RAT LIVER LACTIC DEHYDROGENASE

I. ISOLATION AND CHEMICAL PROPERTIES OF THE CRYSTALLINE ENZYME*

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The fractionation of the proteins of liver, which contains what Edsall (1) has referred to as an "incredible multiplicity" of enzymes, has been attempted with considerable success by several groups of investigators (1-13). The procedures employed have involved for the most part salting-out and solvent processes with careful control of pH, ionic strength, temperature, and protein concentration. As a result of the studies cited above, crystalline catalase (4-6, 10) and glutamic acid dehydrogenase (7-9) have been obtained from bovine liver and crystalline alcohol dehydrogenase from equine liver (11).

The work reported in the present series of papers adds yet another crystalline enzyme to the growing list: rat liver lactic dehydrogenase (14, 15). It seems probable that a number of chemically well defined liver enzymes may soon be in hand for a careful study of physicochemical properties, amino acid composition, and specificity requirements for co-enzymes and substrates. The accumulation of such information coupled with a precise localization of each enzyme is considered to be vital to a thorough understanding of the metabolic reactions which take place in the liver cell.

A preliminary examination of the enzyme, lactic dehydrogenase,1 in rat liver extracts had shown that it was quite stable (16) and might lend itself

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Paper II of this series, on the physicochemical characterization of rat liver lactic dehydrogenase, will appear in a forthcoming issue of the Journal of Physical Chemistry.

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1 Lactic dehydrogenase is abbreviated as LDH; oxidized diphosphopyridine nucleotide, DPN⁺; and reduced diphosphopyridine, DPNH.
to fractionation in view of the availability of a convenient spectrophotometric assay for activity. This paper presents a method for the isolation of crystalline LDH from rat liver and a description of its chemical properties. Another report will describe the details of the physicochemical characterization of the purified enzyme.

LDH was first described in muscle by Meyerhof (17) and the enzyme system further characterized by Szent-Györgyi (18, 19), von Euler (20), Boyland (21, 22), and Green (23) and their coworkers. The crystalline muscle enzyme was prepared by Straub from ox heart in 1940 (24) and by Kubowitz and Ott from rat skeletal muscle and from the Jensen rat sarcoma in 1943 (25). Recently a number of properties of LDH have been reported (26-32).

**EXPERIMENTAL**

**Enzyme Concentration**—Although the lactate-pyruvate equilibrium is strongly in favor of lactate (25), LDH activity can be conveniently estimated by measuring the initial rate of formation of DPNH under a standard set of conditions. In the equation

\[ \frac{d[DPNH]}{dt} = k \times E \text{ per ml.} \]

E per ml. denotes enzyme concentration in arbitrary units.

In practice the LDH assays were carried out in 1.0 cm. quartz cuvettes in the Beckman model DU spectrophotometer. The final concentrations in the cuvette were as follows: sodium DL-lactate, 0.11 M; DPN+, 1.5 \times 10^{-4} M; 0.03 M sodium diethyl barbiturate buffer at pH 8.6. The reaction was initiated by adding the DPN+ component of the solution rapidly with stirring. The final volume was usually 3.6 ml. and the temperature approximately 25°. Time intervals in seconds were measured for optical density increments of 0.005 at 340 mμ. The rate, 0.005/Δt, was nearly constant during the first 2 minutes of the reaction if Δt was in the range of 5 to 20 seconds. If the value of k is taken to be 10^{-3}, the expression for the computation of enzyme concentration is

\[ E \text{ per ml.} = 10^3 \times \frac{0.005}{\Delta t} = 5/\Delta t \]

With the set of conditions as given, 1 unit of LDH was defined as that amount per ml. in the cuvette which would effect a change in optical density of 0.005 in 5 seconds at the onset of the reaction.

