Determination of Dissociation Constants of Coenzymes and Abortive Ternary Complexes with Rabbit Muscle Lactate Dehydrogenase from Fluorescence Measurements*

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Studies on the mechanism of substrate interaction with rabbit muscle lactate dehydrogenase have recently been reported (1). These investigations, which were primarily kinetic in nature, indicated that nicotinamide adenine dinucleotide and reduced nicotinamide adenine dinucleotide must add to the enzyme before the binding of substrates pyruvate and L-lactate. It was also noted that ternary complexes of lactate dehydrogenase, coenzymes, and substrates are short-lived relative to dissociation of the binary complexes.

Many of the definitive conclusions alluded to in the earlier study (1) were made on the basis of kinetic experiments carried out with and without product (2, 3). Equations derived to evaluate such experiments assumed the formation of abortive ternary complexes, enzyme-NAD-pyruvate and enzyme-NADH-L-lactate (1). Ternary complexes of these types have been assumed for other enzyme systems from kinetic (1, 2, 4, 5), spectrophotometric (6), and spectrofluorometric studies (7–9). The possible formation of abortive ternary complexes in kinetic studies of product inhibition and their effect has recently been suggested (1, 2, 4).

It is obviously desirable to correlate various parameters obtained from kinetic experiments with those determined by other means. In the latter case, a homogeneous enzyme preparation of known molecular weight is a prerequisite for such determinations. Hayes and Velick (10) were able to measure certain dissociation constants for glyceraldehyde 3-phosphate dehydrogenase by employing the technique of high speed centrifugation. Takcnaka and Schwert (11) utilized a similar procedure in the case of heart muscle lactate dehydrogenase. More recently, investigators have taken advantage of alterations in the fluorescence spectra of enzymes and substrates to evaluate dissociation constants of binary and abortive ternary complexes (7–9, 12, 13).

The purpose of the present report is to present data obtained from fluorescence experiments of rabbit muscle lactate dehydrogenase. The results lend support to the mechanism of substrate and enzyme interaction proposed earlier (1).

**EXPERIMENTAL PROCEDURE**

Materials—Lactate dehydrogenase was a product of Boehringer and Sons and the sodium salt of NADPH was purchased from Sigma Chemical Company. Sodium pyruvate and zinc L-lactate were obtained from the California Corporation for Biochemical Research and Pfannstiehl Laboratories, respectively. NAD was purchased from Pabst Laboratories.

**Determinations—** Procedures for the analysis of pyruvate and lactate have been described elsewhere (1). Protein was determined colorimetrically (14) with a solution of albumin (Armour and Company) as a standard. Zinc L-lactate was converted to the acid by passage through Dowex 50 (H+) (15) and neutralized with sodium hydroxide.

The sodium salt of NAD was purified on DEAE-cellulose by gradient elution with Tris-Cl buffer. The reduced form of the nucleotide was prepared enzymatically with yeast alcohol dehydrogenase and isolated as the sodium salt (16). The ratio of absorbancies at 260 μμμ and 340 μμμ was 2.4.

Fluorescence measurements were made in an Aminco-Bowman spectrofluorometer equipped with an Electro Instruments X-Y recorder. The cell housing of the instrument was modified to contain a water jacket through which warm water was passed to maintain a constant temperature. Spectra were taken directly from the electrometer for calculation of dissociation constants. A recorder tracing of the enzyme-NAD and enzyme-NADH-L-lactate complexes was made from a recorder tracing.

Additions of reagents to 1.0-ml buffer solutions in cuvettes were by means of micropipettes. All calculations were corrected for dilution of solutions and fluorescence of reagents. The slit widths of the spectrofluorometer were combinations of sizes recommended by the manufacturer to be used with a 1/2 inch photomultiplier slit width. The spectrofluorometer was equipped with an RCA 1P28 photomultiplier tube, and the wave length scale of the fluorescence monochrometer was calibrated with a mercury vapor lamp (American Instrument Company, Inc.).

Velocity measurements were made in a fluorometer (17) and activity of lactate dehydrogenase was determined from the lactate side of the reaction from initial reaction velocity tracings. Absorbancy determinations were carried out in a Beckman model DU spectrophotometer.

