

Fluorometric Studies of the Coupling of Reduced Diphosphopyridine Nucleotide to Lactic Dehydrogenase from Rabbit Skeletal Muscle

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Boyer and Theorell¹ in 1956 reported a shift in the fluorescence spectrum of DPNH* on binding to liver alcohol dehydrogenase. Since that time similar results have been described for many other pyridine nucleotide-requiring dehydrogenases²⁻⁷. Winer *et al.* have reported a shift in the fluorescence spectrum of DPNH when it is bound to beef heart lactic dehydrogenase⁶ as well as to the lactic dehydrogenase from rabbit skeletal muscle⁷. The observations concerning the skeletal muscle enzyme could not be confirmed by other workers⁸, but have now been confirmed in this laboratory. We have also found that these enzyme preparations have the peculiar property of totally quenching the fluorescence of the first part of DPNH added, whereas the fluorescence of subsequently added DPNH is increased. The nonfluorescing part of the coenzyme still exists, however, in its reduced form.

The change in the fluorescence spectrum of DPNH is further strengthened upon addition of sodium oxalate to the skeletal muscle LDH-DPNH complex. Similar results have been obtained for beef heart LDH⁹.

Experimental Rabbit skeletal muscle LDH was obtained from C. F. Boehringer und Soehne, G.m.b.H. ** The enzyme, which was assayed according to the method of Pfeleiderer and Jeckel¹⁰, was recrystallized from ammonium sulfate as described by Beisenhertz *et al.*¹¹ Protein concentrations were estimated spectrophotometrically at 280 m μ ($\epsilon_{280} = 1.27 \text{ cm}^2$

* Abbreviations: LDH, lactic dehydrogenase. DPNH, reduced diphosphopyridine nucleotide.

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$\times \text{mg}^{-1}$)¹¹. The molecular weight was assumed to be 100 000. Alcohol dehydrogenase from horse liver was a gift from Dr. C. L. Woronick of this institute whereas DPNH and sodium pyruvate were purchased from the Sigma Chemical Co., sodium oxalate, reagent grade, from J. T. Baker Chemical Co., and isobutyramide from Eastman Kodak Co. The titrations were performed at 23.5° in sodium phosphate buffers, pH 7.0, $\mu = 0.1$ (unless otherwise indicated), using a spectrophotofluorometer which previously has been described¹². The exciting wavelength was 340 m μ and the fluorescence was recorded at 410 m μ . Emission maxima in the fluorescence spectra are uncorrected for variations with wavelength in the intensity of the exciting light and sensitivity of the photocell. The correction would move

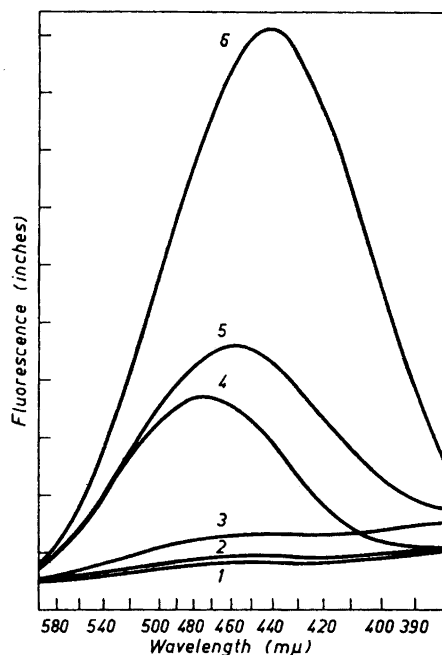


Fig. 1. Fluorescence emission spectra of the DPNH-LDH complex and the DPNH-LDH-oxalate complex. Curve (1): sodium phosphate buffer, $\mu = 0.1$, pH 7.0. Curve (2): 0.01 M sodium oxalate. Curve (3): LDH, 0.107 mg/ml. Curve (4): 1.17×10^{-6} M DPNH. Curve (5): LDH, 0.107 mg/ml plus 1.17×10^{-6} M DPNH. Curve (6): LDH, 0.107 mg/ml plus 1.17×10^{-6} M DPNH plus 0.01 M sodium oxalate. Activating wavelength 340 m μ . Temp. 23.5°. Final volume 2 ml.

the maxima about 15 $m\mu$ towards shorter wavelengths on this apparatus. Electrophoreses and ultracentrifugation were carried out in the Spinco Model H and Model E apparatus, respectively. CM-W cellulose for column chromatography was prepared according to Peterson and Sober¹³, the ethanoldrying step, however, being excluded.