**Protein Concentration and Specific Activity**—The specific enzyme activity (E/P) of liver fractions was expressed as the ratio of the enzyme concentration in units per ml. to the absorption value at 280 mμ (37) and pH 7 to 8. 1 per cent rat liver protein solutions as determined by micro-Kjeldahl analysis showed calculated absorption values of 15 to 20 for most of the early fractions studied.
Electrophoresis—As an additional control in fractionation, active enzyme fractions were routinely examined in the electrophoresis instrument. The enzyme was identified with a component showing a mobility toward the anode of 0.5 to 1.0 X 10⁻⁸ cm.² volt⁻¹ sec⁻¹ in potassium phosphate buffer of ionic strength 0.1, pH 7.8 at 0.8°. Contaminating proteins all showed higher mobility under these conditions. The trailing component in the descending arm of the cell was sampled and showed significantly increased specific activity. The ratio of the amount of this component to the total protein as measured by area analysis was proportional to the specific activity of the whole fraction at progressive stages in the purification of the enzyme.

Fractionation

The steps for the isolation of LDH from rat liver are outlined in Table I, along with the yield and purity of the active fractions. All fractionation processes were conducted at 0° or below, with standard techniques to avoid protein denaturation (35). The electrophoretic patterns for two of the fractions are presented in Fig. 1.

Step I—For every 100 gm. of minced liver from adult, albino rats of both sexes and varying strain were added 20 gm. of ice and 80 ml. of 0.5 M NaCl. The mixture was homogenized for 2 minutes at half maximal speed in the Waring blender. Then 100 ml. of 0.5 M NaCl were added and the homogenate was allowed to stand at 0° for 30 minutes with occasional stirring. The homogenate was transferred to a stainless steel beaker which was secured in a controlled low temperature bath. Next, 200 ml. of 50 volumes per cent ethanol (1 part absolute ethanol, 1 part 0.5 M NaCl), cooled to −10°, were added slowly with mechanical stirring at a rate not in excess of 50 ml. per hour. During this time the temperature of the mixture was permitted to fall from 0–5°. The mixture was stirred continuously for 30 minutes after ethanol addition and then centrifuged at 800 X g for 2 hours at −5°. The final ethanol concentration was 20 volumes per cent, pH 6.5.

Step 2—The clear, red supernatant solution, S-1, was dialyzed against 0.5 M NaCl for 36 hours at 0°. The dialyzed solution was brought to an

² The Tiselius apparatus was manufactured by Frank Pearson Associates, New York. The electrophoresis cell was of 11 ml. capacity and the electrode assembly was the design of Albery (34). The authors gratefully acknowledge the assistance of Robert L. Fischer in performing some of the electrophoretic analyses.

³ The authors wish to express their appreciation to Dr. D. A. McGinty and his associates of the Research Department, Parke, Davis and Company, Detroit, for collecting large quantities of rat liver. Liver from untreated rats and from young male rats 24 hours after hypophysectomy served equally well as a source of LDH, whether used fresh or after storage under deep freeze conditions.
ammonium sulfate concentration of 0.3 gm. per ml. at 0º by slowly sifting in 35.3 gm. of the salt per 100 ml. of dialyzed S-1. Centrifugation in this step, as in most subsequent steps, was performed at $800 \times g$ for 2 hours.

**Table I**

**Essential Conditions for Fractionation at Each Step**

The average specific enzyme activity ($E/P$) and the expected cumulative yield are presented with each enzymatically active fraction.

<table>
<thead>
<tr>
<th>Step No.</th>
<th>Conditions for fractionation</th>
<th>Analysis of active fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EtOH</td>
<td>Ammonium sulfate</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>0.3</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>0.03</td>
</tr>
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<td>3</td>
<td>30</td>
<td>0.05</td>
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<td>4</td>
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<td>5</td>
<td>20</td>
<td>0.03</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
<td>0.03</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0.03</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>0.03</td>
</tr>
<tr>
<td>9</td>
<td>0.3</td>
<td>7.8</td>
</tr>
</tbody>
</table>

**Fig. 1.** The electrophoretic patterns of two representative liver fractions are presented to demonstrate the increase in relative concentration of the LDH component (vertical arrows) and the corresponding increase in specific enzyme activity ($E/P$). At 0.8º, pH 7.8, ionic strength 0.1, phosphate buffer (pattern A) and at pH 8.6, ionic strength 0.1, diethyl barbiturate buffer (pattern B), the enzyme displayed the lowest mobility ($\mu = 0.5 \text{ to } 1.0 \times 10^{-4} \text{ cm.}^2 \text{volt}^{-1} \text{sec.}^{-1}$) toward the anode among the liver proteins present.

