BIOORGANIC CHEMISTRY, 1, 207-233 (1971)

Addition Products of Diphosphopyridine Nucleotides with Substrates of Pyridine Nucleotide-Linked Dehydrogenases[1]

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Received March 8, 1971

Preparation of the reduced and oxidized forms of addition compounds from diphosphopyridine nucleotide and a number of carbonyl compounds are described; these include pyruvate, pyruvic ethylester, oxaloacetate, α-ketoglutarate, acetaldehyde, butyraldehyde, and α-ketobutyrate. The adducts are closely related to the DPN–acetone and the DPN–dihydroxyacetone adducts, both in their chemical and spectral properties. The chemical structures of the reduced and oxidized adducts have been determined and information concerning the mechanism of formation of the adducts is presented.

The lactate, malate, glutamate, and alcohol dehydrogenases show a unique specificity for the reduced adducts; the specificity was found to be related to the normal oxidized substrate utilized by the various enzymes. The inhibition is competitive with DPNH and noncompetitive with the substrate. Oxidized adducts produce considerably less inhibitory effects than the reduced adducts. Evidence is presented showing that the reduced adducts bind to the enzymes in a manner similar to that of the reduced coenzyme. On the basis of the high selectivity of the dehydrogenases for the adducts, a model is proposed which shows the spatial relationship between the reduced coenzyme and the oxidized substrate on the enzyme in the transition state. From this model, a general reaction mechanism is postulated for the pyridine nucleotide-dependent dehydrogenases, which may explain the stereospecificity of the hydrogen transfer with respect to the coenzyme as well as the substrate.

INTRODUCTION

In a preceding communication (*1*) we described the formation and the properties of abortive ternary complexes, which are formed by certain pyridine nucleotide-linked dehydrogenases with the oxidized pyridine nucleotides and their oxidized substrates. We presented evidence indicating that in such complexes the substrate is bound to the coenzyme. This binding occurs at the 4-position of the nicotinamide ring, and possesses covalent characteristics. The data suggested a possible nucleophilic attack by the pyruvate at the 4-position of the DPN^+ that could be facilitated by the enzyme.

Such addition products of substrate-related nucleophiles with DPN^+ have been known for some time. Burton and Kaplan (*3*) described the addition of dihydroxyacetone and glyceraldehyde to the DPN^+ molecule. Dolin and Jacobson (*4*) and Burton

[1] Publication No. 785 from the Graduate Department of Biochemistry, Brandeis University. This work was supported in part by research grants from the American Cancer Society (P77K) and the National Cancer Institute of the National Institutes of Health (CA-03611).

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and Kaplan (*5*) reported the formation of a DPN–acetone adduct. These additions also occurred at the 4-position of the nicotinamide ring. More recently, Lee et al. (*6*, *7*) reported the preparation of adducts of DPN^+ with fluoropyruvate as well as with pyruvate.

The acetone adduct of DPN^+ occurs in a reduced form (*5*), which is easily oxidized by air or certain oxidizing agents into an oxidized form. A reduced form of the DPN–pyruvate adduct was not observed by Lee et al., probably because an oxidizing agent (K-ferricyanide) was present during the preparation of the compound. Recently, however, Ozols and Marinetti (*8*) reported the preparation of the reduced as well as the oxidized form of the DPN-pyruvate adduct. Chemical structures for both forms were proposed by these authors on the basis of nmr and infrared spectral analyses. The structure proposed by these authors for the reduced form (which is identical to the structure presented here in Fig. 4), is somewhat similar to the structure of the DPN–pyruvate complex that we obtained after the denaturation of the abortive ternary complex of LDH–DPN^+-pyruvate (*1*).

It, therefore, became of interest to prepare the reduced form of the DPN-pyruvate adduct in a highly purified form, to determine its chemical structure, and to investigate the effects of this compound on LDH.[3] A comparison between the structure of the reduced DPN-pyruvate adduct and that of the coenzyme–substrate moiety of the lactate dehydrogenase abortive ternary complex could provide more information about the mechanism of action of LDH. Furthermore, since a mechanism similar to that of LDH is probably operating in other pyridine nucleotide-dependent dehydrogenases (*9*, *10*), it became of interest to prepare the reduced adducts of DPN^+ and some oxidized substrates of these dehydrogenases. We have investigated the possible relationship between the reduced adducts and the enzymatically formed coenzyme–substrate derivatives obtained from the abortive ternary complexes.

MATERIALS AND METHODS

Materials

Pyridine nucleotides were obtained from P-L Biochemicals, Milwaukee, Wisconsin; sodium pyruvate was purchased from Sigma Corp., oxaloacetate from Nutritional Biochemicals, and α-ketoglutarate from Calbiochem. DE-11 cellulose was obtained from Reeves-Angel, and Sephadex is a product of Pharmacia.

Crystalline chicken H_4 lactate dehydrogenase was prepared in our laboratory as described by Pesce et al. (*11*); mitochondrial malate dehydrogenase from chicken hearts was prepared by the method of Kitto et al. (*9*); and dogfish liver glutamate dehydrogenase was obtained by the method of Corman et al. (*10*). Alcohol dehydrogenases from yeast and equine liver were purchased from Boehringer and Sohne, New York.

All other reagents were purchased in the purest form available from commercial sources.

Methods

Preparation of reduced DPN–pyruvate adduct. Seven tenths millimole (500 mg) of DPN^+ and 7 mmoles (770 mg) of sodium pyruvate were dissolved in 3 ml of water, and the pH was adjusted to pH 11.0 with the addition of 1 *N* sodium hydroxide. The solution was left standing at room temperature for 1 hr. During this time the solution turned deep yellow. At the end of the incubation period the pH was made neutral with

[3] Abbreviations used: DPNase, diphosphopyridine nucleotidase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; GDH, glutamate dehydrogenase; ADH, alcohol dehydrogenase.

the addition of 1 *N* hydrochloric acid. The solution was diluted with 300 ml of water, and 10 ml of 0.1 *M* ammonium bicarbonate was added to the solution. The resulting solution was then applied to a DE-11 cellulose column (1.7 × 100 cm), which was equilibrated with 0.01 *M* ammonium bicarbonate. The column was developed using a linear gradient of 0–4% sodium chloride in 0.01 *M* ammonium bicarbonate. Fractions of 10 ml were collected, and the absorption at 260 and 340 mμ of each fraction was determined.

Figure 1 shows the elution pattern of a typical column. The first three peaks represent nicotinamide, pyruvate, and adenosine diphosphoribose, respectively. The fourth

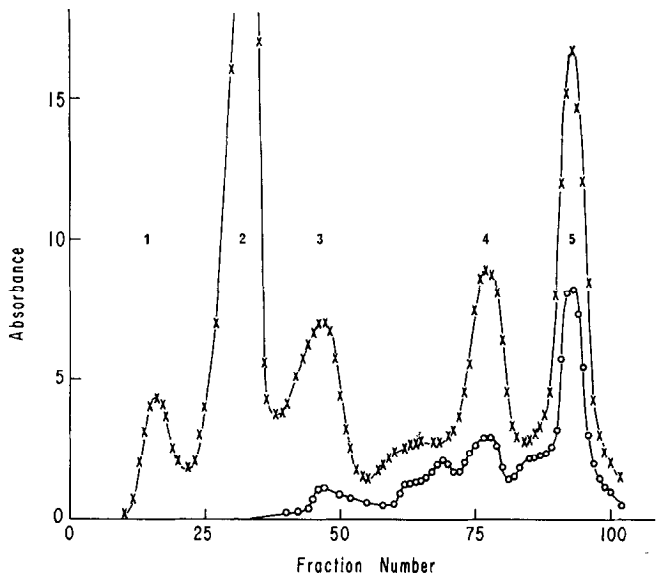


FIG. 1. Elution pattern of the reaction products of DPN^+ and pyruvate from a DE-11 cellulose column. Gradient: 0–4% NaCl in 0.01 *M* ammonium carbonate. ×---× absorbance at 260 mμ; ○---○ absorbance at 340 mμ.

peak represents the DPN–pyruvate adduct in the oxidized form, and the fifth peak shows the reduced form of the same compound. The appropriate fractions were pooled, and 1 ml of 1 *M* barium acetate was added to the solution. Cold acetone was then added till a concentration of 50% was achieved; the solution was then left standing overnight at −20°. The formed precipitate was collected by centrifugation. The faint yellow paste was resuspended in cold acetone and washed twice with acetone and twice with anhydrous ether at 0°. The preparation was then dried in a vacuum desiccator. Whenever possible, all operations were performed in the absence of oxygen. The compound was stored under nitrogen or under high vacuum, and remained stable for about two weeks at −20°.

The barium salt of the reduced DPN–pyruvate adduct can be redissolved in water by adding a few crystals of sodium sulfate. The formed barium sulfate may then be removed by centrifugation.

Preparation of other reduced DPN adducts. In addition to the DPN-pyruvate adduct, we have prepared the reduced DPN adducts of oxaloacetate, acetaldehyde, α-ketoglutarate, butyraldehyde, α-ketobutyrate, and pyruvic ethylester. A reduced DPN–pyruvate compound containing α-DPN was also prepared. The procedure given for the

reduced DPN–pyruvate adduct was used for the preparation of all other reduced compounds. The reduced DPN adducts are faintly yellow in solution as well as in the solid state, and were stored routinely in high vacuum at –20°.

