Reactions of Lactic Dehydrogenase from Various Rabbit Organs with Antirabbit Muscle Lactic Dehydrogenase*†

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(Printed in U.S.A.)

(Received for publication, June 26, 1959)

Within recent years considerable interest has arisen in the differentiation of functionally similar enzymes from different organs of the same species, or from the same organ of different species (1-4). Immunochemical procedures have been applied successfully by Schlamowitz (3) to the differentiation of alkaline phosphatases, and by Henion and Sutherland (2) to the differentiation of phosphorylases. The present study is concerned with an attempt to differentiate among the lactic dehydrogenases from different rabbit organs by means of their reactions with antirabbit muscle lactic dehydrogenase.

EXPERIMENTAL

Enzyme Assay—Lactic dehydrogenase activity was determined in the Beckman spectrophotometer at 340 mμ. One-tenth to 0.5 ml of appropriately diluted enzyme was added to a cuvette with a light path of 10 mm containing 0.1 ml of 0.0025 M DPNH, 0.3 ml of 0.01 M sodium pyruvate, and 0.15 M phosphate buffer to a final volume of 3.0 ml. The final concentration of pyruvate used was necessary to obtain optimal activity for a given amount of enzyme. One unit of enzyme was defined as that amount which will cause a decrease of 0.001 in optical density in one minute at 37°C. Initial velocities were determined during the zero order portion of the reaction.

Buffered Albumin—A 0.15% solution of human serum albumin (Cutter) in 1/30 phosphate buffer, pH 7.4, was used as diluent for all lactic dehydrogenase preparations except where otherwise specified.

Enzyme—Crystalline rabbit muscle lactic dehydrogenase ( Worthington Biochemical Corporation, Freehold, New Jersey) had a specific activity of about 2.0 × 10⁴ units per mg of protein and showed one protein staining area when subjected to paper electrophoresis. All dilutions of enzyme were made in 1/30 phosphate buffer containing 0.15% albumin, except where otherwise specified.

Protein Determinations—Protein was determined by the biuret method of Robinson and Hogden (5), or, when only a small amount was available, by the method of Lowry et al. (6). In both cases crystalline bovine serum albumin was used as a standard.

Antienzyme—White leghorn roosters weighing from 800 to 900 g were immunized with rabbit muscle lactic dehydrogenase adsorbed on aluminum hydroxide which was prepared according to Holford et al. (7). A suspension of crystalline enzyme, 0.1 ml, containing 2.5 mg of protein was diluted with 0.9 ml of 0.02 M phosphate buffer, pH 7.4, and 1.5 ml of aluminum hydroxide gel suspension was added giving a concentration of 0.1 mg of enzyme per ml. Less than 5% of the enzyme remained unadsorbed, and 100% of the activity was recovered when the gel was eluted with 0.2 M phosphate buffer, pH 7.4. A total of 28 mg of alum-adsorbed enzyme was injected into each of 4 roosters via the wing veins. Injections were given on alternate days, and contained increasing amounts of enzyme according to the following schedule: four doses each of 0.5, 1.0, 1.5 and 2.0 mg and two doses of 4.0 mg. After immunization the animals weighed 1.5 to 1.6 kg.

Because of the relatively small amount of serum readily obtainable on clotting of chicken blood, blood was withdrawn into heparinized syringes. Three days after completion of the course of immunization, 0.02 ml plasma of each of the four roosters was tested for ability to inhibit the activity of 100 units of rabbit muscle lactic dehydrogenase. Two of the plasmas showed high antienzyme activity, inhibiting approximately 60 and 90% of the enzyme. The respective animals were exsanguinated one week after the last immunizing dose, and the plasmas were preserved with 0.1% phenol. After a 3 week rest, each of the two remaining roosters received an injection of 4.0 mg of the alum-adsorbed lactic dehydrogenase, but this procedure did not increase the low inhibiting action (15%) of their plasmas. The plasmas were stored separately, in larger amounts in the Deep Freeze at -15° to -20°, and in smaller amounts in the cold room at 4°. In the course of storage for several weeks, precipitates, probably fibrin, formed; these were centrifuged off before precipitin tests were set up. During the 72-hour incubation period used for these tests, the amount of protein precipitated in the control containing plasma from the immunized rooster but no added muscle lactic dehydrogenase did not differ significantly from the amounts precipitated in controls containing human serum and no or varying amounts of the rabbit muscle lactic dehydrogenase. It was not likely, therefore, that precipitation of fibrin during the incubation period was significant enough to alter the values for

* Presented in part at the Annual Meeting, Federation of American Societies for Experimental Biology, Atlantic City, April 1959.
† This work has been supported in part by the following: American Cancer Society Grants No. P-163 and P-184A; Grants No. DRG 332B and 332C from the Damon Runyon Memorial Fund for Cancer Research, and by a research grant, C-4321 (CI81), from the National Cancer Institute, National Institutes of Health, Public Health Service.