**RESULTS**

Preparation and Analysis of Lactate Dehydrogenase—The commercial preparation of lactate dehydrogenase was found to have an absorbancy ratio of 1.18 at A₂₆₀: A₃₄₀. To 50 mg of the enzyme suspension contained in 10 ml were added 10 ml of saturated ammonium sulfate, pH 7.9. (The salt had previously been

* K. Dalziel, personal communication.
recedrized from a solution containing EDTA.) After centrifuga-
tion of the suspension for 20 minutes at 15,000 \(\times g\), the
resulting precipitate was dissolved in 4.5 ml of 0.1 m sodium
phosphate, \(pH\) 7.6. The ratio of absorbancies at 280 \(\mu m\) and 260
\(\mu m\) was found to be 1.45. The enzyme solution was finally
diluted in a rocking dialyzer through which 2 liters of 0.1 m sodium
phosphate buffer, \(pH\) 7.6, were passed. The slightly turbid
solution was centrifuged as above, after which the ratio of ab-
sorbancies at 280 \(\mu m\) and 260 \(\mu m\) was determined to be 1.62.
All manipulations of the enzyme were at 3°.

A solution of lactate dehydrogenase (0 \(mg\) per ml) was ana-
alyzed for homogeneity in the Spinco model E ultracentrifuge in
0.1 m sodium phosphate buffer, \(pH\) 7.6. It appeared to migrate
as a single component over a 50-minute period in the AN-D
rotor at 59,780 r.p.m. at temperatures below 14°. A slower
moving component appeared after approximately 30 minutes at
higher temperatures above 20°. This effect was accompanied by an
obvious turbidity of the protein solution and probably represents
denaturation of the active enzyme.

Molecular weight determinations were made at 3° in synthetic
boundary cells by the method of sedimentation velocity. Analy-
sis of the diffusion constant data indicated a single protein com-
ponent in the enzyme preparation. Concentrations of 3, 4, and
6 \(mg\) per ml of lactate dehydrogenase were subjected to analysis
in the ultracentrifuge. There was no evidence of molecular
weight alteration with change in protein concentration in the
range studied. The results of these experiments are presented in
Table I. The molecular weight of the enzyme was calculated
to be 132,000 and the extinction coefficient at 280 \(\mu m\), 1.62 \(\times\)
10^5 \(M^{-1} cm^{-1}\), was found to be 1.45. The enzyme solution was finally dia-
lyzed in a rocking dialyzer through which 2 liters of 0.1 m sodium
phosphate buffer, \(pH\) 7.6, were passed. The slightly turbid
solution was centrifuged as above, after which the ratio of ab-
sorbancies at 280 \(\mu m\) and 260 \(\mu m\) was determined to be 1.62.
All manipulations of the enzyme were at 3°.

Table I

<table>
<thead>
<tr>
<th>Protein concentration (mg/ml)</th>
<th>(s_{20,w} \times 10^{13})</th>
<th>(D_{20,w} \times 10^{10})</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>7.443</td>
<td>4.97 ± 0.19*</td>
</tr>
<tr>
<td>4</td>
<td>7.307</td>
<td>5.41 ± 0.27</td>
</tr>
<tr>
<td>6</td>
<td>7.432</td>
<td>5.01 ± 0.65</td>
</tr>
</tbody>
</table>

* Standard deviation of the mean.
Dissociation Constants of Coenzymes and Abortive Complexes

The data of Fig. 1 were treated analogously to those described for the titration of Fig. 2. At infinite NADH concentration, 

\[(\alpha' - \beta) = \frac{F}{E_0}\]

where \(E_0\) equals total enzyme concentration. The data for Fig. 1 were used to calculate \(\beta' : \beta\) assuming a molecular weight of 132,000 for lactate dehydrogenase. The values obtained for \(\beta' : \beta\) were 4.45, 3.61, and 3.08, for three, four, and five binding sites per mole of enzyme, respectively. These findings which are shown in Table II led to the conclusion that the rabbit muscle enzyme, like heart muscle lactate dehydrogenase (12, 13), contains four coenzyme binding sites per mole of enzyme.