Oxidation of the Reduced DPN–Pyruvate Adduct

One hundred milligrams of phenazine methosulfate were added to a solution of 500 mg of the reduced DPN–pyruvate adduct in 50 ml of water, and the mixture was stirred for 3 hr at room temperature. The resulting dark-yellow solution was filtered, diluted with 500 ml of 0.005 *M* ammonium bicarbonate, and applied to a DE-11 cellulose column. The column was developed with a 0–2% NaCl gradient in 0.005 *M* ammonium bicarbonate. The optical density was monitored at 260 and 370 mμ. The first peak that eluted from the column and absorbed at 370 mμ contained the oxidized adduct, whereas the second peak represented the phenazine methosulfate. The appropriate fractions were pooled and the solution of the oxidized adduct was acidified to pH 1.5 with 4 *N* HCl. Cold acetone was added until a concentration of 90% was obtained, and the suspension was kept overnight at –20°. The formed precipitate was separated from the supernatant fluid by centrifugation and washed twice with cold acetone and twice with anhydrous ether. The preparation was then dried in a vacuum desiccator.

The oxidation of all other DPN adducts was performed in an identical manner.

Spectrophotometry

Absorption spectra were recorded with a Cary Model 14 recording spectrophotometer, using 3-ml quartz absorption cells with a 10-mm light path.

Fluorescence spectra were obtained with a Zeiss ZMF-4C spectrophotofluorometer. All fluorescence spectra were measured from solutions that had an optical density of 0.100 at the excitation wavelength. The spectra were corrected for the sensitivity of the photomultiplier tube at the various wavelengths.

Nuclear magnetic resonance spectra were obtained with a high resolution Varian HRSC-1X superconducting solenoid 220 MHz nmr system. The measurements were made with solutions containing approximately 0.1 *M* of the compound in D_2O. The compounds were lyophilized twice from D_2O and dissolved in D_2O immediately prior to use. 2,2-Dimethyl-2-silapentane-5-sulfonate was employed as an internal standard.

Infrared spectra were recorded with a Perkin–Elmer Model 257 infrared spectrophotometer. The materials were dispersed in Nujol, and sandwiched between two NaCl disks.

The rates of formation of the reduced DPN-pyruvate adduct were recorded with an Aminco-Morrow stopped-flow apparatus, equipped with a fluorescence attachment. The data was stored on a Tektronix storage oscilloscope type R564B.

Chemical Analyses

Ribose was determined by the orcinol procedure (*12*, *13*). Inorganic phosphate and total phosphate were determined by the method of Fiske and SubbaRow (*14*). Analyses for the presence of carbonyl groups were carried out by the phenylhydrazine method, as described by Friedemann (*15*).

Enzymatic Assays

Lactate dehydrogenase. The following were contained in a 3-ml volume; 300 μmoles of potassium phosphate buffer, pH 7.0; 1.0 μmole of sodium pyruvate; 0.35 μmoles of DPNH, and a sufficient amount of enzyme to produce a decrease in optical density at 340 mμ of between 0.100 and 0.200 in 60 sec.

Malate dehydrogenase. The following ingredients were present in a total volume of 3 ml: 300 μmoles of potassium phosphate buffer, pH 7.5; 1.0 μmole of neutralized oxaloacetic acid; 0.35 μmoles of DPNH; and a sufficient amount of enzyme to produce a decrease in optical density of between 0.100 and 0.150/min at 340 mμ.

Glutamate dehydrogenase. In a total volume of 15 ml the following were present: 1500 μmoles of Tris–acetate buffer, pH 8.0, containing 1×10^{-5} *M* EDTA; 0.20 μmoles of DPNH; 750 μmoles of α-ketoglutarate; 500 μmoles of ammonium acetate; and a sufficient amount of enzyme to yield a decrease in optical density at 340 mμ of 0.020–0.050/min. These assays were performed in quartz cells with a 5-cm light path.

Alcohol dehydrogenase. Present in a total volume of 3 ml there were 300 μmoles of Tris–HCl buffer, pH 7.5; 0.35 μmoles of DPNH; 50 μmoles of acetaldehyde; and enough enzyme to produce a decrease in optical density at 340 mμ of 0.100–0.200/60 sec.

All enzymatic assays were performed at room temperature. Readings were taken every 15 sec for a period of 2–5 min. The initial rates were assumed to represent the activities of the enzymes.

RESULTS

Optical Properties of Reduced and Oxidized Adducts

Reduced adducts display absorption spectra with maxima around 260 and 340 mμ. These spectra bear a significant resemblance to the absorption spectrum of reduced DPN. Figure 2 presents the absorption spectrum of the reduced DPN–pyruvate adduct.

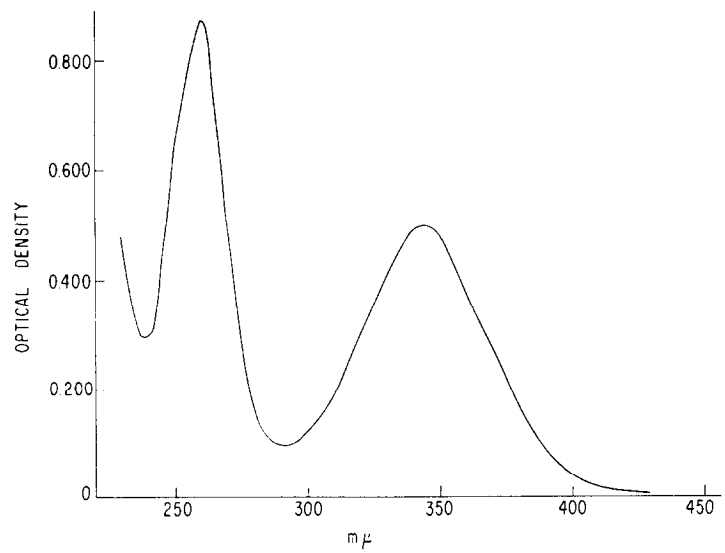


FIG. 2. Absorption spectrum of the reduced DPN–pyruvate adduct.

The absorption band at 340 mμ has a rather broad maximum, which is also present in the spectra of the other reduced adducts. Small shifts in λ_{max} occur between the various reduced adducts, dependent on the attached nucleophilic reagent. Table 1 summarizes the wavelength of maximum absorption for the reduced adducts which were prepared during this study.

TABLE 1

ABSORPTION MAXIMA OF THE DPN ADDUCTS

Adduct	Reduced adduct (mμ)	Oxidized adduct (mμ)		
		pH 1	pH 7	pH 12
β-DPN–pyruvate	340	360	360	415
α-DPN–pyruvate	350	365	375	410
β-DPN–pyruvate ethylester	345	370	370	418
β-DPN–oxaloacetate	343	370	373	415
β-DPN–acetaldehyde	345	358	358	405
β-DPN–α-ketoglutarate	347	375	370	415
β-DPN–butyraldehyde	344	375	373	419
β-DPN–α-ketobutyrate	344	379	375	420

A determination of the molar extinction coefficient at 340 mμ was carried out with the reduced DPN-pyruvate compound. The concentration of the reduced adduct was determined by ribose and phosphate analyses. The value for the molar extinction coefficient at 340 mμ was found to be 8.1×10^3.

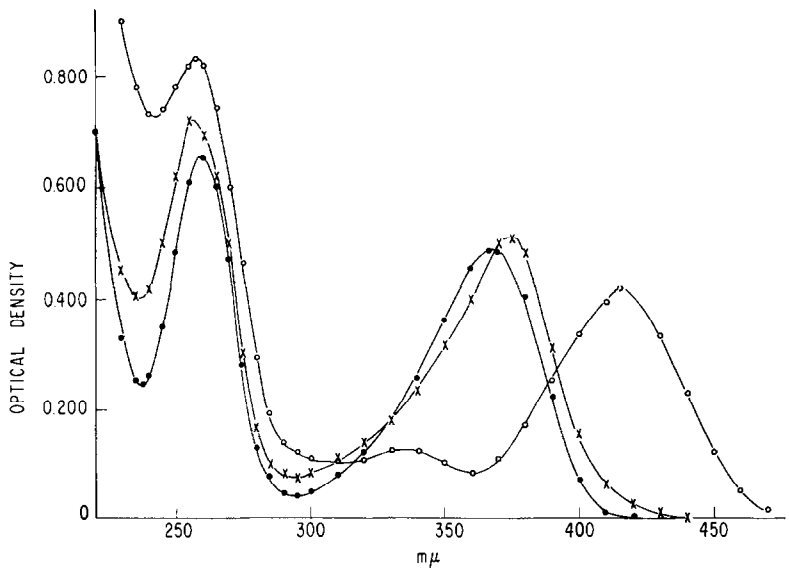


FIG. 3. Absorption spectrum of the oxidized DPN–pyruvate adduct. ●---●, at pH 1.0; ×---×, at pH 7.0; ○---○, at pH 13.0.