Dr. William L. Money, personal communication.
December 1959

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the precipitin reaction. For purposes of convenience the partially fibrin-free plasmas from the immunized roosters will be referred to as antisera. Less than 50% of the lactic dehydrogenase activity in the antisera could be destroyed by heating to 56° for 45 minutes. They were used, therefore, without heating, and appropriate corrections were made for their lactic dehydrogenase activity. With the exception of the results in Fig. 2, all the data reported here were obtained with the antiserum showing 90% inhibition in the test system.

RESULTS

The serum of untreated roosters did not inhibit the activity of the muscle lactic dehydrogenase. It was also found that the presence of normal rooster or human serum prevented the inactivation of dilute solutions of the enzyme incubated for periods of 1 to 3 days at 4°. For this reason human serum was included in the control experiments when the inhibition of the enzyme by antiserum was measured.

Reaction of Rabbit Muscle Lactic Dehydrogenase with Antienzyme—Varying amounts of rabbit muscle lactic dehydrogenase ranging from 0.25 to 500 μg, dissolved in 0.2 ml of M/15 phosphate buffer, pH 7.4, were added to 0.2 ml of undiluted antiserum in small plastic centrifuge tubes. Control tubes contained 0.2 ml of normal human serum and enzyme. All experiments were set up in triplicate. After 72 hours at 4° the tubes were centrifuged at 800 g and the supernatants were carefully decanted. Aliquots of the supernatant solutions from two of each set were appropriately diluted, and lactic dehydrogenase activity was determined. The precipitate in one of each set of tubes was washed twice with 1.0 ml portions of 0.9% NaCl solution. No appreciable enzymic activity was obtained in these washings. The precipitates, suspended in 0.9% NaCl solution, were also inactive. Treatment of portions of the suspensions with 1.0 M phosphate buffer, pH 8.0, or with 30% NaCl did not result in any recovery of enzymic activity. The precipitates from the two remaining tubes in each set were washed twice with 1.0-ml portions of 0.9% NaCl solution, and the protein contents were determined by the method of Lowry et al. (6).

Fig. 1A shows the inhibiting effect of the homologous antiserum on varying concentrations of muscle lactic dehydrogenase. Inhibition of enzymic activity by 0.2 ml of antiserum was complete up to 10 μg of enzyme. Beyond this the extent of inhibition decreased and became unmeasurable above 80 μg of enzyme. The precipitation of protein with varying concentrations of enzyme is typical of an antibody-antigen interaction (Fig. 1B). The extent of precipitation increased up to 40 μg of the antigen (enzyme). Beyond this, precipitation decreased with increasing amounts of enzyme, and became very small at amounts above 300 μg of enzyme. Approximately 110 μg of protein precipitated in the controls containing human serum and varying amounts of rabbit muscle lactic dehydrogenase as well as in controls containing antiserum without any added muscle lactic dehydrogenase. This value was taken as a base line from which the precipitation of the enzyme-antienzyme complex was measured.

The time course of the inhibition of the muscle lactic dehydrogenase by antienzyme is shown in Fig. 2. The reaction is time-dependent and is complete within 4 hours for the concentrated system and within 24 hours for the dilute system. The same degree of inhibition was reached whether the enzyme-antienzyme mixture was diluted for activity determination immediately or at the end of the incubation period.

Marucci and Mayer (8) have reported that the extent of inhibition of urease by its homologous antiserum was independent of the concentration of the reactants at any given ratio of antibody to enzyme, but was a function of the ratio of antibody to enzyme. When rabbit muscle lactic dehydrogenase in concentrations ranging from 0.25 to 5.0 μg per ml was incubated with varying amounts of homologous antiserum, the extent of inhibi
Inhibition of lactic dehydrogenase from rabbit organs by antirabbit muscle lactic dehydrogenase

Two hundred units of enzyme were incubated with 0.02 ml of antiserum or normal human serum in a total volume of 2.0 ml. Percentage of inhibition is calculated as described in the text.

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Specific activity (Units/ml)</th>
<th>Inhibition (specific activity %)</th>
<th>Enzyme form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>3.6</td>
<td>32 (2 ± 4)</td>
<td>Unpurified</td>
</tr>
<tr>
<td>Liver</td>
<td>1.7 x 10^4</td>
<td>69 ± 5</td>
<td>Purified</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.4 x 10^4</td>
<td>25 ± 4</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>1.1 x 10^4</td>
<td>9 ± 3</td>
<td></td>
</tr>
<tr>
<td>Muscle</td>
<td>1.8 x 10^4</td>
<td>81 ± 5</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td>43 ± 1</td>
<td></td>
</tr>
</tbody>
</table>

1:1 serum and muscle (single determination).