It was possible to evaluate the dissociation constant \((K_{ER})\) for lactate dehydrogenase and NADH from the equation 

\[K_{ER} = \frac{(E_0 - ER)(R_0 - ER)}{ER}\]

where 

\[ER = \frac{F - BR_0}{(\alpha' - \beta)}\]

and the data shown in Figs. 1 and 2. The value obtained for this constant was 2.1 \(\mu\)M from Fig. 1, and 2.4 \(\mu\)M from Fig. 2. The points shown for the titration experiments are experimental points. The curves were obtained from theoretical points assuming the calculated dissociation constants. The values for \(\beta\) and \(\beta'\) are shown in the legends to the figures.

It is noteworthy that the fluorescence spectrum of NADPH did not appear to be altered in the presence of lactate dehydrogenase (3 \(\mu\)M NADPH and 3 \(\mu\)M enzyme). This observation correlates well with the finding of Mehler et al. (20) that NADPH is less than 1% as active kinetically as NADH with the rabbit muscle enzyme.

It was desirable to correlate the dissociation constants evaluated from measurements of nucleotide fluorescence with those obtained from protein fluorescence and its quenching by NADH. In Fig. 3 are shown results obtained when protein fluorescence at 350 nm was determined upon the addition of the coenzyme substrate. It was possible to determine the dissociation constant from these experiments in a manner similar to that described above. Here, however,

\[F = \beta E_0 + \beta' ER\]

A double reciprocal plot of 1/fluorescence versus 1/NADH con-
The present study are in reasonable agreement with kinetic experiments. It would appear that the results of the kinetic experiments reported earlier (1) gave a value of 0.93 mM for this constant.

Correlation of Binding Sites with Enzymatic Activity—It would appear from the preceding discussion that a fairly good correlation exists between the dissociation constants determined kinetically (1) for lactate dehydrogenase and coenzymes, and those obtained from fluorescence titrations. Frieden (22) has reported poor agreement between dissociation constants calculated by these two procedures for glutamic dehydrogenase. A priori, there is no reason to believe that the four coenzyme binding sites are all associated with enzymatic activity. It was desirable, however, to attempt to correlate enzymatic activity with lactate dehydrogenase binding sites. To achieve this end, enzyme solutions were heated in a water bath, cooled in an ice bath, and then divided into two aliquots for analysis. One aliquot was used to determine the number of binding sites as indicated in the legend to Fig. 1 while the second was diluted with ice-cold 1% plasma albumin solution (Armour and Company) and its activity calculated from kinetic experiments.

The results obtained from these studies are illustrated in Table III. Cursory examination of these data indicates that heat inactivation leads to a progressive loss of both enzymatic activity and the number of binding sites for coenzyme. Furthermore, there appears to be an good correlation between the two. It might therefore be concluded that enhancement of NADH fluorescence in the presence of lactate dehydrogenase may be related to enzymatic activity.

**Table III**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time of incubation</th>
<th>% Active sites</th>
<th>% Activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>28°</td>
<td>5</td>
<td>4.00</td>
<td>100</td>
</tr>
<tr>
<td>41</td>
<td>5</td>
<td>3.20</td>
<td>80</td>
</tr>
<tr>
<td>50</td>
<td>5</td>
<td>2.36</td>
<td>50</td>
</tr>
<tr>
<td>60</td>
<td>1</td>
<td>0.52</td>
<td>10</td>
</tr>
</tbody>
</table>

* Calculated from titrations with NADH as indicated in Fig. 1.
† Determined in an assay mixture containing 1.47 X 10⁻² M L-lactate, 1.28 X 10⁻⁴ M NAD, and 0.05 M sodium phosphate buffer, pH 6.9, at 28°.
Estimation of Dissociation Constants of Abortive Ternary Complexes—Abortive ternary complexes thought to be formed in a number of enzymatic reactions have been suggested from kinetic (1, 2, 4, 5), spectrophotometric (6), and spectrofluorometric (7-9) investigations. It was of interest to attempt to determine the dissociation constants of these complexes fluorometrically and correlate such results with those evaluated kinetically (1).

It was observed that protein fluorescence, when measured at 350 μμ, was not affected by levels of pyruvate which might be expected from kinetic experiments to form a complex of the type enzyme-NAD-pyruvate. It was also found that pyruvate, when added to a solution of lactate dehydrogenase and NAD in which protein fluorescence was partially quenched by the coenzyme, caused additional quenching of the protein fluorescence.