The absorption spectra of the oxidized adducts are strongly pH dependent. At neutral pH, absorption maxima are observed in the 260- and 370-mμ regions. At an acidic pH the latter maximum usually shows a slight shift toward a shorter wavelength. At alkaline pH a large red shift occurs, and an absorption maximum appears at about 410 mμ, which is usually accompanied by a shoulder, or a less intense maximum around 340 mμ. These results are also summarized in Table 1. Figure 3 shows the absorption spectra of the oxidized DPN–pyruvate adduct at several pH values. The observed spectra are similar to those observed by Dolin and Jacobson (*16*) for the

oxidized form of the DPN–acetone adduct. The pK_a, determined from the change in absorption at 360 and 415 mμ with pH, was 9.6 for the oxidized DPN–pyruvate compound. Dolin and Jacobson (*16*) reported a pK_a of 9.5 for the oxidized acetone adduct. It is assumed that this pK_a represents the ionization of the enol form of the nicotinamide carbonyl group, as indicated in Fig. 4.

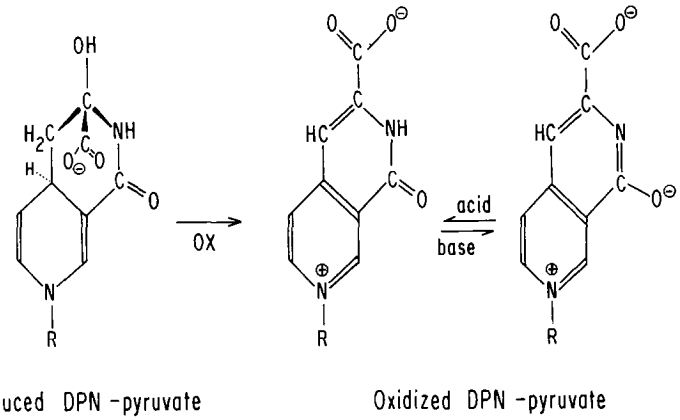


FIG. 4. Structure of the reduced and oxidized forms of the DPN–pyruvate adduct.

Adducts prepared during this investigation possess fluorescent properties that are similar to those reported for the DPN–acetone complex (*16*). The reduced adducts show a single fluorescence maximum around 475 mμ when excited at their absorption maxima. The fluorescence spectra of the reduced adducts resemble the fluorescence spectrum of DPNH and are independent of pH above pH 7.

TABLE 2

FLUORESCENCE MAXIMA OF THE ADDUCTS

Adduct	Reduced form (mμ)		Oxidized form (mμ)					
			pH 1		pH 7		pH 12	
	A[a]	B	A	B	A	B	A	B
β-DPN–pyruvate	345	480	365	440	370	440 510	415	515
α-DPN–pyruvate	350	470	360	430	360	430 500	400	500
β-DPN–pyruvate ethylester	345	475	370	430	370	430 500	410	500
β-DPN–oxaloacetate	340	480	370	430	370	430 490	410	500
β-DPN–acetaldehyde	350	480	350	430	350	430 480	390	500
β-DPN–α-ketoglutarate	350	475	380	460	370	460	420	520
β-DPN–butyraldehyde	340	475	370	460	370	450 520	420	520
β-DPN–α-ketobutyrate	350	470	370	470	370	470	420	520

[a] A: excitation maxima; B: emission maxima.

The fluorescence spectra of the oxidized adducts in 0.1 *N* HCl show a single maximum around 440 mμ when these compounds are excited at their absorption maximum (see Table 2). The fluorescence maximum shifts to about 510 mμ when the solutions are brought to pH 12. At a neutral pH usually two emission maxima are observed. Figure 5 shows the fluorescence spectra of the oxidized DPN–pyruvate compound. A determination of the pK_a, from the change in fluorescence at 440 and 510 mμ with pH, also resulted in a value of 9.6 for the oxidized DPN–pyruvate compound.

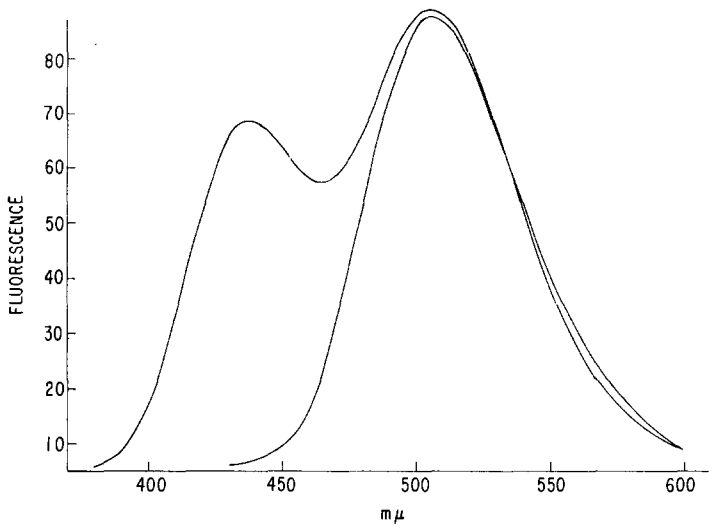


FIG. 5. Fluorescence spectra of oxidized DPN–pyruvate adduct at pH 7.0 and 13.0. Excitation was at 360 mμ and 415 mμ, respectively. The sensitivity of the fluorometer was decreased by a factor 10 at pH 13.0 as compared to that at pH 7.0.

The spectral properties of the reduced adducts thus indicate that the nicotinamide moiety in these compounds is probably present in the dihydropyridine form.

Stability of the Reduced and Oxidized Adducts

Reduced adducts are very sensitive to oxidation and decomposition. In solutions above pH 7 the compounds are relatively stable when kept cold and under nitrogen. The pH sensitivity of the reduced adducts is similar to that of DPNH. Decomposition occurs in acid solutions, and the absorption at 340 mμ decreases, while a new absorption maximum is formed at about 290 mμ. Such changes in absorption have also been observed with acidified solutions of DPNH (*17*). The reduced adducts may be stored in the solid form as barium salts, with oxygen excluded. Under these conditions the compounds are stable for several weeks.

Oxidized adducts are less sensitive to decomposition and they may be prepared in the solid form with little difficulty. The solid oxidized adducts are normally stored at 5° in a vacuum desiccator. In acidic solutions the oxidized adducts are stable at temperatures up to 50°. In alkaline solutions (at room temperature) a slow decomposition occurs which is indicated by a slow decrease in absorption at 410 mμ. This decomposition is accelerated at higher temperatures. A solution of 1×10^{-4} *M* of the oxidized DPN–pyruvate compound is completely destroyed after boiling for 10 min at pH 12, with the concomitant formation of an absorption band at 310 mμ. The products of the

decomposition of the oxidized DPN–pyruvate adduct in alkaline solutions have not yet been identified.

Structure of the DPN–Pyruvate Adduct

Table 3 summarizes the results of an analysis of the ribose and phosphate content of the oxidized DPN–pyruvate compound. The data are presented as moles of ribose and phosphate per mole of adduct (mol wt = 809). DPN^+ was analyzed for comparative purposes. The results indicate that the ribose and phosphate content of the oxidized adduct are identical to that of DPN^+.

TABLE 3

RIBOSE AND PHOSPHATE CONTENTS OF THE OXIDIZED DPN–PYRUVATE ADDUCT

	DPN^+ (moles/mole)	DPN–pyruvate adduct[a] (moles/mole)
Phosphate	1.81	1.82
Ribose	2.14	2.09

[a] A molecular weight of 809 was used for the oxidized DPN–pyruvate adduct.

To ascertain the presence of an unaltered adenine moiety in the adduct, the following experiments were performed. Two milligrams of the oxidized DPN–pyruvate adduct were dissolved in 1 ml of 0.1 *M* sodium phosphate buffer, pH 7.5, and a small amount of snake venom phosphodiesterase was added. The solution was incubated at 37° for 30 min. The incubation mixture was lyophilized, and chromatographed on Whatman No. 1 paper with isopropanol–1% ammonium sulfate, 2:1. Another sample of the lyophilized material was chromatographed on the same paper with isobutyric acid–concentrated ammonia–water, 66:1:33, pH = 3.7. On both chromatograms one of the products of the reaction moved with an R_f value identical with that of 5′-adenylic acid. The other product could not be identified with any of the known pyridine nucleotides. These results suggest that the adenine moiety of the DPN^+ molecule remains unchanged during the reaction with pyruvate.

Both the reduced and the oxidized forms of the DPN–pyruvate adduct were sensitive to hydrolysis by snake venom phosphodiesterase, at a rate comparable to that of DPN^+. In both cases, one of the hydrolysis products was identified as 5′-adenylic acid.

A further characterization of the structure of the adduct was achieved by an investigation of the reactivity of the nicotinamide moiety. One milligram of the reduced as well as the oxidized form of DPN–pyruvate adduct was treated with DPNase from *Neurospora* or pig brain at 37° for 30 min in phosphate buffer, pH 7.5. The products were then subjected to paper chromatography. Each adduct yielded a single spot after treatment, with an R_f value identical to that of the starting material. This indicates that no hydrolysis of the adducts had occurred. The amount of enzyme used was adequate to hydrolyze 1 μmole of DPN^+ in 2 min under the given conditions.