* Values in this column represent the mean ± the average deviation of the results obtained with three animals. (Four sets of determinations except in the case of spleen where only three determinations are represented).

† Crystallized 2 times.

The influence of substrates on the inhibition of enzymes by homologous antiserum has been studied in several instances (2, 9, 10, 11). Mansour et al. (11) were unable to detect any effect on the inhibitory action of rooster antiserum to rabbit muscle lactic dehydrogenase by pre-incubation of the enzyme with pyruvate, lactate, DPN, or DPNH. We have also been unable to demonstrate any influence of pyruvate or DPNH on the inhibition of rabbit muscle lactic dehydrogenase by its homologous antiserum, as is shown in the following experiment. Two ml of 50-fold diluted antienzyme was added to 3.0 ml of buffer containing 2.5 mg of enzyme and pyruvate to give a final concentration of 0 to 30 mM. Samples were withdrawn at zero time and again after 1 hour and 24 hours incubation at 4°. Enzyme activity was determined at these times, suitable allowances being made for the substrate present in the incubation mixture. After 24 hours incubation the inhibition by the antiserum was 65% ± 3% in the absence and in the presence of pyruvate at the various concentrations tested. Similar experiments showed that DPNH in concentrations up to 0.5 mM also had no effect on the inhibition of the lactic dehydrogenase by its homologous antiserum.

Comparison of Inhibiting Effect of Antirabbit Muscle Lactic Dehydrogenase on Lactic Dehydrogenases from Various Rabbit Organs—Rabbit liver, heart, and kidney were partially perfused with 0.9% NaCl, and homogenized in a Waring Blender with a volume of distilled water equal to twice the weight of the organ; insoluble material was removed by centrifugation. Spleen and skeletal muscle were treated similarly, but without perfusion. Serum was also studied. A portion of each extract and of the serum were purified as follows: the pH was adjusted to 5.2 with 2 N acetic acid and the enzyme was adsorbed on an equal volume of calcium phosphate gel prepared according to McFarland (12). The gels were then washed once with a volume of water equal to the volume of extract used, and once with an equal volume of 0.1 M phosphate buffer, pH 7.8. The enzyme was then eluted with two successive 0.5 volumes of 0.2 M phosphate buffer, pH 7.8. The gel eluates were readjusted to pH 5.3 with 2 N acetic acid, and solid ammonium sulfate was added to a final concentration of 0.3 g per ml. The precipitates were collected by centrifugation and dissolved in 0.1 M phosphate buffer, pH 7.8, containing 0.001 M EDTA. The specific activities of the partially purified enzyme from each organ and of the unpurified extracts are given in the second and fourth columns of Table I.

Portions of the purified and unpurified preparations were diluted with buffered albumin, and aliquots containing about 200 units of enzyme activity were mixed with 0.02 ml of antiserum or human serum in buffered albumin to give a final volume of 2.0 ml. After standing at 4° for 48 to 72 hours, the mixtures were centrifuged, and the activity in the supernatant fluids was determined.

The data presented in Table I represent the averages of the results obtained with unpurified organ extracts from three different rabbits, and with purified enzyme from one organ of one animal. The values given in columns 3 and 5 were calculated from the activities in the presence of antiserum compared with those of the control mixtures containing human serum. There was no significant difference between the inhibition of the unpurified preparation and that of the corresponding purified preparation except, possibly, in the case of serum and kidney. The level of anti-enzyme that inhibited about 80% of muscle lactic dehydrogenase in the test system inhibited about 70% of enzyme from liver, 25% of that from kidney, 43% of that from spleen, and only 9% of the enzyme from heart. Only 33% of the lactic dehydrogenase in rabbit serum was inhibited by the anti-enzyme. The inhibition of a 50:50 mixture of the dehydrogenases from rabbit serum and rabbit muscle was equal to the calculated value, i.e. the average of the inhibition of enzyme from each source by anti-rabbit muscle lactic dehydrogenase.