In Fig. 5 are shown data which were used to evaluate the dissociation constant of the oxidized abortive ternary complex (EOS). In this experiment, approximately 25% of the enzyme fluorescence was quenched by NAD, after which small amounts of pyruvate were added and fluorescence quenching recorded. Pyruvate in this case serves to remove enzyme-NAD, which in turn results in a decrease in the concentration of free enzyme so as to maintain KEO constant. Calculations for the dissociation constant of the oxidized abortive ternary complex, KEOs, were made using Equation 3 below and substituting Eo, Os, KEO, KEOs for Eo, Es, KEO, and KEOs, respectively. KEO was taken to be 0.91 mM. The value for β (molar emittance of enzyme-NAD-pyruvate) was obtained by extrapolating the data of Fig. 5 to infinite pyruvate concentration. Pyruvate at infinite concentration, in the presence of NAD, was found to quench lactate dehydrogenase fluorescence approximately 88%. β and β' (molar emittances of enzyme and enzyme-NAD) were evaluated as indicated above.

Pyruvate, like NAD, absorbs light at 305 μμ. The apparent quenching of protein fluorescence by both pyruvate and NAD was taken into consideration before plotting the data shown in Fig. 5.

The average dissociation constant found for the complex enzyme-NAD-pyruvate was 0.30 mM from the data of Fig. 5. The value of this complex reported from kinetic experiments was 0.20 mM (1).

Fluorescence enhancement of enzyme-NADH spectra upon the addition of reduced substrate has been observed with a number of enzyme systems (7-9). It was noted that L-lactate was capable of augmenting the fluorescence of the binary rabbit muscle lactate dehydrogenase-NADH spectrum. This effect of reduced substrate is illustrated in Fig. 6. The spectra have been corrected for small but significant fluorescence contributions of the buffer, enzyme, and L-lactate. It can be seen from the figure that L-lactate enhances and also shifts the spectrum of the binary complex approximately 10 μμ to shorter wave lengths.

It was possible, on the basis of these observations of alterations of the binary complex spectrum upon addition of reduced substrate, to calculate the dissociation constant of the enzyme-NADH-L-lactate complex. The graph in Fig. 7 depicts results obtained when L-lactate was added to a solution containing lactate dehydrogenase and NADH. In this experiment the enzyme concentration exceeded that of NADH by a factor of 8.06. By using 2.5 μμ as the dissociation constant for the

![Fig. 5](https://example.com/fig5.png)

Fig. 5. Plot of fluorescence intensity at 350 μμ versus molar concentration of pyruvate. Measurements were made on solutions containing 0.05 mM sodium phosphate buffer, pH 6.9, 0.4 μμ lactate dehydrogenase, and 0.5 mM NAD. The solutions were activated with light at 350 μμ. Fluorescence readings were corrected for buffer and pyruvate fluorescence, dilution by added pyruvate, and light absorption due to NAD and pyruvate. The dissociation constant for the enzyme-NAD-pyruvate complex was calculated from equations indicated in the text and was found to be 0.3 μμ. The points shown in the figure were determined experimentally. The curve was obtained from theoretical points assuming the experimentally determined dissociation constant. The values for β, β', and β'', determined as indicated in the text, were 54.6 × 10^{-4} M^{-1} cm^{-1}, 16.4 × 10^{4} M^{-1} cm^{-1}, and 6.6 × 10^{4} M^{-1} cm^{-1}, respectively.

![Fig. 6](https://example.com/fig6.png)

Fig. 6. Plot of fluorescence (inches) intensity versus wavelength (millimicrons). Measurements were made with an X-Y recorder. The solutions were activated with light at 350 μμ, and contained 0.05 mM sodium phosphate buffer, pH 6.9. The lower curve represents 21.3 μμ NADH (corrected for fluorescence of buffer). The middle curve represents 21.3 μμ NADH plus 1.8 μμ lactate dehydrogenase (corrected for buffer and enzyme fluorescence). The upper curve represents 21.3 μμ NADH, 1.8 μμ lactate dehydrogenase, and 0.161 μμ L-lactate (corrected for buffer, enzyme, and L-lactate fluorescence). All recordings were corrected for dilution.
binary complex it was possible to estimate the amount of free enzyme. At infinite L-lactate concentration the amount of free enzyme could not decrease by more than 2.5%.