**Step 3**—The resulting brown precipitate, P-2, was redissolved in a volume of water one-tenth as large as the volume of S-1 employed in Step 2. The solution was dialyzed against 0.03 m NaCl for 36 hours at 0º, the pH, after dialysis, was adjusted to 5.8 with dilute acetic acid, and the ethanol concentration was brought to 5 volumes per cent at −3º.

**Step 4**—After centrifugation, the enzyme remained in the supernatant...
solution, S-3, and was reprecipitated in fraction P-4 by bringing the conditions to 30 volumes per cent of ethanol, ionic strength 0.03, pH 5.8, and \(-10^\circ\).

**Step 5**—Precipitate P-4 was redissolved in sufficient phosphate buffer (ionic strength 0.1, pH 7.8) to bring the solution to a volume equal to that of P-2 in Step 3. To this was added an equal volume of 60 volumes per cent of ethanol. The final conditions were 30 volumes per cent of ethanol, ionic strength 0.05, pH 7.8, and \(-10^\circ\).

**Step 6**—Precipitate P-5 was resuspended in phosphate buffer at a volume one-half as large as the solution of P-4. The conditions were then brought to 20 volumes per cent of ethanol, ionic strength 0.05, pH 7.8, and \(-8^\circ\).

**Step 7**—The resulting precipitate, P-6, was again suspended in phosphate buffer and brought to the same set of conditions as in Step 6, but the final volume was reduced by half.

**Step 8**—Precipitate P-7 was resuspended in approximately 3 volumes of phosphate buffer (ionic strength 0.03, pH 7.8) and centrifuged at 25,000 \(\times g\) for 30 minutes. The active residue was similarly reextracted three or four times until all the brown color was removed. The combined extracts, S-8, contained a variable amount of enzyme which could be recovered, if warranted, by reprecipitation with ethanol and treating as described previously. The final residue, P-8, was suspended in about 2 volumes of 1.0 m ammonium sulfate (made up in 0.1 m phosphate buffer, pH 7.8) and was stored for 12 hours at \(0^\circ\) to assure complete solution of the enzyme. The residue remaining after this period was removed by centrifugation and discarded if no further enzyme could be extracted with 1.0 m ammonium sulfate. If the enzyme dissolved in 1.0 m ammonium sulfate did not possess the maximal purity index (E/P = 8400), the solution was exhaustively dialyzed against phosphate buffer (ionic strength 0.05, pH 7.8) and then reprecipitated at 20 volumes per cent of ethanol and the precipitate again carried through the extraction steps.

**Step 9**—The pure enzyme was brought into a very concentrated solution by first precipitating at 0.3 gm. per ml. of ammonium sulfate concentration and then redissolving in the smallest possible volume of 1.0 m ammonium sulfate. A dialysis bag containing sufficient saturated ammonium sulfate (at pH 7.8) to bring the enzyme solution to 0.3 gm. per ml. was then immersed in the solution at \(0^\circ\) and mechanically rotated very slowly over a period of 12 hours. Crystallization was evident by microscopy and by the display of a sheen in the suspension on stirring. Recrystallization did not improve the specific activity (E/P = 8400) nor the size of the crystals. The crystals were extremely minute (Fig. 2), requiring a magnification of 1500 \(\times\), and were difficult to photograph, since the refractive index was almost identical with that of the suspending medium. Methylene blue
staining was not particularly helpful. The average total yield of enzyme was about 30 per cent. 1 kilo of whole liver yielded 2 million units or 200 mg. of crystalline LDH.

