STUDIES ON LACTIC DEHYDROGENASE OF HEART

II. A COMPOUND OF LACTIC DEHYDROGENASE AND REDUCED PYRIDINE NUCLEOTIDE*

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(Received for publication, June 9, 1952)

Although the shift in the absorption band of DPNH upon the addition of ADH is sufficient to be readily measurable in the ordinary spectrophotometer (1), we have previously been unable to detect a similar shift in the presence of yeast ADH or glutamic dehydrogenase. However, the existence of such a shift has been observed in respiring yeast cells and bacteria (2), and thus there can be little doubt as to the generality of this phenomenon. This paper describes the successful detection and preliminary study of a compound of DPNH and LDH that has a number of properties in common with the one formed by ADH (1, 3).

Preparations

These are described in Paper I (4). The bulk of these experiments was carried out with the “unresolved” LDH.

Method

In a search for spectrophotometric evidence for the binding of LDH by DPNH, several improvements of the technique used for the study of the ADH-DPNH complex (3) were necessary. The LDH-DPNH complex is more highly dissociated and is therefore much more difficult to detect. First, the present experiments were carried out at 5°, since some increase of the affinity of LDH for DPNH would be expected at the lower temperature. Also, turbidity changes caused by the protein component were minimized by operating at 5°. Secondly, in order to add very large amounts of DPNH (up to 70 µM) without causing a deflection of the spectropho-

* This research was supported in part by a grant from the Division of Research Grants and Fellowships, United States Public Health Service, and from the Office of Naval Research.
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LDH = lactic dehydrogenase; ADH = horse liver alcohol dehydrogenase; DPNH = reduced diphosphopyridine nucleotide.

* Chance, B., unpublished observations.
tometric trace, the alternating current output of the photomultiplier was connected only to the alternating current amplifier of Fig. 1 of Theorell and Chance (3) (see also Chance (5)), and not to the first vibrating switch. Thirdly, the total light absorption by the DPNH was so great that the slits of the monochromator were reset to compensate for the loss of light intensity after 2 or 3 additions of \( \sim 8 \mu\text{M} \) DPNH. With these modifications, we can detect a difference in the light absorption at 328 and 353 m\( \mu \) of log \( I_0/I = 0.0008 \) in the presence of DPNH absorption as great as log \( I_0/I = 0.400 \).

In addition to these titration studies, we have recorded the spectrum of the LDH-DPNH complex by a sensitive spectrophotometer that rapidly plots the difference of optical density of two cuvettes with an error of about \( 2 \times 10^{-4} \) in the region 310 to 390 m\( \mu \) (6).

**EXPERIMENTAL**

*Titrations with LDH*—The effect of added DPNH upon the optical density changes at 328 and 353 m\( \mu \) in the presence of varying amounts of LDH is shown in Fig. 1. The blank run with zero LDH shows that the absorption of DPNH alone causes little deflection of the spectrophotometric readings. If LDH is present, there is a progressive increase in the optical density change up to a limit set by the LDH concentration. The optical density change divided by the initial LDH concentration corresponds to an increment of molecular extinction coefficient of 2.0 and 2.3 cm.\(^{-1}\) \( \times \) mm\(^{-1}\) for 6.1 and 3.1 m\( \mu \) LDH respectively. This value is in good agreement with the corresponding value for the ADH-DPNH complex, 2.4 cm.\(^{-1}\) \( \times \) mm\(^{-1}\) (1). The type of complex formed with LDH would seem to be similar to that formed with ADH, at least on a spectroscopic basis. In this case, the stoichiometry of the reaction LDH and DPNH is 1:1, inasmuch as we used the LDH molarity to calculate the values 2.0 and 2.3 cm.\(^{-1}\) \( \times \) mm\(^{-1}\).

The added DPNH concentration that gives half maximum concentration is about 10 m\( \mu \), over 3 times the stoichiometric amount; thus we cannot independently determine the number of DPNH molecules bound per LDH molecule under these experimental conditions. But on the basis of the 1:1 stoichiometry indicated by the spectrophotometric data, we may calculate apparent dissociation constants of 7.5 and \( 7 \times 10^{-6} \) m for 3.1 and 6.1 m\( \mu \) LDH respectively at 5\( ^\circ \).

In order to investigate further the stoichiometry of the reaction, higher concentrations of LDH were used, but, for some reason that we are unable to explain, the apparent dissociation constant increased and unsatisfactory results were obtained.

*Spectrum of LDH-DPNH*—An approximation to the spectrum of the
LDH-DPNH complex was obtained in the recording spectrophotometer (6) by using the following procedure. 12 μM LDH are placed in one cuvette and its absorption is plotted relative to the buffer solution in the other cuvette in the region 315 to 390 mμ (Spectrum A). Equal volumes of DPNH are then added to both cuvettes to give a final concentration of 21 μM. The resultant spectrum (B) is recorded and represents the shift of the DPNH band caused by LDH binding, plus the absorption of the LDH protein. Thus the difference of Spectra B and A gives the spectrum of the LDH-DPNH complex as plotted3 in Fig. 2. Although the shift of the DPNH band shown in Fig. 2 is only 15 per cent of the total

![Fig. 1](http://www.jbc.org/)

**Fig. 1.** The titration of LDH with DPNH. The optical density changes at 353 mμ are subtracted from those at 328 mμ as DPNH is added to the LDH solutions of the indicated concentrations. The values of the abscissa are for added DPNH. A control experiment is included (△). 5°, pH 7.15, 0.15 M phosphate buffer (Experiment 947b).

