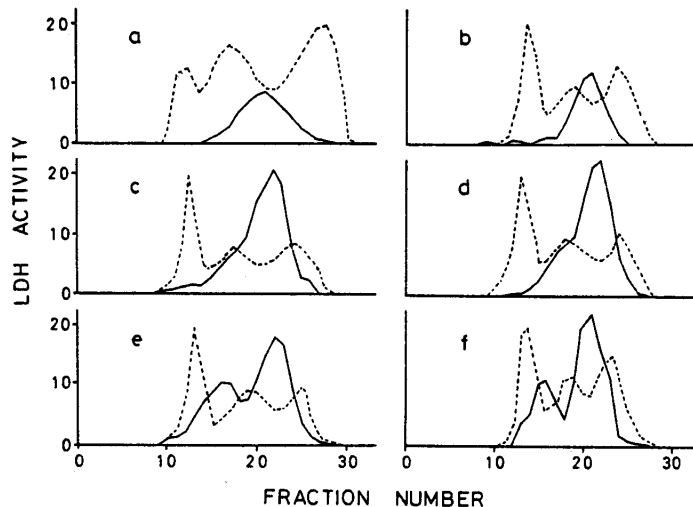


Fig. 4. Chromatographic patterns of LDH in mixtures of normal or heated patient serum with normal or patient's hepatic LDH. LDH is indicated by the solid, and proteins by the broken lines. a, heated normal serum; b, heated patient's serum; c, heated normal serum + normal hepatic LDH; d, heated normal serum + patient's hepatic LDH; e, heated patient's serum + normal hepatic LDH; and f, heated patient's serum + patient's hepatic LDH.

Differences of physicochemical properties between Type-I and Type-II

Although the Type-I was quite stable at 50°C, the Type-II was inactivated progressively as shown in Fig. 5.

The LDH activity of the Type-I was maximum at the concentration of 1.4 mM of pyruvate, but was inhibited strongly by the excessive amounts of pyruvate. The activity of the Type-II was highest at the concentration of 2.7 mM of pyruvate, and did not change significantly beyond this concentration (Fig. 6).

The apparent Michaelis-Menten constants for pyruvate concentration were

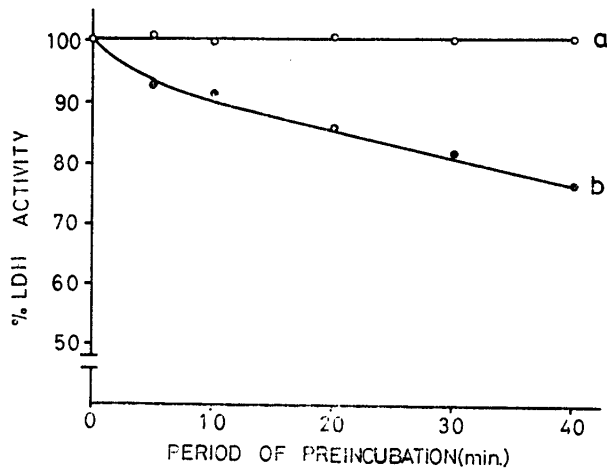


Fig. 5. Heat inactivation of Type-I (a) and Type-II (b). The LDH activities of both types were measured before and after heating for 40 min at 50°C. The activity after heating is expressed as per cent of the original activity.

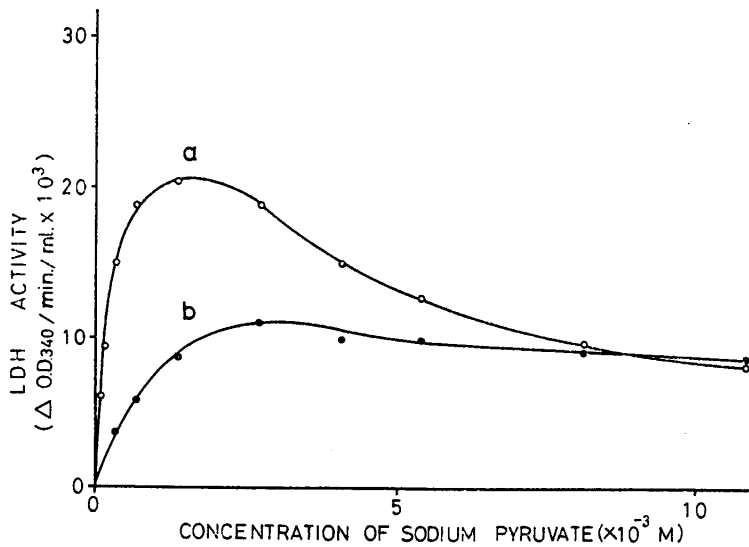


Fig. 6. Effects of substrate concentration on Type-I (a) and Type-II (b) activities. The concentration on the abscissa indicates the final concentration of the substrate ranging from 0.087 mM to 10.87 mM in the reaction mixture.