**Chemical Characterization of Lactic Dehydrogenase**

The dry weight of the purified enzyme was determined with exhaustively dialyzed samples which were then dried either by heating for 2 weeks in a vacuum oven at 110° or by lyophilizing for 12 hours. The percentage composition of nitrogen by both methods after salt correction or salt removal was quite similar, giving an average value of 14.8 gm. of nitrogen per 100 gm. of dry protein. The absorption spectrum was typical of that for most proteins having an absorption maximum at 280 mμ (pH 6 to 8). No absorption peak was observed at 340 mμ, the region of maximal absorption for reduced DPN. The specific absorption coefficient at 280 mμ for a 1 per cent protein solution (1 gm. of protein per 100 ml. of solution) was 12.58. An enzyme solution with a calculated optical density of 1.0 at 280 mμ had an enzyme concentration of 8400 units per ml.; i.e., the maximal specific enzyme activity of the pure enzyme. 1 mg. of enzyme is equivalent to 10,500 units of enzyme activity.

The pH of an enzyme sample dialyzed for several days against 0.1 M NaCl was 6.4 at 10°. This value compared favorably with the isoelectric point of 6.3 determined in electrophoresis experiments. A complete solubility study of the purified enzyme was not performed, since it was impossi-
ble to obtain the enzyme in the dry state without denaturation. However, if a partial precipitation of enzyme in solution was accomplished by bringing the ammonium sulfate concentration to 0.27 gm. per ml., the precipitated material, the supernatant solution, and the initial solution all showed the maximal specific activity.

The enzyme was stable in the pH and temperature range employed in the fractionation procedure. There was a gradual loss of activity at values less than pH 5.0 or greater than pH 8.5. The pure enzyme was stored as a concentrated solution in 1.0 M ammonium sulfate or in 0.5 M NaCl at 0° for periods of a month or more. Attempts to lyophilize solutions of the enzyme without loss of activity were unsuccessful.

The results of determinations of the amino acids in acid hydrolysates of the enzyme are presented in Table II. The analyses were performed by microbiological methods (33, 36). Tryptophan was estimated from absorption data of the whole protein after correcting for the content of tyrosine (37). The amino acids found by analysis accounted for 93.5 percent of the total protein weight and represented at least 1065 amino acid residues per enzyme molecule. No analysis was made for alanine and hydroxyproline. The summation of the atoms of amino acid nitrogen per molecule of enzyme (calculated) was 1323. This compared with the value of 1332 atoms of nitrogen per molecule of enzyme as determined separately by Kjeldahl analysis. The most striking aspect of the amino acid analysis was the high content of leucine, valine, and isoleucine. The values presented must be considered to be subject to the errors of the microbiological procedures.

Kinetics of Lactic Dehydrogenase System

Kinetic studies of the conversion of lactate to pyruvate in the presence of purified rat liver LDH as represented by the over-all equation

\[
\text{LDH} \quad \text{Lactate + DPN}^+ \rightarrow \text{pyruvate + DPNH + H}^+
\]

were undertaken in an attempt to determine the classical Michaelis-Menten constants (38) for a two substrate system. The preliminary data presented here demonstrate that the maximal initial reaction velocity \((V_M)\) and the Michaelis-Menten constant \((K_M)\) for one substrate are dependent on the concentration of the second substrate. This interdependence of substrate concentrations was recently demonstrated by Theorell and Bonnichsen (39) and Theorell and Chance (40) with equine liver alcohol dehydrogenase and by Schwert and Hakala (29) for a bovine heart LDH preparation.

* The authors are greatly indebted to Dr. L. M. Henderson and Mrs. Jean Ogren of the Division of Biochemistry for performing these analyses.
In the present study with rat liver LDH, initial reaction velocities \( (V_i) \) were measured at 24° as the increment in optical density at 340 m\( \mu \) per unit time at the onset of the reaction with LDH and the substrates D,L-lactate and DPN+.