Results and discussion. On binding of DPNH to LDH from rabbit skeletal muscle, the emission maximum for DPNH is shifted from 478 $m\mu$ to 460 $m\mu$, and the intensity of the emitted light is increased (Fig. 1). In the presence of 0.01 M sodium oxalate this effect is very much increased (*cf.* Fig. 1). The fluorescence maximum is shifted another 20 $m\mu$ towards shorter wavelengths and the maximal fluorescence intensity is increased about 3 times. In 1959 Winer and Schwert⁷ reported the same results concerning the binary complex, whereas Shifrin and Kaplan⁸ were not able to find any changes in the fluorescence spectrum of DPNH on binding to this enzyme. The spectral shift first found by Winer and Schwert, and now confirmed by us, should enable determinations of dissociation constants of LDH-coenzyme complexes¹⁴. However, upon titration of LDH with concentrations of DPNH which were small compared with the LDH concentration, the first added coenzyme gave smaller increments in the fluorescence intensity than that produced by free DPNH alone (Fig. 2). By decreasing the concentra-

tion of DPNH in each addition, it could be seen that the fluorescence is completely quenched for amounts of DPNH corresponding to about 1/10 or less of the total concentration of LDH. Six times recrystallization of the commercial enzyme preparation did not change this ratio, nor did prolonged dialysis against phosphate buffer, pH 7.0, $\mu = 0.1$. There was a possibility that the disappearance of fluorescence could be caused by oxidation of DPNH. Therefore the following experiments were made. The supernatant from heat-inactivated enzyme did not increase the quenching. Adding liver alcohol dehydrogenase and isobutyramide, which from a very tight complex with DPNH¹⁴, to an LDH-DPNH solution, liberated all the added DPNH in its reduced form. This means that the DPNH was not oxidized by any substrate that may have been present as an impurity in the enzyme preparation. Moving-boundary electrophoresis of the enzyme in phosphate buffers, pH 6.0–8.0, $\mu = 0.1$, gives an asymmetric peak which does not separate into definite peaks even after 24 h. The asymmetry corresponds to about 15 % of impurity which is in good agreement with the results of Ankel *et al.*¹⁵ In the ultracentrifuge, the protein appears homogeneous at pH 7.1, $\mu = 0.1$. On chromatography of the enzyme on CM-W cellulose with stepwise elution with phosphate buffers, pH 6.0–6.9, $\mu = 0.05$, the protein exhibits a very pronounced "tailing" with no sharp protein peak in the eluate. In one experiment, some of the enzyme which was eluted at pH 6.8, $\mu = 0.05$, upon titration in the same buffer, showed a considerable increase in the relative proportion of nonfluorescent complex formed with DPNH (nonfluorescent complex: fluorescent complex $\sim 1:1$).

A small fraction with a typical nucleic acid absorption spectrum was removed on the CM-W cellulose column. This fraction had no influence on the ability of LDH to quench the fluorescence of DPNH. Performing the chromatography a second time, an enzyme fraction was obtained which caused no detectable quenching of DPNH, but already from the beginning gave an increase in fluorescence. This enzyme fraction was obtained by an elution procedure similar to that in the first experiment. The specific activities of these enzyme preparations were about the same as those for the unchromatographed enzyme. The errors in the estimations of the specific activities of the chromatographed