The fluorescence (F) observed in Fig. 7 is due to three species, NADH (R), enzyme-NADH (ER), and enzyme-NADH-L-lactate (ERS). This may be expressed as follows

\[ F = \beta R + \beta' ER + \beta'' ERS \]  

where \( \beta, \beta', \) and \( \beta'' \) represent the appropriate “molar emissions” (13). From the expressions

\[ R_0 = R + ER + ERS \]

and

\[ K_{BR} = \frac{(E_f)(R_f)}{ER} \]

the following equation is obtained

\[ F = \beta'' R_0 + \left( \frac{K_{BR}(\beta - \beta'')}{E_f} + (\beta' - \beta'') \right) \]  

where \( R_0 \) represents total NADH, \( E_f \), free enzyme, \( ER \), enzyme-NADH, and \( K_{BR} \), the dissociation constant of the binary complex.

From the foregoing, it can be seen that

\[ K_{BR} \frac{R_f - ER - ERS = E_f}{ER} \]

and similarly

\[ \frac{S_f}{K_{ERS}} = \frac{ERS}{ER} \]

where \( S_f \) is taken to be L-lactate and \( K_{ERS} \), the dissociation constant for ERS. Thus

\[ \frac{ER}{E_f} = \frac{R_f}{1 + \frac{K_{BR}}{E_f} + \frac{S_f}{K_{ERS}}} \]

By substituting this expression into Equation 2 in place of \( ER \), the following equation which permits calculation of \( K_{ERS} \) from the data of Fig. 7 is obtained.

\[ F = \beta'' R_0 + \left( \frac{K_{BR}(\beta - \beta'')}{E_f} + (\beta' - \beta'') \right) \frac{R_0}{1 + \frac{K_{BR}}{E_f} + \frac{S_f}{K_{ERS}}} \]  

The value obtained for the dissociation constant of the reduced abortive ternary complex was 38 mM.

The figure reported for this constant from kinetic studies of the lactate dehydrogenase system varied between 14.4 mM and 0.6 mM (1).

**DISCUSSION**

The question necessarily arises in studies of the type presented in this report as to whether one is in fact measuring dissociation constants associated with enzymatic activity. Clearly, the possibility of nonspecific substrate binding to the enzyme cannot be discounted. That the outlined experiments may in fact be a measurement of dissociation constants associated with enzymatic activity is suggested by two lines of evidence. Firstly, it seems quite unlikely that values for four enzyme-substrate dissociation complexes could, from kinetic and fluorometric experiments, agree so well for lactate dehydrogenase, simply by chance. Secondly, lactate dehydrogenase does not appear to alter the fluorescence spectrum of NADPH. This nucleotide is relatively inactive with the enzyme compared to NADH (20).

As pointed out by Velick (12) from studies of two other anerobic dehydrogenases, one may take advantage of changes either of protein or NADH fluorescence in measuring dissociation constants of enzyme-coenzyme complexes. Good agreement was obtained when either of these parameters was measured in the case of the enzyme-NADH complex with the rabbit muscle enzyme. This observation would appear to place more credence on those experiments involving NAD in which only alterations of protein fluorescence could be measured.

The findings presented in the present investigation would appear to support many of the conclusions alluded to from kinetic studies of lactate dehydrogenase (1). The good agreement of the data obtained between the two experimental approaches would appear to support the mechanism originally proposed for lactate dehydrogenase (1), i.e. a “Theorell-Chance” (21) type of mechanism. Although it is theoretically possible to exclude other pathways of enzyme and substrate interaction which give rate expressions of the “Theorell-Chance” type by employing the Haldane relationship as suggested by Alberty (23), or the equations relating rate constants advanced by Dalziel (24), the data for lactate dehydrogenase were not precise enough for one to use these equations to draw definitive conclusions. Kinetic studies on the effect of product inhibition suggested the type of interaction between lactate dehydrogenase and its substrates outlined above (1). The present findings, particularly those relative to the abortive ternary complexes,
would seem to strengthen those postulations concerning the treatment of kinetic data obtained from studies involving reaction products (2).

Up to the time of the present investigation the rabbit muscle enzyme was reported to be electrophoretically inhomogeneous (26). Carlström has reported the commercial enzyme preparation to be homogeneous in the ultracentrifuge but inhomogeneous by other criteria (19). It would appear that the enzyme has now been purified to the point of electrophoretic homogeneity.