The method was identical with the analytical procedure described in the first section of this paper with the exception that \( V_i \) was expressed as \( \Delta[DPNH] \) per second, where an optical density change of 0.005 is equivalent to an increase in DPNH concentration of \( 8.0 \times 10^{-7} \) M as calculated from the recently determined specific absorption coefficient of \( 6.22 \times 10^6 \) sq. cm. per mole (41).

The components added to the 1.0 cm. quartz cuvette were 0.5 ml. of enzyme solution, 1.0 ml. of Veronal buffer at pH 8.8, 1.0 ml. of D,L-lactate solution, and 0.5 ml. of DPN+ solution, the last at zero time. At the final volume of 3.0 ml., the pure

### Table II

Amino Acid Analysis of Lactic Dehydrogenase

The amino acid content of an acid hydrolysate of purified LDH was determined by microbiological assay. Tryptophan was estimated from the absorption of the enzyme at 280 m\( \mu \). The combined content of cystine and cysteine was expressed as cysteine. The moles of amino acid residues per mole of protein were calculated on the basis of a molecular weight for the enzyme of 126,000.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Gm. amino acid residue per 100 gm. protein</th>
<th>Moles amino acid residue per mole protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine</td>
<td>11.6</td>
<td>129</td>
</tr>
<tr>
<td>Valine</td>
<td>10.7</td>
<td>136</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>7.4</td>
<td>82</td>
</tr>
<tr>
<td>Glycine</td>
<td>4.3</td>
<td>95</td>
</tr>
<tr>
<td>Alanine</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Serine</td>
<td>6.2</td>
<td>00</td>
</tr>
<tr>
<td>Threonine</td>
<td>2.7</td>
<td>34</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.1</td>
<td>20</td>
</tr>
<tr>
<td>Cystine</td>
<td>1.3</td>
<td>16</td>
</tr>
<tr>
<td>Cysteine</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.7</td>
<td>23</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.5</td>
<td>19</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>3.3</td>
<td>22</td>
</tr>
<tr>
<td>Proline</td>
<td>5.1</td>
<td>66</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Lysine</td>
<td>8.8</td>
<td>87</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.8</td>
<td>39</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.7</td>
<td>16</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>10.3</td>
<td>113</td>
</tr>
<tr>
<td>Glutamic *</td>
<td>8.0</td>
<td>78</td>
</tr>
</tbody>
</table>

| Total            | 93.5                                     | 1065                                      |

* No analysis made.
enzyme solution had a calculated optical density at 280 \text{nm} of 0.00012, which represented a concentration of $9.50 \times 10^{-5}$ mg per ml or $7.57 \times 10^{-10}$ mole of enzyme per liter. The concentrations of DL-lactate and DPN$^+$ were varied. In Fig. 3 and Table III lactate concentration is expressed as moles of L-lactate per liter, since it was found in systems in which the L isomer was employed that the $V_i$ was the same as if twice the concentration of DL-lactate had been used. The DPN$^+$ preparation was 73 per cent pure as analyzed by the method of Hogeboom and Barry (42).

![Graph](http://www.jbc.org/)

**Fig. 3.** $V_{ML}$ as a function of [DPN$^+$] and $V_{MD}$ as a function of [L-lactate]. In Curve A, $1/[\text{DPN}^+]$ (upper abscissa) is plotted against $1/V_{ML}$. In Curve B, $1/\text{[L-lactate]}$ (lower abscissa) is plotted against $1/V_{MD}$. The reciprocals of the maximal velocities have the same dimensions and are represented together on the ordinate. Slopes and intercepts were calculated by the method of least squares.