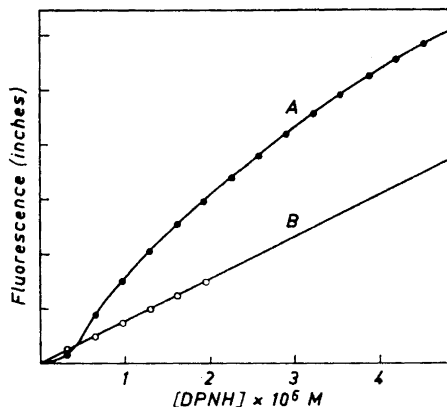


Fig. 2. Titration of LDH with DPNH (A). DPNH blank (B). The concentration of LDH is 0.087 mg/ml.

LDH are, however, quite large, because the eluted enzyme is very dilute, and the determinations of protein concentrations therefore inexact. If the enzyme is eluted in a more concentrated form no fractionation is produced. Higher specific activities are then obtained, but the large variations in the quenching of the DPNH fluorescence are not obtained.

These observations could be explained in several ways, a few of which will be mentioned. The LDH preparations used contain a protein impurity, which has a higher affinity for DPNH than has LDH, and it quenches the fluorescence of DPNH on complexing. A second explanation is that a small molecule is bound to the enzyme, which together with enzyme and DPNH forms a non-fluorescent ternary complex. The enzyme could also contain two different binding sites, one binding the coenzyme tightly and quenching its fluorescence, along with another site which binds it more loosely and enhancing its fluorescence. There is also a possibility that the conformation of the LDH is changed upon chromatography, thereby unmasking hidden binding sites. Polymerization or depolymerization could also explain our observations.

The present results might possibly explain the diverging results of different workers on this enzyme.

Work is continued in this laboratory to purify the enzyme and to determine some of its physical-chemical properties.

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1. Boyer, P. D. and Theorell, H. *Acta Chem. Scand.* **10** (1956) 447.
2. Duysens, L. N. M. and Kronenberg, G. H. M. *Biochim. et Biophys. Acta* **26** (1957) 437.
3. Theorell, H. and Langan, T. A. *Acta Chem. Scand.* **14** (1960) 933.
4. Langan, T. A. *Acta Chem. Scand.* **14** (1960) 936.

5. Winer, A. D. and Schwert, G. W. *Biochim. et Biophys. Acta* **29** (1958) 424.
6. Winer, A. D., Novoa, W. B. and Schwert, G. W. *J. Am. Chem. Soc.* **79** (1957) 6571.
7. Winer, A. D., Schwert, G. W. and Millar, D. B. S. *J. Biol. Chem.* **234** (1959) 1149.
8. Shifrin, S. and Kaplan, N. O. *Advances in Enzymol.* **22** (1960) 337, p. 366.
9. Winer, A. D. and Schwert, G. W. *J. Biol. Chem.* **234** (1959) 1155.
10. Pfeleiderer, G. and Jeckel, D. *Biochem. Z.* **329** (1957) 370.
11. Beisenhertz, G., Boltze, H. J., Bücher, Th., Czok, R., Garbade, K. H., Meyer-Arend, E. and Pfeleiderer, G. *Z. Naturforsch.* **8 B** (1953) 555.
12. Theorell, H. and Winer, A. D. *Arch. Biochem. Biophys.* **83** (1959) 291.
13. Peterson, E. A. and Sober, H. A. *J. Am. Chem. Soc.* **78** (1956) 751.
14. Winer, A. D. and Theorell, H. *Acta Chem. Scand.* **14** (1960) 1729.
15. Ankel, H., Bücher, Th., Czok, R., Schmiedtberger, R. and Schulze, H. Unpublished results, cf. Ref.¹⁴.
16. Czok, R. and Bücher, Th. *Advances in Protein Chem.* **15** (1960) 315, p. 367.

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Correction to "Microdetermination of Polyenoic Fatty Acids" *

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On p. 529, last line (the eqn. for diene fatty acids) the term $-k'_5 \times 0.961$ is incorrect and should read $-k'_5 \times 0.091$.

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