Addition of potassium cyanide or sodium bisulfite to the reduced or oxidized forms of the DPN–pyruvate adduct did not result in any changes in the absorption and fluorescence spectra or in any changes in their R_f values. This indicates that the adduct is probably unable to react with these reagents.

These results suggest that the addition of pyruvate to DPN^+ results in alterations of the pyridine moiety of DPN^+; these alterations are expressed by the inability of DPNase to hydrolyze the nicotinamide–riboside linkage, and by the decreased reactivity of the 4-position of the nicotinamide ring.

The structure of the reduced DPN–pyruvate adduct was also investigated by nmr and infrared spectral analysis. Figure 6 shows the high resolution 220 MHz nmr spectra

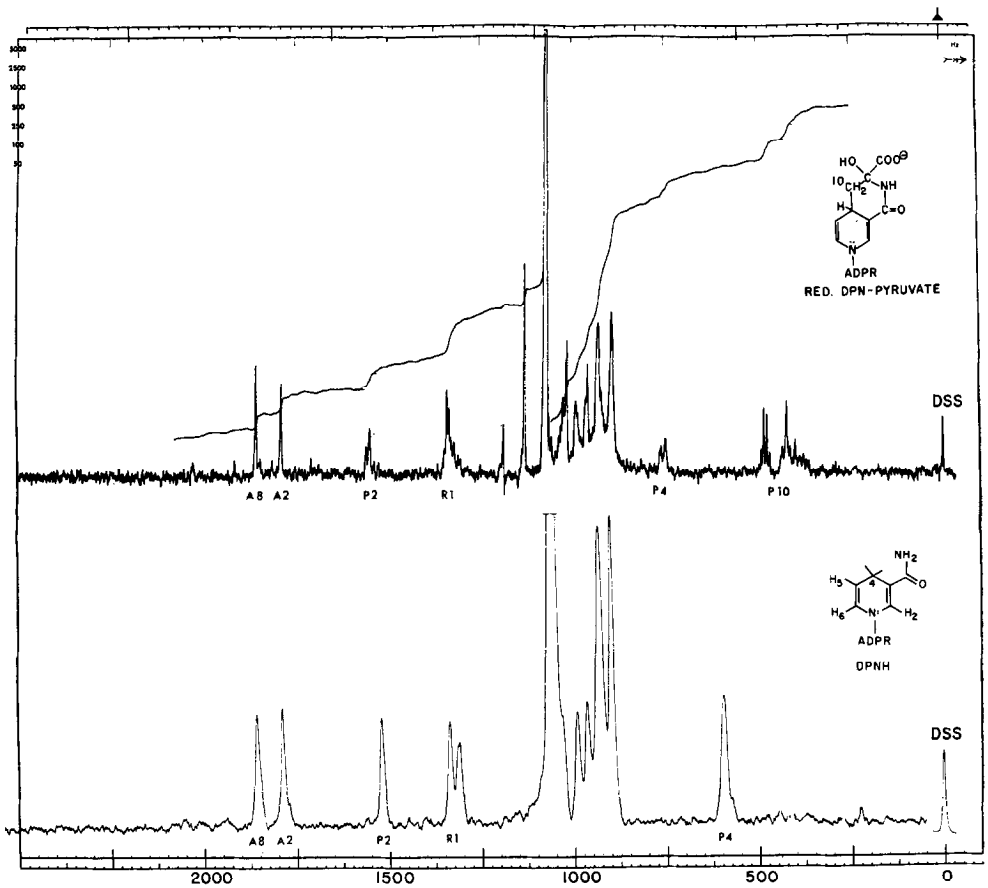


FIG. 6. Nuclear magnetic resonance spectra (220 MHz) of DPNH and of reduced DPN–pyruvate adduct. Peaks: A8, adenine C_8H; A2, adenine C_2H; P2, dihydropyridine C_2H; R1, (adenine)-ribose C_1H and (dihydropyridine)-ribose C_1H; P4, dihydropyridine C_4H; P10, the methylene group at C_{10} of the reduced DPN–pyruvate adduct.

of DPNH and of the reduced DPN–pyruvate adduct. The spectra are remarkably similar, and distinctly different from the nmr spectrum of DPN^+. The position of the resonance frequencies of the pyridine ring hydrogens indicates clearly that the reduced DPN–pyruvate compound possesses a dihydropyridine configuration. A shift to lower fields of the resonance frequencies of the C_8H of the adenine ring, the C_2H of the pyridine ring, and the C_1H of the pyridine ribose in the reduced adduct, as compared to DPNH, indicates a less effective diamagnetic shielding in the DPN–pyruvate (Table 4). This in turn seems to suggest that there is a lesser interaction between the base pairs in the adduct molecule than between the base pairs in DPNH (*18*).

TABLE 4

CHEMICAL SHIFTS OF THE ADENINE AND DIHYDROPYRIDINE PROTONS OF THE REDUCED DPN–PYRUVATE ADDUCT, BEFORE AND AFTER TREATMENT WITH PHOSPHODIESTERASE, IN COMPARISON WITH THOSE OF DPNH

	DPNH	Red. DPN–pyruvate adduct	Red. NMN–pyruvate adduct + 5′-AMP
Adenine			
C_8H	1857	1857	1866
C_2H	1789	1791	1798
Ribose-C_1H	1335	1338	1337
Pyridine			
C_2H	1552	1558	1590
C_4H			
A	584	758	817
B	559		
Ribose-C_1H	1311	1338	1383
Methene group	—	423 483	455 520

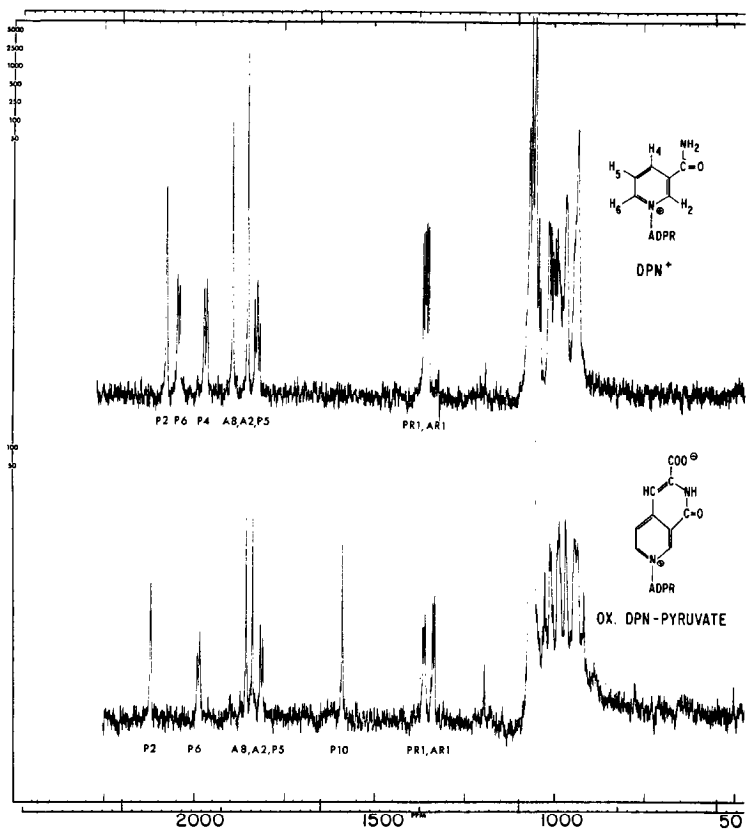


FIG. 7. Nuclear magnetic resonance spectra of DPN^+ and the oxidized DPN–pyruvate adduct. Peaks: P2, pyridine C_2H; P6, pyridine C_6H; P4, pyridine C_4H; A8, adenine C_8H; P5, pyridine C_5H; A2, adenine C_2H; P10, the vinyl proton at C_{10} in the oxidized DPN–pyruvate adduct; PR1, (pyridine)-ribose C_1H; AR1, (adenine)-ribose C_1H.

A further shift of these hydrogens to lower fields is observed when the DPN–pyruvate molecule is cleaved by snake venom phosphodiesterase (Table 4). The magnitudes of these shifts seem to indicate that the stacking of the pyridine and adenine rings in the reduced adduct is similar to that in DPNH (*19*).