In Fig. 3 are plotted the values$^5$ of $1/V_{ML}$ versus $1/[\text{DPN}^+]$ (Curve A) and the values of $1/V_{MD}$ versus $1/\text{[lactate]}$ (Curve B). Examination of Fig. 3 clearly shows the dependence of the $V_M$ of one substrate on the concentration of the second substrate. As the concentrations of DPN$^+$ and L-lactate approach infinity, the values of $V_{ML}$ and $V_{MD}$, respectively, approach the same limiting value. From the intercept in Fig. 3, the limiting value of $V_M$ was calculated to be $4.15 \times 10^{-7}$ mole of DPNH per liter.

$^5$ $V_{ML}$ values are the maximal initial reaction velocities obtained when the DPN$^+$ concentration was constant and the lactate concentration was varied. $V_{MD}$ values are the maximal initial reaction velocities obtained when the lactate concentration was constant and the DPN$^+$ concentration was varied.
per second. The limiting value for the turnover number was 32,900 moles of DPNH per minute per mole of enzyme at 24° in Veronal buffer at pH 8.8.

The data presented in Table III also demonstrate the dependence of $K_{MD}$ and $K_{ML}$ on the concentrations of lactate and DPN+, respectively. The kinetic equations of Theorell and Chance predict a relationship between the specific reaction velocity constants of their proposed three-step dehydrogenase system and the limiting values of $V_M$ and $K_M$ (40). In this respect the data presented here seemed to fit the equations of Theorell and Chance, although no enzyme-coenzyme complex could be detected by

**Table III**

Summary of Experiments on LDH Kinetics

These data were obtained at 24° by measuring the initial rate of formation of DPNH spectrophotometrically at 340 mμ. $V_M$ and $K_M$ values were determined by varying the concentration of one substrate at four different concentrations of the second substrate. $V_M$ is expressed as moles of DPNH per second, $K_{ML}$ as [l-lactate], and $K_{MD}$ as [DPN+]. The turnover number is expressed as moles of DPNH per minute per mole of enzyme.

<table>
<thead>
<tr>
<th>[DPN+] × 10^-4</th>
<th>$V_M$ × 10^-7</th>
<th>Turnover No.</th>
<th>$K_{ML}$ × 10^-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.44</td>
<td>0.65</td>
<td>5,100</td>
<td>2.82</td>
</tr>
<tr>
<td>0.88</td>
<td>1.11</td>
<td>8,800</td>
<td>2.56</td>
</tr>
<tr>
<td>1.75</td>
<td>1.78</td>
<td>14,100</td>
<td>2.23</td>
</tr>
<tr>
<td>3.50</td>
<td>2.44</td>
<td>19,300</td>
<td>1.96</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>[l-Lactate] × 10^-2</th>
<th>$V_M$ × 10^-7</th>
<th>Turnover No.</th>
<th>$K_{MD}$ × 10^-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.66</td>
<td>2.21</td>
<td>17,500</td>
<td>3.49</td>
</tr>
<tr>
<td>3.30</td>
<td>2.94</td>
<td>23,300</td>
<td>3.08</td>
</tr>
<tr>
<td>6.60</td>
<td>3.34</td>
<td>26,500</td>
<td>2.66</td>
</tr>
<tr>
<td>13.30</td>
<td>3.65</td>
<td>28,900</td>
<td>2.55</td>
</tr>
</tbody>
</table>

spectrophotometry. Very recently a complex of DPNH and LDH (Straub preparation from beef heart) was demonstrated by Chance and Neilands (32) by employing the sensitive spectrophotometric technique previously applied to the study of liver alcohol dehydrogenase. A more detailed analysis of kinetic data is required to define these relationships in the mechanism of action of LDH from rat liver. Although the DPN+ preparation employed here was 73 per cent pure, the possibility that impurities may influence the substrate interdependence cannot be rejected.

