The Electrophoretically Distinct Forms of Mammalian Lactic Dehydrogenase

I. DISTRIBUTION OF LACTIC DEHYDROGENASES IN RABBIT AND HUMAN TISSUES*

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In a preliminary note (1) it was reported that at least two serologically and electrophoretically distinct lactic dehydrogenases were present in rabbit tissues. Nisselbaum and Bodansky (2) recently reported similar serological differentiation of lactic dehydrogenases from various rabbit organs. Heterogeneity of this enzyme derived from different tissues of mammalian species has also been shown by the use of diphosphopyridine nucleotide analogs (3). The lactic dehydrogenases of human sera and various animal tissues have been separated by elution from a column of Hyflo Super Cel with decreasing concentrations of ammonium sulfate (4) and electrophoretically (4–14) into several distinct components. Several other enzymes have also been shown to exist in multiple forms, not only within a single organism but even within a single tissue (13). Markert and Mösler (13) suggested that these distinguishable molecular types of enzymes be called isozymes, a terminology we shall follow.

In the present investigation the cross reactions of lactic dehydrogenases derived from various rabbit and bovine tissues with antibodies produced against the lactic dehydrogenases of rabbit skeletal muscle and bovine heart muscle were studied. Starch gel electrophoresis was used to separate the lactic dehydrogenase isozymes of rabbit and human tissues. It will be shown that each tissue has a characteristic distribution of these isozymes and that the isozyme patterns of rabbit and human tissues have many similarities.

EXPERIMENTAL PROCEDURE

Lactic Dehydrogenase Assay—Activity was determined by measuring the rate of optical density decrease at a wave length of 340 mu resulting from the oxidation of DPNH in the presence of 0.00084 m sodium pyruvate and a suitable dilution of enzyme (15). All measurements were made with a Beckman DU spectrophotometer at pH 7.4 and 30°. One unit of LD was defined as the amount of enzyme required to produce a decrease in optical density of 0.001 per minute under the above conditions.

Antilactic Dehydrogenase Production—Antibody inhibitive to commercially purified rabbit muscle LD (Nutritional Biochemicals Corporation) was produced in chickens and purified by fractional precipitation with ammonium sulfate as previously described (16). Antibody was similarly prepared against commercially purified bovine heart muscle LD (Worthington Biochemical Corporation). The degree of inhibition of LD by anti-LD was expressed as the percentage inhibition of 40 ± 4 units of LD per cuvette when two cuvettes were simultaneously prepared with and without anti-LD and correction applied for the trace of LD activity in the anti-LD preparation (16). One unit of anti-LD was defined as the amount required to inhibit 50% of the activity of the homologous LD under these test conditions.

Tissue Extracts—All rabbit tissues employed for electrophoresis studies were taken from rabbits killed by exsanguination. These included a transplantable carcinoma, Vx-2, originally developed by Dr. Peyton Rous and in its 135th and 136th transplant generations. Tissue extracts were prepared by grinding tissues, within 1 hour of the death of the animal, in barbital buffer, ionic strength = 0.05, pH 8.6, with a motor driven Potter-Elvehjem, Teflon pestle, tissue grinder. The extracts were clarified by centrifugation. Erythrocytes and plasma were separated from heparinized blood by centrifugation; the erythrocytes washed and lysed by treatment for 30 seconds in a Raytheon sonic oscillator. Human tissues were obtained from two autopsies performed 12 and 14 hours after death from cancer and nephritis, respectively. These tissues were immediately frozen and kept frozen until extracted as described for the rabbit tissues. Human plasma and erythrocytes were separated from heparinized blood of normal subjects.

Nitrogen content of the tissue extracts was determined by a micro-Kjeldahl procedure (17). Corrections were made for the nitrogen content of the barbital buffer, which was small in relation to the total amounts of nitrogen in the extracts.

Electrophoresis—Zone electrophoresis, with the use of a gel prepared from specially hydrolyzed starch and barbital buffer (ionic strength = 0.05, pH 8.6), was used. Most separations were made with the gel in a horizontal position and the crude extracts applied at the bottom of the gel. The electrophoresis was continued until the migration of a reference marker (bovine serum albumin) was complete. Some separations were made with the gel in a vertical position (18). The electrophoresis was performed at 400 volts at 4°C. The horizontal gel was then stained for protein (19) and the vertical gel was stained for lactic dehydrogenase (20) with the aid of an ultraviolet transilluminator.