The spectrum of the reduced DPN–pyruvate adduct also indicates the presence of a methylene group, which is absent in DPNH. Also, only one hydrogen atom is present at the 4 position of the nicotinamide ring. These data indicate that a condensation

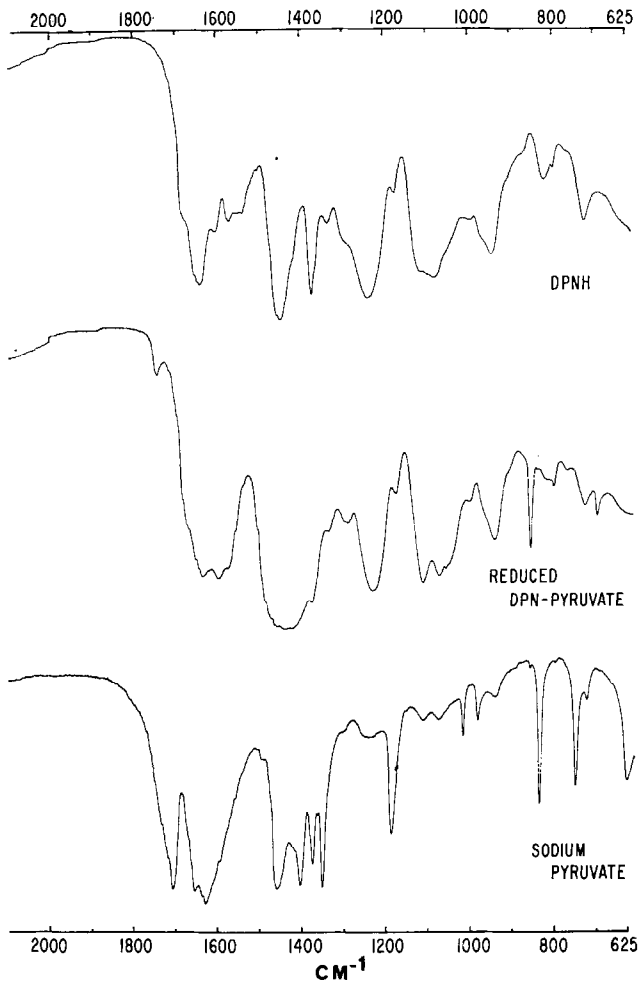


FIG. 8. Infrared spectra of DPNH (top), the barium salt of the reduced DPN–pyruvate adduct (middle), and sodium pyruvate (bottom). The solid samples were suspended in Nujol.

may have taken place between the methyl group of the pyruvate and the C_4 of the pyridine ring. Such an arrangement is in agreement with previously published structures for the reduced DPN adducts (*3–5*).

The nmr spectra of DPN^+ and of the oxidized DPN–pyruvate adduct are presented in Fig. 7. The spectra of the pyridine ring in the oxidized adduct is similar to that in DPN^+. The methylene group of the pyruvate moiety contains only one hydrogen and

is shifted far downfield, suggesting that its environment is of a conjugated double-bond character. This indicates that a double bond may exist between the C_2 and C_3 of the pyruvate moiety, which may be formed by the elimination of water. The assignment of the peaks in the nmr spectra was based upon earlier work in our laboratory (*20–22*).

Further information about the structure of the reduced DPN–pyruvate compound was obtained with the use of infrared spectroscopy. Figure 8 shows the infrared spectra of pyruvate, DPNH, and the reduced DPN–pyruvate adduct from 625 to 2000 cm^{-1}. The spectra indicate that the carbonyl group of the pyruvate molecule, which shows a strong absorption band at 1708 cm^{-1}, is absent in the reduced DPN–pyruvate adduct. Furthermore, the strong absorption bands at 1400 and 1600 cm^{-1} suggest that the carboxylate ion is present in the reduced DPN–pyruvate molecule.

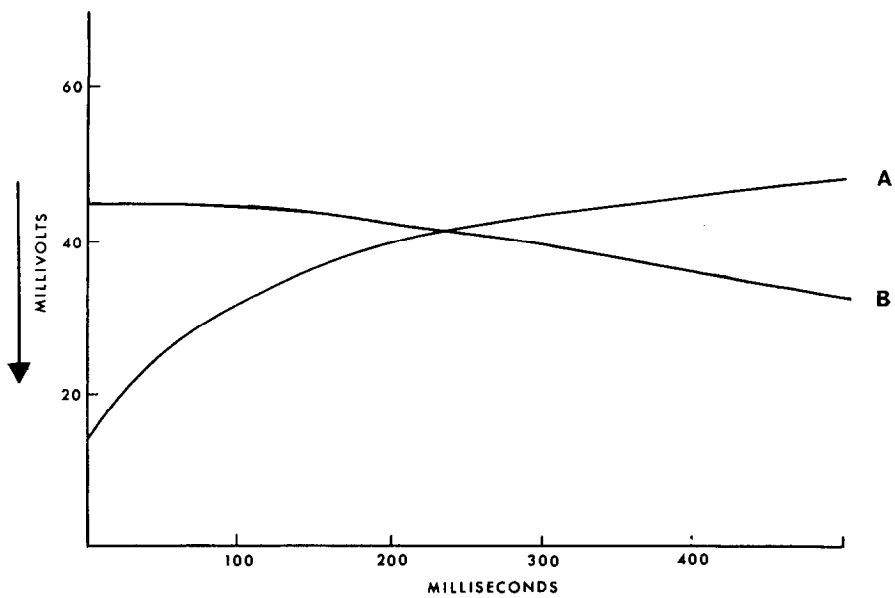


FIG. 9. Initial rates of the increase in absorption at 340 mμ (curve A) and the increase in fluorescence at 450 mμ (curve B). Excitation was at 340 mμ. Sweeptime: 50 msec/cm. Signal: 10 mV/cm. The arrow points in the direction of increasing light intensity. Syringe 1: 10 m*M* pyruvate + 10 m*M* DPN^+ in water. Syringe 2: 2 *N* NaOH.

The absence of the carbonyl group in the pyruvate moiety of the reduced adduct indicates that an interaction may have taken place between the carbonyl group and the amide group of the nicotinamide moiety. Such an interaction would lead to the formation of a second six-membered ring, as shown in Fig. 4. The weak absorption band at 1125 cm^{-1} may indicate the presence of a tertiary alcohol group in the reduced adduct; however, a firm identification of this band must be postponed until appropriate model compounds have been analyzed.

Colorimetric analyses by the Friedemann procedure (*15*) for the presence of ketones were also carried out to verify the absence of a carbonyl group in the oxidized and reduced adducts. No ketone group could be detected by this method. Sodium pyruvate and an equimolar mixture of DPN^+ and sodium pyruvate were used as control samples. The results indicate that the carbonyl group of pyruvate is lost as the result of adduct formation.

The formation of the reduced DPN adduct from DPN^+ and pyruvate in an alkaline medium may be followed by stopped-flow techniques. Figure 9 shows the initial rates that are observed when an equimolar solution of DPN and pyruvate (1×10^{-2} *M* in water) is mixed with an equal volume of 2 *N* NaOH. The absorption maximum at 340 mμ forms immediately, whereas the appearance of the fluorescence at 450 mμ shows a considerable lagtime. These data thus suggest that the primary reaction in the formation of the reduced adduct is the nucleophilic attack by pyruvate at carbon 4 of the nicotinamide ring. Preliminary evidence from our laboratory as well as from other

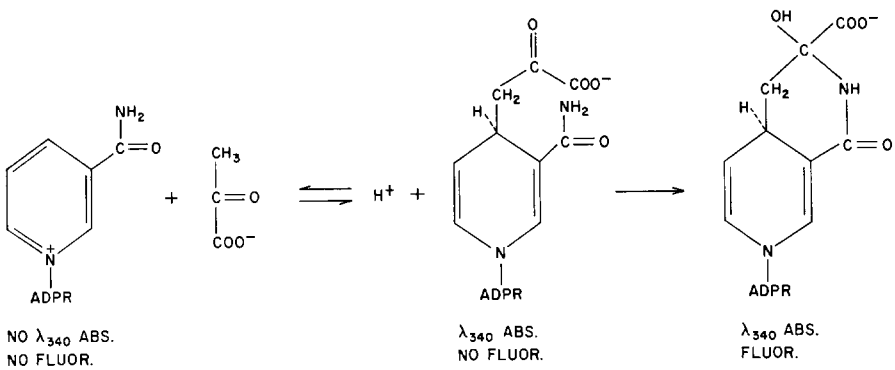


FIG. 10. Schematic presentation of the reactions leading to the formation of the reduced DPN–pyruvate adduct.

groups (*26*) indicates that the enol form of pyruvate may be the reactive species. The attack by pyruvate at the 4 position of the nicotinamide is then followed by the attack of the amide nitrogen on the carbonyl carbon, resulting in a ring closure. The sequence of events is presented in Fig. 10. The data suggest clearly that the intermediate form absorbs at 340 mμ, but does not fluoresce. When (AcPy) DPN^+ is used to replace DPN^+ in this experiment, no fluorescent compound is formed. This may indicate that the fluorescence of the reduced DPN adduct is a consequence of ring closure.

Binding of the Adducts to Dehydrogenases

Addition of the reduced DPN–pyruvate adduct to a solution of chicken H_4 LDH results in a quenching of the protein fluorescence at 340 mμ. Figure 11 shows the decrease in fluorescence of a solution of chicken H_4 LDH upon the addition of small amounts of the reduced adduct. The concentration of the reduced DPN–pyruvate compound was determined by its absorption at 340 mμ, using a molar extinction coefficient of 8.1×10^3. The curve indicates that a maximal quenching of the tryptophan fluorescence is obtained when four moles of the reduced compound are bound per mole of enzyme.

Similar results were obtained when the fluorescence of the reduced DPN–pyruvate adduct was monitored during this experiment. A significant enhancement in the adduct fluorescence occurs when it is bound to the enzyme. The saturation curve also led to the conclusion that four moles of the reduced DPN–pyruvate compound are bound per mole of LDH (Fig. 12).