Miller (26) has reported that beef heart lactate dehydrogenase dissociates at concentrations below 2 mg per ml. The lowest concentration of the rabbit muscle enzyme studied in the ultracentrifuge was 3 mg per ml. Quite possibly the skeletal muscle enzyme will depolymerize in more dilute solutions.

Thomson and Darling (27) recently investigated the mechanism of substrate interaction with rabbit muscle lactate dehydrogenase. These authors carried out kinetic experiments with NADH and α-NADHP and observed a significant decrease in ϕ₀, H/φ₀ D with lactate dehydrogenase. (The significance of ϕ₀ as defined by Dalziel (24).) The authors suggested that these data appear to exclude the “Theorell-Chance” mechanism and substrates in which the enzyme-substrate ternary complexes are kinetically significant ternary complexes instead (27).

It is the opinion of this author that the approach used by Thomson and Darling (27) may serve to establish the binary complex mechanism under certain conditions; however, it cannot be employed to exclude this mechanism in preference to a ternary complex mechanism. φ₀ as defined by Dalziel (24) is equal to 1/k₃₂ + 1/k₃₄ for an ordered enzyme and substrate pathway leading to a kinetically significant ternary complex. For the “Theorell-Chance” mechanism k₃₄ > k₃₂, and φ₀ is thus equal to 1/k₃₂. In the presence of α-NADHP, k₃₄ will decrease in the ternary complex mechanism, thus φ₀ D will increase. In the “Theorell-Chance” sequence k₃₄ must also decrease in the presence of α-NADHP; however, it is not possible to predict whether this decrease will result in k₃₄ being much greater than k₃₂, or in the same range as k₃₂. In the former case φ₀ H/φ₀ D will equal unity. In the latter instance the ratio will decrease. For these reasons, it is felt the evidence presented by Thomson and Darling (27) does not permit one to exclude the mechanism originally proposed for lactate dehydrogenase (1).

It is of interest to note that when the number of NADH binding sites was estimated by the procedure of Velick (12) for rabbit muscle lactate dehydrogenase a value of from three to five sites per mole of enzyme was obtained. If one differentiates Equation 5 of Winer et al. (13) to determine the equation of the line where coenzyme equals zero and sets this equation equal to

\[ F = (β' - β)nE₀ \]

it is apparent that

\[ R₀ = nE₀ + K_{ER} \]

Here F, β', β, n, E₀, R₀, and K_{ER} represent fluorescence due to enzyme-NADH, molar emittance of enzyme-NADH, molar emittance of NADH, number of binding sites per mole of enzyme, total enzyme concentration, total coenzyme concentration, and the dissociation constant of the enzyme-NADH complex, respectively. Thus, one can determine the number of binding sites per mole of enzyme from the break in the titration curve only when \( K_{ER} \ll nE₀ \). The dissociation constants of the binary complexes studied by Velick (12) were sufficiently low for the application of this approach; however, it was not valid in the case of rabbit muscle lactate dehydrogenase in which \( K_{ER} \) is relatively high.

**Summary**

Commercially obtained lactate dehydrogenase from rabbit muscle after treatment with ammonium sulfate followed by dialysis was found to be homogenous in the ultracentrifuge. The molecular weight of the enzyme was calculated to be 132,000. It migrated as a single protein when subjected to various types of electrophoresis.

The dissociation constants of the enzyme-coenzyme binary complexes and of the enzyme-reduced coenzyme-reduced substrate and enzyme-oxidized coenzyme-oxidised substrate ternary complexes were determined from fluorescence titrations. The enzyme does not cause an alteration in the spectrum of NADPH. Lactate dehydrogenase appears to contain four coenzyme binding sites per mole of enzyme.

The results are consistent with data from kinetic experiments which suggested a compulsory binding sequence of enzyme and substrates in which the enzyme-substrate ternary complexes are short-lived relative to the enzyme-substrate binary complexes.

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**References**

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Determination of Dissociation Constants of Coenzymes and Abortive Ternary Complexes with Rabbit Muscle Lactate Dehydrogenase from Fluorescence Measurements
Herbert J. Fromm


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