The abbreviations used are: LD, lactic dehydrogenase; anti-LD, antibody inhibitive to lactic dehydrogenase; LD₁–LD₇, slowest to fastest electrophoretically migrating forms of lactic dehydrogenase, respectively; RₐHₑ, distance of electrophoretic migration relative to the migration front of bovine hemoglobin.
extracts inserted into a slit in the gel with a supporting medium of starch granules (18). Vertical starch gel electrophoresis as described recently by Smithies (19), in which no supporting medium was used at the origin, was used in some separations with similar results, but proved to be less satisfactory with large samples.

A few milligrams of twice crystallized bovine hemoglobin (Nutritional Biochemicals Corporation) were mixed with each sample. The LD content of this preparation was negligible. This colored protein provided a visible indicator of the progress and sharpness of the electrophoretic migration. It migrated approximately one-half the distance of the rabbit and human serum albumins and served as a convenient control protein for comparing different separations.

Most separations were made in gels 17 x 21 x 0.8 cm. The gel was connected to bridge solutions of barbital buffer, ion strength \(= 0.1\), pH 8.6, by wads of filter paper saturated with the bridge buffer. Melted white petrolatum at approximately 50° was layered over the gel to prevent evaporation. Electric power was supplied by a Reco D.C. power unit of 750 volts capacity through carbon electrodes to the bridge solutions. A voltage drop across the gel of 3 to 4 volts per cm was used. All separations were made in a refrigerated room at 4°.

About after 18 hours' electrophoresis the power supply was disconnected and the petrolatum removed from the surface of the gel. One strip of gel was cut in the direction of the migration and the distance from the origin to the front of the bovine hemoglobin measured along this cut surface. The remainder of the gel was cut into 0.3-cm strips at right angles to the direction of migration. These strips were placed into numbered test tubes and frozen. The LD isozymes appeared to be stable for several days while frozen in the gel. The gels were thawed as soon as convenient and macerated in an approximately equal volume of a solution of 1.0 mg of \(\alpha\)-amylase per ml of 0.067 M phosphate buffer, pH 7.0, which was also 10% saturated with ammonium sulfate. This high salt concentration was used, since the slowest migrating rabbit LD isozyme was relatively unstable in dilute salt solutions. After 30 minutes' incubation at room temperature the residual starch was removed by centrifugation. The LD activity of the clear supernatant fluid in each tube was determined as described above.

Recovery tests on the LD's in a mixture of rabbit liver and heart extracts were performed with the use of the vertical electrophoresis procedure, in which the volume of extract added to the slit was accurately measured. Enzymes recovered in the supernatant fluids after the action of \(\alpha\)-amylase on the starch strips accounted for 55% or more of the total enzyme added. When the enzyme remaining in the starch residue was included, total recovery exceeded 85%. It was concluded, therefore, that the procedures used resulted in an approximately correct representation of the relative proportion of LD isozymes in the various extracts.

RESULTS

The percentage inhibition of the lactic dehydrogenases derived from various rabbit and bovine sources by antilactic dehydrogenases revealed both species and tissue differences in serological specificity of these enzymes (Table I). Antibody produced against rabbit skeletal muscle LD, while strongly inhibitive to its homologous enzyme at a concentration of 2.5 units per cuvette (76% inhibition), caused only a trace of inhibition of the LD from rabbit erythrocytes and leukocytes. The rabbit plasma LD was inhibited to an intermediate degree, as was the LD in an extract from the transplantable Vx-2 tumor. The fact that the LD in a crude extract of rabbit skeletal muscle was inhibited as strongly as was the purified muscle LD showed that the presence of other proteins in the crude extracts was not responsible for the lower inhibition levels observed with the LD from some sources. None of the LD's from bovine sources was markedly inhibited by 2.5 units of this anti-LD preparation per cuvette. In fact, purified bovine heart muscle showed slightly increased activity in the presence of this anti-LD γ-globulin.

The antiovine LD preparation similarly demonstrated some, but considerably less, species specificity of LD since equal concentrations inhibited the LD from rabbit sources less than the LD from bovine sources, although the former enzyme was inhibited to an appreciable extent. This anti-LD did not appear to show tissue specificity of LD's except that a lower per cent inhibition was obtained with the LD from rabbit plasma than from other rabbit sources. It had previously been shown that when this enzyme was combined with its coenzyme (DPNH) before the addition of anti-LD, the equilibrium of LD and anti-LD was greatly delayed (16). In the possibility that sufficient coenzyme may have been present in the plasma to lower the percentage inhibition obtained in these tests the determinations were repeated with plasma dialyzed against 1.8% NaCl. This dialysis increased the inhibition level to approximately 30%, suggesting that coenzyme protection of the LD was responsible for the lower inhibition values obtained. Dialysis of the other crude samples did not result in an altered percentage inhibition by anti-LD.