The binding to LDH is specific for the reduced DPN–pyruvate adduct. No effect on the protein fluorescence and the coenzyme fluorescence is observed when the oxidized DPN–pyruvate adduct is added to LDH at comparable concentrations. Also, no effect

on the LDH fluorescence is observed when the reduced DPN–oxaloacetate, DPN–pyruvic ethylester, α-DPN–pyruvate, or DPN–acetaldehyde adducts are added to the enzyme. A similar specificity was found for the reduced DPN–oxaloacetate compound with respect to chicken heart mitochondrial MDH. The fluorescence of yeast alcohol

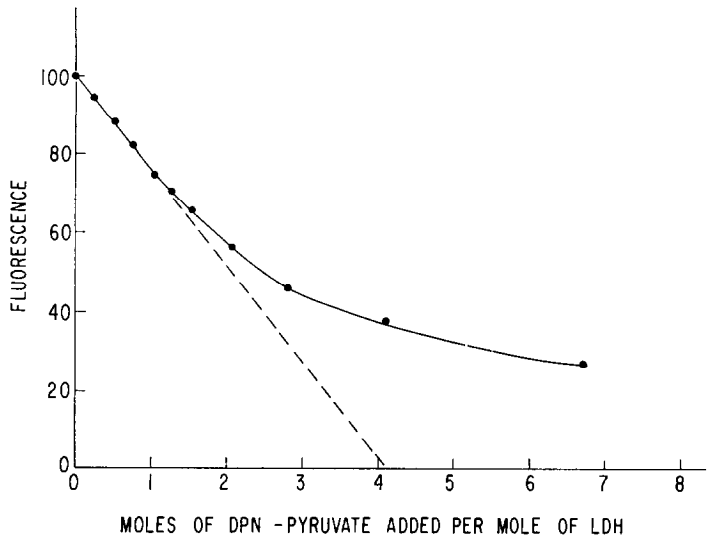


FIG. 11. The quenching of the protein fluorescence of LDH by the reduced DPN–pyruvate adduct. LDH: 1.5×10^{-3} μmoles in 3 ml of 0.1 *M* phosphate buffer, pH 7.0. A series of additions of 0.02-ml quantities, each containing 4.0×10^{-4} μmoles of the reduced DPN–pyruvate compound, were carried out, and the fluorescence was measured after each addition. Excitation was at 288 mμ; emission at 340 mμ.

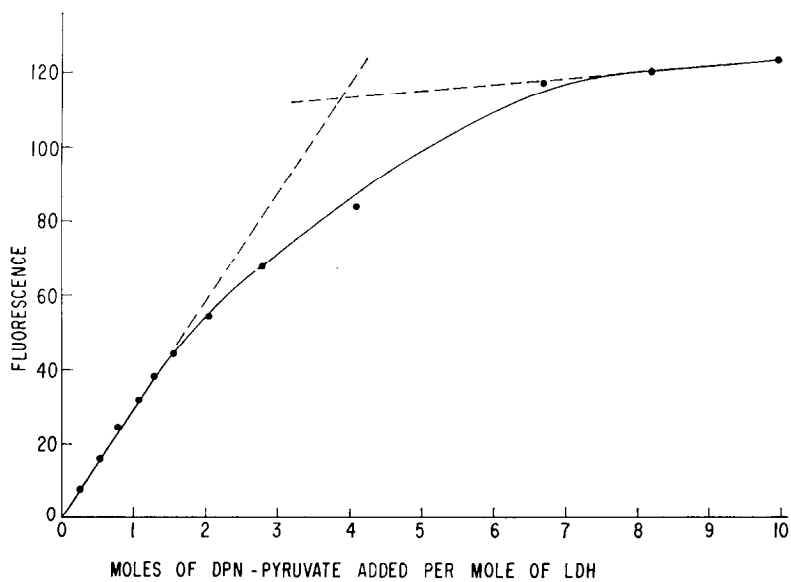


FIG. 12. The enhancement of the fluorescence of the reduced DPN–pyruvate adduct upon binding to LDH. The experimental conditions were as given in Fig. 11. Excitation: 340 mμ; emission: 450 mμ.

dehydrogenase is significantly quenched only by the reduced DPN–acetaldehyde adduct, and the reduced α-ketoglutarate adduct specifically quenches the fluorescence of dogfish liver glutamic dehydrogenase. The fluorescence of liver alcohol dehydrogenase is affected only to a small extent by the reduced DPN–acetaldehyde, but a significant quenching is obtained when the reduced DPN–butyraldehyde adduct is added to this enzyme.

These results revealed that the dehydrogenases show a high degree of specificity toward the reduced adduct which contains their specific substrate. The specificity toward β-DPN^+ was demonstrated by the fact that the reduced α-DPN–pyruvate adduct is inert for all lactate dehydrogenases tested.

The equilibrium constant for the dissociation of the LDH–DPN–pyruvate complex to chicken H_4 LDH and reduced DPN–pyruvate adduct was calculated from the fluorescence titration curves (*23*). The value obtained at pH 7.0 was 2.5×10^{-7} *M*.

None of the oxidized adducts had any significant effect on the fluorescence of the investigated enzymes at the concentrations employed for the reduced adducts. Higher concentrations of the oxidized adducts resulted in a quenching of the protein fluorescence; however, the specificity of the enzymes for a particular adduct was greatly diminished. These results with the oxidized compounds are in agreement with the observations of Lee and Winer (*24*), who reported that the oxidized DPN–pyruvate adduct binds equally well to chicken H_4 LDH and to pig heart MDH. The dissociation constants determined by these authors were 4.6×10^{-5} *M* for the LDH and 5.1×10^{-5} *M* for the MDH.

Inhibition Studies with Reduced DPN Adducts

A similar specificity of the dehydrogenases for a particular reduced adduct could be demonstrated when the effects of the reduced adducts upon the catalytic activity of the enzymes were investigated. Such studies were performed by adding a suitable amount of an adduct solution to the enzymatic assay mixtures (described in Methods) and observing the inhibitory effect of the adduct on the rate of the reaction. Table 5 summarizes the percentage of inhibition that was observed when 2×10^{-5} *M* of one of the

TABLE 5

INHIBITION OF VARIOUS DEHYDROGENASES BY REDUCED ADDUCTS[a]

Adduct	Chicken H_4 LDH (%)	Pig heart mitochondrial MDH (%)	Equine liver ADH (%)	Yeast ADH (%)	Dogfish liver GDH (%)
β-DPN^+–pyruvate	59	1	0	1	7
α-DPN^+–pyruvate	0	0	0	0	0
β-DPN^+–pyruvate ethylester	0	0	0	0	0
β-DPN^+–oxaloacetate	5	44	0	0	5
β-DPN^+–acetaldehyde	4	5	10	71	0
β-DPN^+–α-ketoglutarate	0	0	0	0	46
β-DPN^+–butyraldehyde	0	0	28	7	0
β-DPN^+–α-ketobutyrate	0	0	0	0	0

[a] Reduced adduct to a final concentration of 2×10^{-5} *M* was added to the assay mixtures described in Methods, and the initial rate was compared to the initial rate of an assay in the absence of adduct. Values are given as percentage of inhibition. In calculating the concentration an ϵ_{max} of 8.1×10^3 was used for all reduced adducts.

reduced adducts was present in the various assay mixtures. These results indicate that each enzyme was only significantly inhibited by the adduct that contains its specific substrate. The LDH system was inhibited about 60% by the reduced DPN–pyruvate compound, whereas the DPN–oxaloacetate and DPN–acetaldehyde adducts showed only slight inhibitory effects. Both the chicken H_4 and the M_4 LDH were inhibited to the same degree by the pyruvate adduct. Chicken mitochondrial MDH was inhibited 44% by the reduced DPN–oxaloacetate adduct. Yeast alcohol dehydrogenase was inhibited 71% by the reduced DPN–acetaldehyde compound and 7% by the reduced DPN–butyraldehyde adduct, whereas the liver enzyme was inhibited more strongly by the