0.32 mM for Type-I and 1.14 mM for Type-II (Fig. 7).

The effect of urea on LDH activity was studied by adding the various amounts of urea to the reaction mixtures. When the ratios of the untreated

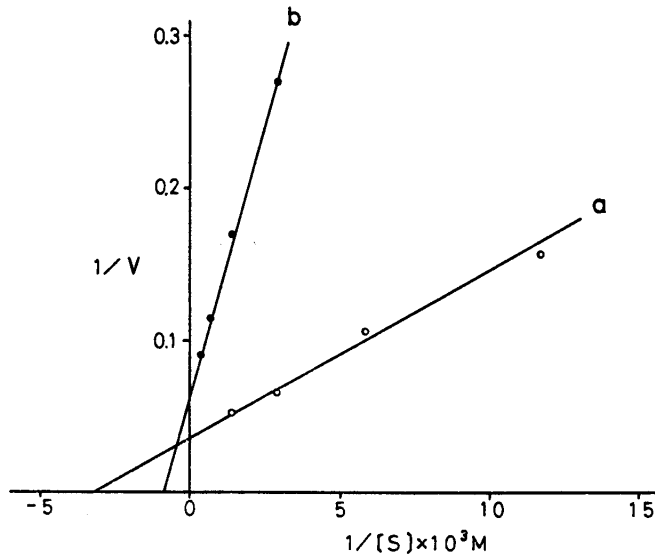


Fig. 7. Determination of the K_m (pyruvate) values for Type-I (a) and Type-II (b). The reciprocal of initial velocity ($1/V$) was plotted against the reciprocal of substrate concentration ($1/S$) according to the Lineweaver-Burk's method.

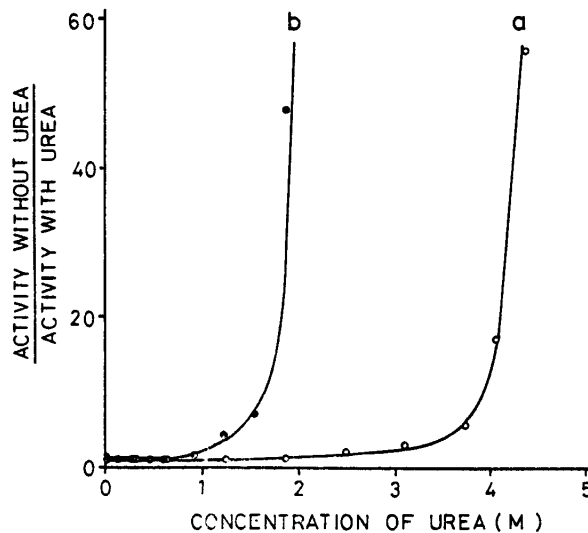


Fig. 8. Inhibition of LDH activities of Type-I (a) and Type-II (b) by urea. The urea concentration on the abscissa indicates the final reaction mixture concentration, ranging from 0.156 M to 4.375 M.

activities against the activities with urea were plotted against urea concentrations, the ratios of the Type-I did not show any significant changes until a

critical concentration of 2.5 M-urea, but beyond this point a sharp inflection of the curve occurred, indicating eventually complete inhibition by urea. However, when the Type-II was examined in a similar manner, the inflection took place at a much lower concentration of 0.9 M-urea (Fig. 8). The physicochemical properties described above were summarized in Table 1 as compared with those of the LDH isolated from human liver and heart in our laboratory.

The molecular weight of the Type-II estimated by the technique of gel filtration using Biogel P-300 was approximately 300,000 as shown in Fig. 9.

TABLE 1. PHYSICOCHEMICAL PROPERTIES OF TYPE-I AND TYPE-II AS COMPARED WITH THOSE OF LDH ISOLATED FROM HUMAN LIVER AND HEART.