**Fig. 2.** An approximate spectrum of the LDH-DPNH complex. 12.7 μM LDH plus 21 μM DPNH, 25°; pH 7.15, 0.15 M phosphate buffer (Experiment 949d-1).

DPNH absorption, we believe that this method is fairly free from artifacts; if unequal volumes of DPNH were added, then a 340 mμ peak would be registered. The actual spectrum has a peak at 330 mμ compared with 325 mμ for the ADH-DPNH complex. The absorption at 350 mμ is 75 per cent of that at 330 mμ, compared with a value of 59 per cent for the ADH-DPNH complex. Thus only a fair agreement of the spectra of the two complexes is obtained.

3 The difference of absorption between the LDH-DPNH complex and LDH was not exactly zero in the region 390 to 400 mμ; the experimental data showed a difference of -0.005 in optical density, corresponding to negative absorption of the complex above 370 mμ and due to the imperfection of our experiment. We have arbitrarily lowered the base line for the experimental data by 0.005 to give the curve of Fig. 2.
The amount of DPNH bound to LDH in this experiment at 25° is much smaller than that calculated from the apparent dissociation constant of \(7 \times 10^{-6} \text{ M}\) obtained at 5° with dilute enzyme.

**DISCUSSION**

The results above clearly indicate that the spectrum of DPNH may be shifted by the protein, LDH, in a manner analogous to that caused by ADH. The LDH-DPNH complex is, however, much more difficult to study experimentally, and we cannot put forward the detailed kinetic data needed to establish that this complex is a rate-limiting or Michaelis complex, as we have done in the case of the ADH-DPNH complex. If indeed these are functionally identical complexes, then the lack of inhibition of LDH activity by \(p\)-chloromercuribenzoate, contrasted to the sensitivity of ADH to this reagent, must be carefully considered in formulations of the mode of binding of DPNH to these proteins (1).

The rather large value of the apparent dissociation constant of the LDH-DPNH complex renders it difficult to detect by ordinary spectrophotometric methods; therefore, our sensitive methods, that permit studies of very dilute enzyme solutions, appear to be essential for this type of investigation.

A large dissociation constant for LDH-DPNH may well be a consequence of the relatively large value for the turnover for lactic acid (~50 sec.\(^{-1}\) at pH 7.0 and 25°) which requires a high dissociation velocity of the LDH-DPNH complex according to the mechanism discussed previously (3). On the assumption that the rate of combination of DPNH with LDH or ADH is the same, the dissociation constants of their DPN compounds would be in the ratio of the values of their turnover numbers with lactate or alcohol respectively (50:1.1 at pH 7.0 and 26° (3)). On this basis the dissociation constant of the LDH-DPNH complex would be \(50 \times 10^{-7}\) or \(5 \times 10^{-6}\) \(\text{M}\) compared with the value of the apparent dissociation constant of \(7 \times 10^{-6}\) \(\text{M}\) that we measure at 5°. In spite of the inequalities of temperature, we can put forward a satisfactory qualitative explanation of the effect. The value of the Michaelis constant (\(K_m\)) for DPNH in the presence of pyruvate must be greater than the dissociation constant, and a value of about \(10^{-5}\) \(\text{M}\) is found in studies of the enzyme activity (4). Therefore, it is suggested that those enzymes that show a high turnover number in DPN reduction may also have a large dissociation constant of their protein-DPNH complex. The latter would be difficult to detect spectrophotometrically and this may be the cause of our current failure to detect a complex of DPNH and yeast ADH.

The experiments reported thus far are based on the “unresolved” enzyme, but we have also identified the DPNH complex in both the “anode”
and "cathode" fractions of LDH. Interestingly enough, the "cathode" fraction forms less than one-half (0.4) as much DPNH complex per mole of protein as does the "anode" fraction (Experiment 949a). This roughly approximates the ratio of the enzymatic activities of the two fractions (8100:12,800 (4)). On this basis, the part of the protein content of the "cathode" fraction that binds DPNH is approximately as active enzymatically as the protein of the "anode" fraction, the "cathode" fraction being less pure.

SUMMARY

A shift in the absorption band of DPNH is caused by the addition of the protein, LDH. The DPNH concentration giving half maximum effect is $7 \times 10^{-6}$ M at 5° (for $<6 \mu$M of "unresolved" enzyme). Our data suggest that this is the dissociation constant of an LDH-DPNH complex in which 1 molecule of protein binds 1 molecule of DPNH in the same manner and with the same molecular extinction coefficient as in horse liver ADH. In view of the extreme difficulty of the experiment, quantitative values are regarded as preliminary.

Addendum—Recent tests in collaboration with Dr. J. Harting show that p-Chloromercuribenzoate neither prevents the binding of DPNH and LDH nor causes the splitting of the LDH-DPNH complex at concentrations of p-chloromercuribenzoate that we find to be quite effective in splitting the ADH-DPNH complex (1).

BIBLIOGRAPHY

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