To illustrate how the extent of inhibition of different organ lactic dehydrogenases by antirabbit muscle lactic dehydrogenase may be used to determine the organ of origin of this enzyme, the hepatotoxic agent CCl₄ was administered to two rabbits. Each animal received 1.0 g of CCl₄ in peanut oil per kg of body weight. This agent has been shown to cause the release of transaminase (13) and phosphohexose isomerase into the circulation (14). Fig. 4A shows that a 40-fold increase in the serum lactic dehydrogenase activity, from a normal value of 300 to 400 units per ml to a maximal level of over 14,000 units per ml, occurred between
the 10th and 20th hours after administration of CCl₂. At the same time the inhibition of the serum enzyme by antirabbit muscle lactic dehydrogenase rose from a normal value of 25 to 30% to a maximal value of 75 to 80% (Fig. 4B), the same extent of inhibition which was obtained with liver lactic dehydrogenase. The degree of inhibition of the serum enzyme by its anti-enzyme remained elevated until the serum enzyme activity returned to normal. Damage to the liver was also indicated by the finding that the serum phosphohexose isomerase level closely paralleled the rise and fall in serum lactic dehydrogenase. A second rabbit treated with CCl₂ gave the same results as are shown in Fig. 4. A control animal injected with peanut oil, the vehicle in which the CCl₂ had been administered, showed no rise in either the serum level of lactic dehydrogenase or its inhibition by antiserum.

**DISCUSSION**

The data presented in Fig. 1 show that at high antibody:antigen ratios rabbit muscle lactic dehydrogenase can be completely inhibited by its homologous antiserum. As the antibody:antigen ratio is decreased lactic dehydrogenase activity in the supernatant increases while the amount of protein precipitated also increases. This behavior in the region of moderate antigen excess is in agreement with the observations of Heidelberger and Kendall (18) on the dye-egg albumin:antibody system and with their Equation 4 expressing the general relationship. Further increases in the antigen concentration causes a decrease in the amount of protein precipitated. Goodman et al. (16) and Banovitz et al. (17) reported that the usual isotonic concentration of 0.15 M NaCl, optimal for the precipitation of antibodies from rabbit and horse sera (18), is not adequate for the precipitation of antibodies to crystalline beef albumin, human γ-globulin, and beef serum from chicken sera at higher concentrations of antigen. If these considerations apply to the antigen:antibody system considered in the present study, the values for the amount of precipitate listed in Fig. 1B would probably be altered at the higher concentrations of enzyme, although the general form of the relationship would remain the same.

All attempts to recover lactic dehydrogenase activity from the antigen:antibody precipitates were unsuccessful, suggesting that at least one of the antigenic sites on the enzyme molecule is identical with the enzymically active center. Henion and Sutherland (2) also showed that the washed precipitates obtained from the reaction of dog phosphorylase with its homologous antiserum were enzymically inactive. In contrast, however, enzymically active precipitates have been reported for the interaction of homologous antisera with dog intestinal phosphatase (19), jack bean urease (8), and liver phosphohexose isomerase (20).

Our finding that the extent of inhibition of rabbit muscle lactic dehydrogenase by antienzyme was dependent solely upon the antibody:antigen ratio is in agreement with the findings of Marucci and Mayer for urease (8), but is contrary to the results of Gregory and Wróblewski (21) who used diluted, unprotected solutions of rabbit muscle lactic dehydrogenase and short periods of incubation of enzyme with antiserum. As is shown in Fig. 2, the reaction between enzyme and anti-enzyme in dilute mixtures is time dependent.

**SUMMARY**

The role of various factors in the inhibition of rabbit lactic dehydrogenases by antirabbit muscle lactic dehydrogenases has been studied. At those conditions under which the anti-enzyme inhibits lactic dehydrogenases of rabbit skeletal muscle and liver almost completely, those of spleen, kidney and serum are affected only moderately, and that of heart slightly. The passage of enzymes from damaged liver into the circulation was reflected...
by a rise in the activity of the lactic dehydrogenase in the serum
and by a marked increase in the inhibition of serum lactic dehy-
rogenase by antienzyme.

Acknowledgment—The authors are indebted to Mr. Harry
Kolbe for his skillful and valuable assistance in these studies.

REFERENCES
2. HENION, W. F., AND SUTHERLAND, E. W., J. Biol. Chem., 224,
477 (1957).
(1953).
5. ROBINSON, H. W., AND HODDER, G. C., J. Biol. Chem., 135,
727 (1940).
6. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RAND-
7. HOLFORD, F. E., LUDDIN, J. B., AND STEVENS, H. W., J. Im-
munol., 46, 47 (1943).
(1948).
13. WROBLEWSKI, F. AND LADE, J. S., Ann. Internal Med., 63,
345 (1955).
15. ROBINSON, H. W., AND HODDER, G. C., J. Biol. Chem., 135,
727 (1940).
16. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RAND-
17. HOLFORD, F. E., LUDDIN, J. B., AND STEVENS, H. W., J. Im-
munol., 46, 47 (1943).
20. LIPSETT, M. N., REISBERG, R. B., AND BODANSKY, O., Arch.
(1958).