Preliminary studies were made relative to optimal pH and substrate specificity. Initial reaction velocities observed for the pyruvate to lactate conversion were maximal at pH 8.4, while for the lactate to pyruvate conversion a reproducible maximum was obtained at pH 8.9. Velocity de-
terminations with systems more alkaline than pH 8.9 were inconsistent, owing probably to denaturation effects. Substitution of $\beta$-hydroxybutyrate, malate, or ethanol in place of lactate in the LDH test system resulted in no increase in optical density at 340 m$\mu$ at pH 8.6.

**Physicochemical Characterization**

A thorough study of the physical properties of LDH was made and will be reported in detail in a separate communication. The principal findings of this investigation are summarized below.

The sedimentation behavior of the purified enzyme was examined in the ultracentrifuge in the range of 0.4 to 1.4 per cent. A single gradient peak displaying a high degree of symmetry and Gaussian form was observed in each experiment. The value of $s_{20,w}$ at infinite dilution was $7.39 \times 10^{-13}$ second. The diffusion coefficient, $D_{20,w}$, evaluated by the maximal height-area method, had a value of $5.8 \times 10^{-7}$ cm$^2$ per second at infinite dilution. The partial specific volume was found to be 0.745 ml. per gm. at 0°C. From these data the anhydrous molecular weight was calculated to be 126,000 and the molar frictional ratio 1.13. It is indicated that the shape of the LDH molecule is somewhere between that of a sphere of 34 per cent hydration and an unhydrated ellipsoid with an axial ratio of 3.4. The ellipsoid form would have a diameter of 60 A and length of 200 A.

Upon electrophoresis the enzyme migrated as a single, symmetrical peak over a pH range of 5.8 to 7.8 in potassium phosphate buffers of 0.1 ionic strength at 0.8°C. The isoelectric point was 6.3.

**DISCUSSION**

The isolation procedure presented in this paper demonstrates again that intracellular enzymes and undoubtedly a host of other tissue proteins are susceptible to separation by the use of organic solvents at low temperature. The first supernatant fluid, S-1, contained many different types of protein in solution, revealing at least ten electrophoretic components. LDH alone made up less than 0.7 per cent of the total protein in this solution. The usefulness of ammonium sulfate was limited, because the solubilities of the enzyme and of other proteins were not sufficiently different in systems of the salt to permit efficient separation of the enzyme.

From a kinetic standpoint rat liver LDH was similar to LDH isolated from other tissues. The LDH system oxidized only the L-lactate isomer, as similarly reported for LDH from heart muscle (27, 31) and from skeletal muscle (25). In recent kinetic studies with LDH from heart muscle Nei\-lands reports $K_M$ values for DPN$^+$ and L-lactate of about $10^{-5}$ and $10^{-3}$, respectively. These are an order of magnitude smaller than the values determined in the present investigation. The turnover numbers for two
fractions separated by electrophoresis of crystalline LDH from beef heart (Fractions A and C of Neilands) were 12,800 and 8100 moles of DPNH per minute per mole of enzyme, respectively, at pH 10 with $8.7 \times 10^{-4}$ M DPN$^+$ and $2.7 \times 10^{-2}$ M sodium m-lactate concentrations (31). Although the buffer and pH in the latter study are different from those employed in the present paper, the turnover numbers lie in the range of values presented in Table III. Since the turnover numbers are frequently employed in comparing the kinetic characteristics of enzymes, it may be suggested that the limiting value of the turnover number at infinite concentrations of substrates should be established as the most suitable reference for this comparison.

SUMMARY

1. A method in which ethanol and ammonium sulfate at low temperatures are employed in the isolation and crystallization of lactic dehydrogenase from rat liver has been described.

2. The nitrogen content, specific absorption coefficient (280 m$\mu$), and amino acid composition of the purified enzyme were determined.

3. Kinetic studies demonstrated that the Michaelis-Menten constants for one substrate were dependent upon the concentration of the second substrate. The values of $K_M$ and $V_M$ for four different concentrations of lactate and oxidized DPN are reported.

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DEHYDROGENASE: I. ISOLATION AND
CHEMICAL PROPERTIES OF THE
CRYSTALLINE ENZYME

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