Extracts obtained from various rabbit and human tissues differed widely in their total LD content. Table II shows the amount of LD extracted from various rabbit tissues. Similar data from human tissues are not included, since the interval between time of death and excision of tissues may have rendered

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**Table I**

<table>
<thead>
<tr>
<th>Source of LD</th>
<th>Inhibition by:</th>
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<tbody>
<tr>
<td></td>
<td>Anti-rabbit skeletal muscle LD*</td>
</tr>
<tr>
<td>Rabbit</td>
<td>%</td>
</tr>
<tr>
<td>Skeletal muscle (purified)</td>
<td>75.7</td>
</tr>
<tr>
<td>Skeletal muscle (crude extract)</td>
<td>77.0</td>
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<tr>
<td>Erythrocytes</td>
<td>4.3</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>8.2</td>
</tr>
<tr>
<td>Plasma</td>
<td>34.4</td>
</tr>
<tr>
<td>Carcinoma (Vx-2)</td>
<td>53.4</td>
</tr>
<tr>
<td>Cow</td>
<td></td>
</tr>
<tr>
<td>Heart muscle (purified)</td>
<td>-5.5</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>5.0</td>
</tr>
<tr>
<td>Plasma</td>
<td>2.8</td>
</tr>
</tbody>
</table>

* Means of three or more determinations. Standard deviations at ≤30% inhibition, ±3.8%; at 30 to 60% inhibition, ±3.0%; at >60% inhibition, ±2.1%.
such quantitative comparisons inaccurate. It will be noted that the enzymatic activity of various solid tissues ranged from 10,000 to 330,000 units of LD per mg extract N, a 33-fold variation. It is apparent that rabbit erythrocytes, although a convenient source of LD since these cells may be easily and quantitatively lysed to release their enzymes, are not richly endowed with this enzyme in comparison to other tissues.

The results of typical electrophoretic separations of LD isozymes from rabbit tissues are shown in Fig. 1. Although replicate determinations revealed minor quantitative variations in the proportions of various electrophoretically distinct LD's, each type of tissue was found to have a typical pattern of these isozymes. A total of five LD isozymes was identified. In order to facilitate the description of results these isozymes are designated, from slowest to fastest migrating forms, as LD₁ to LD₅. In Fig. 1 the graphs have been arranged in a series from the tissue containing only the slowest migrating isozyme (skeletal muscle) to the tissues containing only the fastest migrating isozyme (erythrocytes and heart muscle). Liver, the Vx-2 tumor, plasma, lung, and brain contained all five isozymes but in different proportions. In some electrophoretic separations of the LD isozymes from Vx-2 tumors the amount of LD₁ exceeded the amount of LD₅, whereas in other samples the relative proportion of these forms was reversed. The low LD levels characteristic of the plasma from normal mature rabbits (Table II) resulted in the LDr isozyme appearing to split into two components. When the human tissues were arranged in order of an increasing content of fast migrating isozymes, they fell into the same sequence as did the rabbit tissues. The RBH values of the human isozymes (Table III) showed that these LD forms were also uniformly spaced in the starch gel, except for a greater spread between the first two isozymes. The human isozymes were all more widely spaced than the rabbit LD isozymes, permitting a sharper separation of the former.

Some notable differences between the distribution of LD isozymes in the human and rabbit tissues were also apparent. Only four of the five LD isozymes were detected in human plasma, whereas all five LD isozymes were found in rabbit plasma. Human erythrocytes and heart muscle contained the three fastest migrating isozymes; these same tissues from the rabbit contained just the fastest migrating form. Human heart muscle contained a quantitatively predominant amount of LD₁.

**DISCUSSION**

The data presented here confirm the reports that electrophoretically (4-14) and serologically (1, 2) distinct forms of lactic dehydrogenase occur in mammalian tissues. The electrophoretic separations indicated that the distribution of isozymes in different tissues varied quantitatively as well as qualitatively. For example, skeletal muscle and heart muscle, both human and rabbit, contained qualitatively distinct isozymes. On the other hand, although human heart muscle contained the same three LD isozymes as did human erythrocytes it had a much greater relative amount of LD₄ than did the erythrocytes. This high proportion of LD₄ in human heart muscle may explain the observation by Vesell and Bearn (9) that sera from patients with myocardial infarction showed a selective elevation of the fastest migrating LD component as separated by zone electrophoresis in a starch block. The results reported here for human erythrocytes agree with the separations Vesell and Bearn (9) reported, but these workers did not observe the presence of the LD₁ isozyme in their serum samples. This isozyme appears to be less stable than the faster migrating forms, which may account for their failure to detect it.