	Type-I	Type-II	LDH isolated from liver	LDH isolated from heart
Heat stability (50°C, 40 min)	100%*	77%*	30%*	99%*
Optimal pyruvate concentration	1.4 mM	2.7 mM	2.7 mM	1.4 mM
Km pyruvate	0.32 mM	1.14 mM	1.11 mM	0.33 mM
Urea inhibition (minimum concentration of urea)	2.5 M	0.9 M	0.9 M	2.5 M

* Activity (%) retained after heating

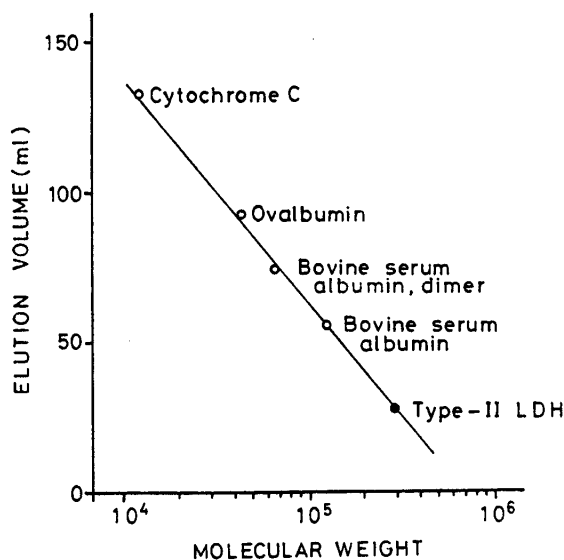


Fig. 9. Determination of the Type-II molecular weight by gel filtration. The standardization curve was drawn by plotting elution volumes of the standard substances against their molecular weights.

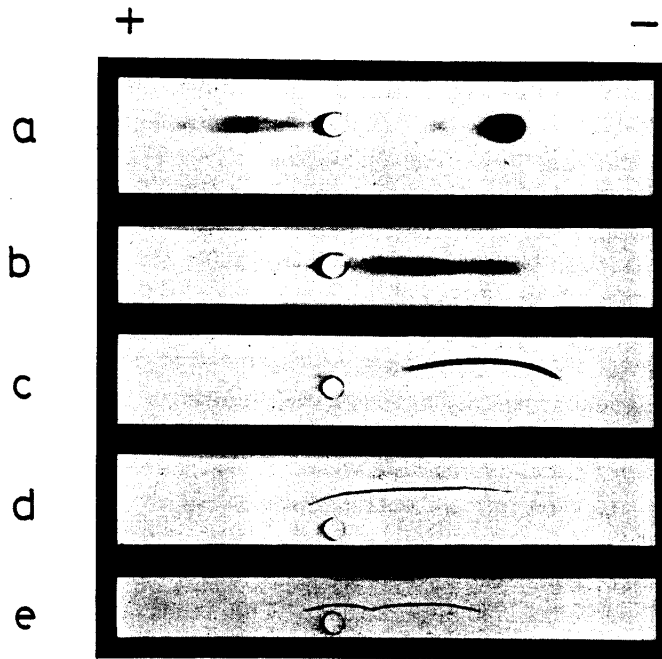


Fig. 10. Immunoelectrophoretic patterns of Type-II fraction. Note the similarity in the electrophoretic mobilities between LDH fraction and the precipitation line with anti-IgG. a, LDH staining; b, protein staining; c, precipitation lines with anti-IgG; d, anti-IgA; and e, anti-IgM.

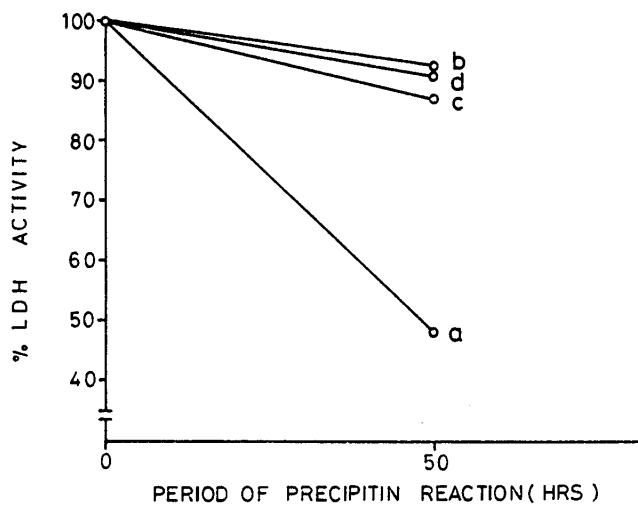


Fig. 11. Alterations of LDH activities in supernatant fluids of mixtures of Type-II with anti-IgG (a), anti-IgA (b), anti-IgM (c) and 0.9% NaCl as control (d).

Immunological properties of Type-II

The protein fraction with almost the same electrophoretic mobility as that of LDH5 formed a distinct precipitation line with anti-IgG, whereas the arc lines with anti-IgA and anti-IgM displaced somewhat anodally from the location of the protein (Fig. 10). The interaction between Type-II and IgG was also studied quantitatively. After aliquots of the Type-II fraction were mixed separately with appropriate amounts of anti-IgG, anti-IgA, and anti-IgM and were allowed to stand for 50 hr at 3°C, the mixtures were centrifuged at 1,400 g for 10 min. The LDH activity in the supernatant fluid of the mixture treated with anti-IgG decreased about 52% of the original activity. However, the activity in the supernatant fluid treated with anti-IgA or anti-IgM did not show any significant changes in comparison with the control mixture containing 0.9% NaCl solution instead of the anti-immunoglobulins (Fig. 11).