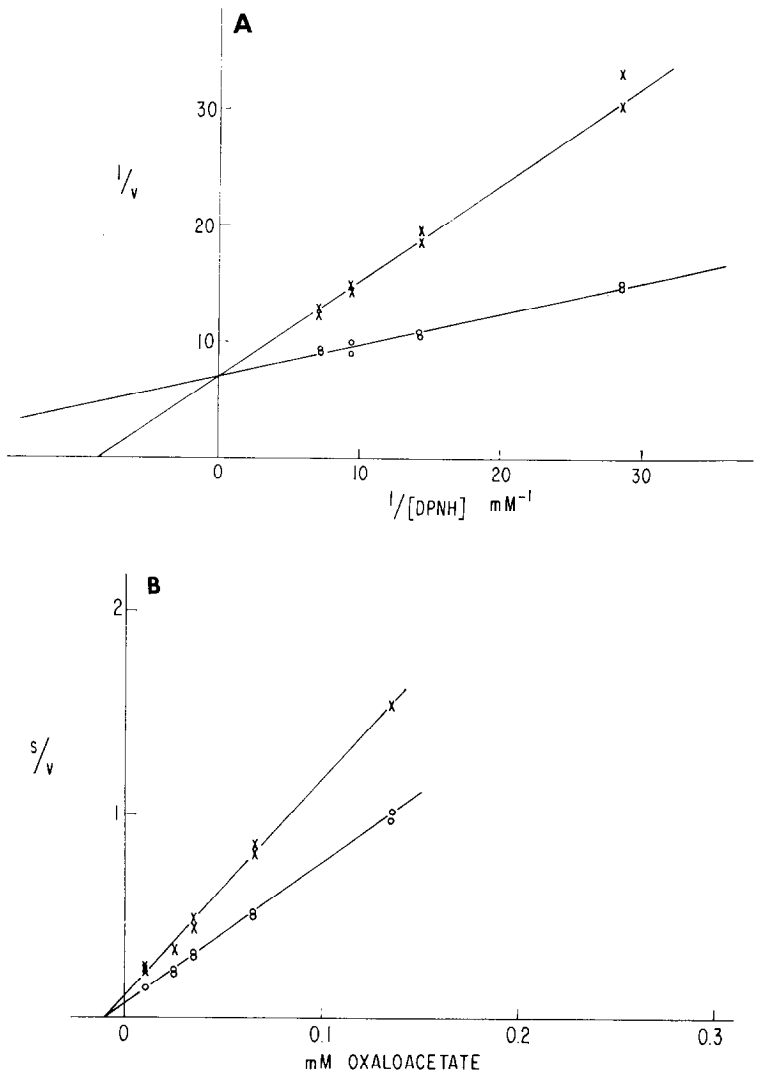


FIG. 13. Lineweaver–Burk plots, showing the inhibitory effects of the reduced DPN–oxaloacetate on MDH. Part A: DPN–oxaloacetate concentration: 8.5×10^{-6} *M*; oxaloacetate concentration: 3.3×10^{-4} *M*. Part B: DPN–oxaloacetate concentration: 8.5×10^{-6} *M*; DPNH concentration: 1.4×10^{-4} *M*. The measurements were made in 0.1 *M* phosphate buffer, pH 7.5.

butyraldehyde adduct (28%) than by the DPN–acetaldehyde compound (10%). These results reflect the differences in the substrate specificity of these enzymes. Glutamate dehydrogenase was inhibited to an extent of 46% by the DPN–α-ketoglutarate compound, whereas the reduced pyruvate and oxaloacetate adducts showed only slight inhibitory effects on this enzyme.[4]

Investigations into the nature of the inhibition by the reduced DPN adducts revealed that the inhibition is competitive with the reduced coenzyme and noncompetitive with the substrates in the lactate, malate, and alcohol dehydrogenase systems. Figure 13 shows the results of this type of investigation of the malate dehydrogenase system using the reduced DPN–oxaloacetate adduct as inhibitor.

Inhibition of the dehydrogenases with oxidized adducts is much less specific than with the reduced adducts. Lee and Winer (*24*) reported that the oxidized DPN–pyruvate adduct inhibits LDH and MDH about equally well, and we have confirmed this finding. Furthermore, we found that the inhibition constants for the oxidized adducts are about an order of magnitude higher than those for the reduced adducts under comparable conditions.

A determination of the inhibition constants for the reduced DPN–pyruvate adduct in the LDH system, from double-reciprocal plots, showed that these constants are somewhat smaller than the K_s for DPNH, indicating that the reduced adduct has a greater affinity for the enzyme than the reduced coenzyme itself. These results are summarized in Table 6.

TABLE 6

INHIBITION CONSTANTS OF REDUCED DPN–PYRUVATE ADDUCT FOR SEVERAL LACTATE DEHYDROGENASES

Enzyme	Specificity	Substrate varied[a]	K_I (M)
Chicken H_4	L-Lactate	DPNH	1.0×10^{-5}
		Pyruvate	3.0×10^{-5}
Chicken M_4	L-Lactate	DPNH	1.0×10^{-5}
		Pyruvate	2.2×10^{-5}
Limulus muscle	D-Lactate	DPNH	0.6×10^{-5}
		Pyruvate	1.2×10^{-5}
Nereis muscle	D-Lactate	DPNH	0.5×10^{-5}
		Pyruvate	1.5×10^{-5}

[a] The values were obtained with the use of double-reciprocal plots. This column indicates which substrate was varied to obtain the plots.

Dr. George L. Long in our laboratory observed that the D-lactate dehydrogenase from *Limulus* muscle and *Nereis* is also strongly inhibited by the reduced DPN–pyruvate adduct (*25*). For comparison purposes, the inhibition constants for these enzymes are included in Table 6. The data indicate that the reduced DPN–pyruvate compound is almost equally effective as an inhibitor for both the D- and L-specific lactate dehydrogenases.

[4] It is of interest to note that when inhibitory levels of DPNH were used (1.5×10^{-4} M), the addition of 2×10^{-5} M of reduced DPN-α-ketoglutarate adduct resulted in an activation of the enzyme. In this manner the inhibition by excess DPNH could be reversed to an extent of 80%. Increasing the concentration of the DPN-α-ketoglutarate compound resulted in decreased activation of the enzyme.

The pH dependency of the inhibition of MDH by reduced DPN–oxaloacetate is shown in Table 7. It should be noted that the inhibition is most pronounced at pH 6.0,

TABLE 7

INHIBITION OF MDH BY REDUCED DPN$^+$–OXALOACETATE ADDUCT AT DIFFERENT pH VALUES AND VARIOUS OXALOACETATE CONCENTRATIONS[a]

	Oxaloacetate concentration (%)		
	2×10^{-4} *M*	5×10^{-4} *M*	2×10^{-3} *M*
pH 6.0	52	61	35
pH 7.5	27	28	23
pH 9.0	11	16	11

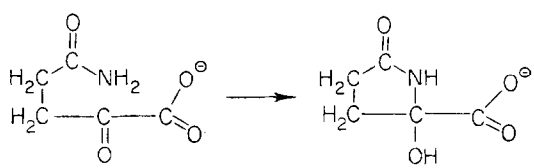
[a] Concentration of adduct: 2.2×10^{-5} *M*. Other concentrations as described in Methods. Buffers: 0.1 *M* citrate, pH 6.0; 0.1 *M* phosphate, pH 7.5; and 0.1 *M* Tris–HCl, pH 9.0.

amounting to well over 50%, whereas an inhibition of only 10% is found at pH 9.0. Intermediate values are observed at pH 7.5. Similar pH effects were found with the chicken H_4 system in the presence of reduced DPN–pyruvate adduct as the inhibitor.

DISCUSSION

The chemical and enzymatic analyses as well as the spectral properties of the reduced DPN–pyruvate adduct show conclusively that alterations have taken place in the nicotinamide portion of the DPN$^+$ molecule as a result of the interaction with pyruvate. These alterations result from an attack by the pyruvate at the 4-position of the nicotinamide moiety, resulting in the formation of a dihydropyridine ring. The amide nitrogen subsequently exerts a nucleophilic attack on the carbonyl carbon of the pyruvate moiety; this reaction results in the formation of a second six-membered ring.

Similar reactions have been shown to occur with related compounds. Hersh and co-workers[5] have investigated the formation of 5-hydroxypyroglutamate from α-ketoglutarate, and have found that the equilibrium of this reaction, which is base catalyzed, is in the direction of ring formation ($K_{eq} = 3 \times 10^{-3}$). Furthermore,



Ludowieg (*26*) studied the interaction of pyruvate with *N*-propyl-nicotinamide in basic solutions. He concluded that the interaction proceeds through an attack by the methyl

[5] Louis B. Hersh, *Biochemistry* (in press).

group of the pyruvate on the carbon at the 4-position of the nicotinamide, and that ring closure occurs through an attack by the amide group on the pyruvate carbonyl group. These studies, together with the evidence presented in this paper, establish a second ring in the structure of the reduced DPN–pyruvate adduct.

Figure 4 shows the structure of the reduced DPN–pyruvate molecule in somewhat more detail. The nicotinamide ring and the amide group lie in one plane. The methylene group may also be approximately in the same plane, whereas carbon atom 2 of the pyruvate moiety is out of the plane toward the front of the ring. The hydroxyl group is in an equatorial position to the second ring, and the carboxyl group is in the axial position.[6] Oxidation of the reduced adducts causes the nicotinamide ring to become aromatic. Furthermore, the equivalents of water are removed, resulting in a double bond between the carbon atoms 2 and 3 of the pyruvate moiety. This causes the entire part of the molecule to become more aromatic and, therefore, more planar. The significance of this change in structure will be detailed later in this discussion.

The results presented in this paper indicate that all the reduced adducts have similar optical and fluorescence properties. The products that are obtained upon oxidation of the reduced compounds are also very similar in their spectral properties. It, therefore, seems reasonable to assume that all the adducts that were prepared during this study possess the same basic structure as the DPN–pyruvate compound, with differences existing only in the side chains of the second six-membered ring. Such an assumption is also supported by the similarities in the stabilities of the reduced and oxidized adducts.