Serological differences between the LD₁ from rabbit tissues were clearly detected with the antirabbit muscle LD. The failure of antibovine heart muscle LD to differentiate between LD₁ derived from various tissues of a single species may have resulted from the presence of more than one LD isozyme in the heart muscle LD preparation used for the production of antibody (5, 7). Thus the antibovine LD may have been polyvalent. Kubowicz and Ott (20) found no serological differences between purified lactic dehydrogenases derived from a rat sarcoma and normal rat muscle.

A comparison of data on the inhibition by antibody of the lactic dehydrogenases in crude extracts of rabbit tissues (Table I) with the patterns of LD isozymes obtained by electrophoresis...
Fig. 1. Distribution of lactic dehydrogenase isozymes in rabbit tissues as separated by starch gel electrophoresis. Position of the reference protein, bovine hemoglobin, is shown below each graph.
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SKELETAL MUSCLE

ERYTHROCYTES

FRACTION NUMBER

FIG. 2. Distribution of lactic dehydrogenase isozymes in human tissues as separated by starch gel electrophoresis. Positions of the origin and the reference protein, bovine hemoglobin, are shown below each graph.

TABLE III

<table>
<thead>
<tr>
<th>Source of enzyme</th>
<th>Rabbit</th>
<th>Human</th>
</tr>
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<tbody>
<tr>
<td>LD number</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.13 ± 0.05</td>
<td>-0.21 ± 0.02</td>
</tr>
<tr>
<td>2</td>
<td>0.54 ± 0.05</td>
<td>0.39 ± 0.03</td>
</tr>
<tr>
<td>3</td>
<td>0.85 ± 0.03</td>
<td>0.75 ± 0.02</td>
</tr>
<tr>
<td>4</td>
<td>1.18 ± 0.03</td>
<td>1.14 ± 0.05</td>
</tr>
<tr>
<td>5</td>
<td>1.51 ± 0.04</td>
<td>1.53 ± 0.05</td>
</tr>
</tbody>
</table>

* In starch gel containing barbital buffer, ionic strength = 0.05, pH 8.6.

(Fig. 1) shows that, at the concentrations used, antibody to rabbit LD$_1$ (skeletal muscle LD) inhibited only a trace amount of LD$_5$ (erythrocyte LD). The fact that the same concentration of this anti-LD gave an intermediate level of inhibition of the LD from the Vx-2 tumor extract and rabbit plasma, agrees with the electrophoretic findings that these samples contained a mixture of isozyme forms and suggests that the isozymes intermediate between the slow and fast migrating forms were inhibited to an intermediate degree by the anti-LD. Similarly, Nisselbaum and Bodansky (2) found that an antirabbit muscle lactic dehydrogenase preparation which inhibited the LD from rabbit skeletal muscle 80% inhibited the LD’s from liver, 70%; spleen, 43%; kidney, 25%; and heart, 9%. These data correlate with the LD isozyme patterns found in these tissues.

The data suggest that it should be possible to identify the origin of LD causing elevated serum LD levels observed in certain disease states (15, 21), by determining the proportion of LD isozymes in the serum, or to detect specific changes in these proportions in the absence of an elevation of the total serum LD level. The apparent increase in the proportion of LD$_2$ and LD$_3$ isozymes in the plasma of rabbits bearing the Vx-2 tumor is pertinent, since these are the two isozymes present in greatest concentrations in this tumor. Immunochemical procedures have been studied for the determination of the tissue of origin of human serum alkaline phosphatase (22) and rabbit serum LD (2).
Studies on the properties and interrelationships of the LD isozymes described here are presented in Paper II of this series.

SUMMARY

Lactic dehydrogenase has been shown to occur in serologically distinct forms in different rabbit tissues. Five electrophoretically distinct forms (isozymes) of lactic dehydrogenase have been isolated from both rabbit and human tissues. Each tissue was found to have a characteristic distribution of these isozymes and quantitative isozyme patterns for nine rabbit tissues and plasma and five human tissues and plasma have been presented. Analogous rabbit and human tissues were found to have similar but not identical isozyme patterns.

REFERENCES

The Electrophoretically Distinct Forms of Mammalian Lactic Dehydrogenase: I. DISTRIBUTION OF LACTIC DEHYDROGENASES IN RABBIT AND HUMAN TISSUES

Peter G. W. Plagemann, Kenneth F. Gregory and Felix Wróblewski