DISCUSSION

Lactate dehydrogenase, a tetramer composed of two different types of subunit, possesses about 135,000 of molecular weight and has been known to be essentially free form in the circulation. The isoenzymes of human serum LDH are able to separate usually into five components by agar-gel electrophoresis. They are designated generally LDH1, LDH2, LDH3, LDH4, and LDH5 from anode to cathode in accordance with their electrophoretic mobilities. However, since Kreutzer and his co-workers (2) reported 5 cases with anomalous LDH zymograms, various types of unusual LDH isoenzyme patterns have been found.

The electrophoretic pattern of the patient with liver cirrhosis in our paper showed an extra band between LDH4 and LDH5 associating with a somewhat wide LDH5 band. This pattern was closely similar to that of the patient with diabetes mellitus reported by Kreutzer *et al.* As a feasible explanation for their electrophoretic irregularity, they supposed the existence of a relatively thermostable factor in the serum.

In recent years several papers have been reported on the cases with abnormal serum LDH patterns, indicating that these abnormalities were due to the binding of LDH with serum IgG or IgA. Biewenga and Feltkamp (13) analyzed sera of 15 patients containing complexes of LDH and IgG, and suggested that the IgG binding to LDH was consisted mostly of IgG3 subclass and that its special structure of the hinge region was an important factor in the formation of the complex. In our case the Type-II possessed higher molecular weight than the normal Type-I and the Type-II behaved on agar gel as the extra and LDH5 bands. These data indicated that the electrophoretic abnormality in this patient's serum could be related to the formation of a macromolecular

LDH. Immunological and chromatographical studies led to the conclusion that the Type-II was in the state combined with IgG in the blood, so that the molecular weight of the complex attained to about 300,000. The binding site of the LDH molecule to IgG is likely to be independent of the active center of the enzyme, since the complex still possesses the enzymatic activity.

Kinetically the macromolecular LDH behaved in a remarkably similar manner to the LDH isolated from human liver in regard to the optimal substrate concentration, the value of Michaelis constant, and the stability against urea. Only the heat stability of the Type-II was somewhat resistant in comparison with that of LDH from human liver. This could be interpreted by a protective effect of the combining immunoglobulin from heat.

The mechanism responsible for the linkage of the LDH to IgG has not been clear, so far. The abnormality is unlikely to be of hereditary nature because of the absence of any demonstrable LDH abnormality in the patient's relatives. The mixtures of the heated patient's serum with the LDH, isolated from the liver of a normal subject or the patient, caused an occurrence of the Type-II fraction, whereas this fraction was not demonstrated from the mixtures of the heated normal serum with the normal or patient's hepatic material. This result suggested that the formation of the LDH-IgG complex could be due to the existence of an abnormal patient's IgG and not due to an abnormality of his hepatic LDH.

Clinical significance of complexes of LDH and immunoglobulin has never been known. Lubrano *et al* (9) reported a total of 76 patients with severe liver diseases, including 42 cases with an extra LDH band (band "T") between LDH4 and LDH5; out of these 42, 21 cases were proved to be liver cirrhosis. And they pointed out that these patients with band "T" generally showed a very poor prognosis. However, they did not investigate the matter of the complex. Biewenga *et al* (13) divided 15 patients with LDH-IgG complex into 3 groups on the basis of differences in the anomalous LDH isoenzyme pattern. The first group was characterized by an anomalous LDH band in the γ -globulin region. The second group showed an anomalous LDH band with β - γ -mobility and the third group demonstrated an extra band between LDH4 and LDH5 whose pattern was similar to the pattern in our case. According to their study, the patients with autoimmune states, such as systemic lupus erythematosus, rheumatoid arthritis and active chronic hepatitis, were included in group I and II, and none of these patients was found in group III. On the contrary, the serum LDH pattern of our case belonged to group III. Nevertheless he exhibited clinical and laboratory signs suggesting the autoimmune state by recurrent autosensitization dermatitis, increased serum immunoglobulins, and the presence of rheumatoid factor in the serum. Therefore, it can be said that

there possibly exists a relationship between the occurrence of the LDH-IgG complexes and an autoimmune disease, although the production of an extra band between LDH4 and LDH5 is not closely related to an autoimmune disease.

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