Binding of the Adducts to the Dehydrogenases

Evidence was obtained that LDH can bind up to four moles of the reduced DPN–pyruvate adduct per mole of enzyme. Furthermore, the binding of the reduced adducts is competitive with the binding of DPNH, as indicated from inhibition studies with the lactate, malate, and alcohol dehydrogenases. These results suggest that the reduced adducts bind to the same binding site on the enzymes as does DPNH. The quenching of the protein fluorescence, resulting from the binding of the reduced adduct, and the enhancement in the reduced adduct fluorescence are quite similar to the changes in fluorescence observed when DPNH is bound to these dehydrogenases. This implies that, upon binding of the reduced adduct, certain structural changes are brought about in the enzyme and in the adduct in a manner that closely resembles the changes ensuing from the binding of DPNH to the dehydrogenase. The position of the reduced adduct molecule on the enzyme is thus probably identical with the position that is normally occupied by the reduced coenzyme.

The Substrate Binding Site

To the best of our knowledge, there is no information available at the present time concerning the position of the substrate molecule on the enzyme, except that the substrate is located somewhere near the nicotinamide ring. However, a more detailed knowledge of the spatial orientation of the coenzyme and the substrate molecules on the enzyme is of fundamental importance to the understanding of the reaction mechanism of the dehydrogenases. The unique specificity of the dehydrogenases for the reduced adducts, demonstrated in Table 5, can be explained only by assuming that the enzymes "recognize" the substrate moieties in the adduct molecules. This implies that

[6] Several stereoisomers may be present in our preparations of the reduced adducts. A new asymmetric center is formed during the closure of the second ring. Furthermore, the remaining hydrogen at the 4-position of the nicotinamide ring may be in either the A or the B position. At the present time we have no information regarding the extent in which these isomers are present in our preparations.

the substrate part of the reduced adduct may be located at the substrate binding site of the enzyme, and may be in a position similar to that which the substrate normally occupies in the transitional complex of enzyme, DPNH, and oxidized substrate during normal catalytic activity.

The same conclusions may be arrived at from a careful consideration of the structure and specificity of the abortive ternary complexes of several dehydrogenases, which have been described in detail elsewhere (*1*, *2*). These complexes of enzyme, oxidized coenzyme, and oxidized substrate are very specific with respect to the substrate moieties; the substrate may be replaced only by compounds that are competitive with the substrate in the dehydrogenase reaction. Evidence has been presented suggesting that a covalent bond may be present between the substrate and the coenzyme moiety in the abortive ternary complex (*1*). Two observations, however, led us to the conclusion that the coenzyme–substrate moiety in such a complex is not identical with the reduced adducts of DPN. First, the enzyme-reduced adduct complex possesses a fluorescence spectrum similar to that of enzyme-bound DPNH, whereas the abortive ternary complex does not fluoresce. Second, the absorption maximum of the abortive ternary complex of LDH, DPN^+, and pyruvate is at 322 mμ, whereas the absorption maximum of the LDH-bound reduced DPN–pyruvate compound is at 340 mμ. These differences may be explained by assuming an open structure for the pyridine–substrate moiety in the abortive ternary complex. The pyruvate is bound to the 4 position of the nicotinamide; however, the attack of the amide group on the carbonyl carbon is in some manner prevented by the protein. The coenzyme–pyruvate moiety present in the ternary complex thus resembles the intermediary nonfluorescent product that was observed during the formation of the reduced DPN–pyruvate adduct (Fig. 10). The shift in the absorption maximum toward a shorter wavelength may be attributed to the fact that the pyruvate moiety in the abortive ternary complex probably has a slightly different orientation with respect to the dihydronicotinamide ring than that of the enzyme-bound reduced adduct (see below). Similar conclusions were also drawn by Coulson and Rabin (*27*) from their studies of the structure of the abortive ternary complex of LDH. These authors found that the reduced adduct formed from pyruvate and $(AcPy)DPN^+$ is a much better inhibitor of LDH than is the reduced DPN–pyruvate. They concluded that the (AcPy)DPN–pyruvate adduct bears a closer resemblance to the transition state of LDH than the DPN-containing adduct does. This is in accordance with our conclusions, since the (AcPy)DPN–pyruvate adduct cannot form the ring compound and has the general structure of the intermediary product shown in Fig. 10.

The structure of the coenzyme–pyruvate moiety of the abortive ternary complex of LDH, which is proposed in this communication, is different from the structure recently proposed by DiSabato (*28*). This is probably due to the fact that this author subjected the complex to treatment with $NaBH_4$ prior to his analyses. It is possible that the reduction of the ternary complex by $NaBH_4$ leads to other reactions (for example, internal rearrangements) which may account for this discrepancy.

The structure of the reduced DPN–pyruvate adduct and that of the DPN–pyruvate moiety of the abortive ternary complex of LDH are compared in Fig. 14A and B. The schematic drawing shows that the carbon backbone of the pyruvate moiety in the rather rigid structure of the reduced adduct is positioned at a right angle with the plane of the dihydropyridine ring. Evidence has been presented in this paper as well as in a previous communication (*1*), suggesting that in the presence of LDH the pyruvate moiety of the reduced DPN–pyruvate adduct, as well as that of the ternary complex, may be located at the substrate binding site. Such an assumption requires the spatial position of the pyruvate moiety, with respect to the nicotinamide ring, to

be similar in both compounds. As indicated in Fig. 14B, the structure of the DPN–pyruvate moiety of the ternary complex (which has significantly more flexibility than the reduced adduct) may closely resemble the structure of the reduced DPN–pyruvate compound when the moiety is bound to the LDH. The spatial structure of the pyruvate moieties in these binary compounds may thus resemble the relative position of the

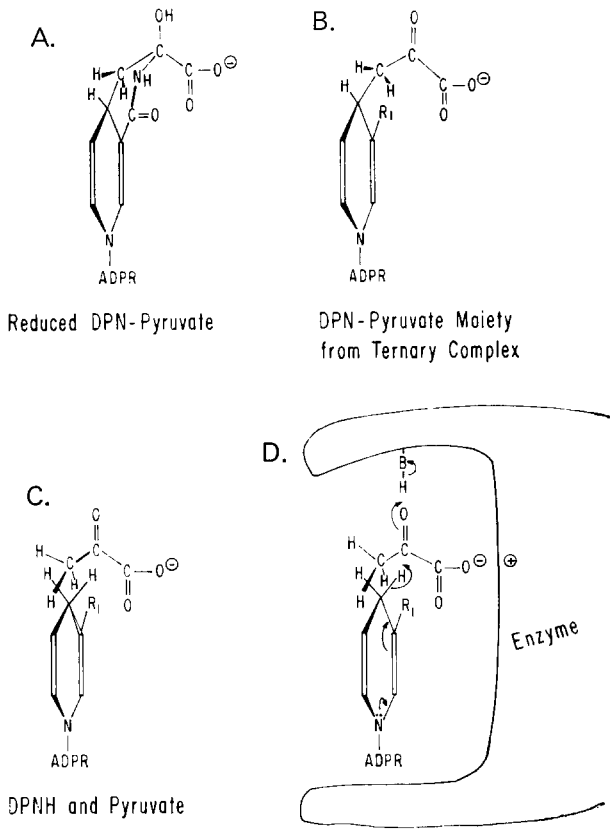


FIG. 14. Schematic presentation of the spatial structure of the reduced DPN–pyruvate adduct (A) and the coenzyme–substrate moiety of the LDH–DPN–pyruvate ternary complex (B). A possible orientation of the DPNH and pyruvate at the active site of LDH is presented (C), as well as a possible mechanism by which the LDH-catalyzed reduction of pyruvate may occur (D).

pyruvate molecule with respect to the nicotinamide ring during the lactate dehydrogenase-catalyzed reaction. A schematic drawing of a model for such a transitional LDH–DPNH–pyruvate complex is presented in Fig. 14C, in which the orientation of the groups closely resembles the structure of the compounds in Fig. 14A and B.

The reduced adducts are easily oxidized, which results, as discussed earlier, in a significant change in structure around the nicotinamide moiety. In view of the above considerations concerning the high sensitivity of the enzymes toward the appropriate structure of the substrate moiety, one would expect to find a dramatic change in the binding constants and the inhibitory properties after oxidation of the adduct. Our results show, indeed, an almost complete lack of binding capacity and virtually no

inhibitory effects for all the oxidized adducts when tested at the same levels at which extensive inhibition was observed with the reduced adduct.

Stereospecificity of Dehydrogenases

The model proposed in Fig. 14C shows a possible sequence by which the stereospecificity for the coenzyme hydrogens may be brought about. The position of the substrate in this model allows only for the A-hydrogen of the DPNH to be transferred to the carbonyl group, whereas the B-hydrogen occupies a relatively remote position. At the active site of a B-specific dehydrogenase, the position of the nicotinamide at the active site may be the mirror image of the position shown in Fig. 14C, namely, with the amide group in front of the carbonyl group. In that case only the B-hydrogen of the dihydropyridine ring can be transferred. The model thus indicates that the stereospecificity for the hydrogens of the nicotinamide ring may be brought about by the proper orientation of the substrate molecule with respect to the reduced coenzyme.

The model as written in Fig. 14C, however, does not explain the stereospecificity of the dehydrogenases with respect to the substrate. The mechanism of the L-lactate dehydrogenases appears to be closely related to that of the D-lactate dehydrogenases; this is indicated by the similarities in their kinetic parameters (K_m, V_{max}, turnover numbers, inhibition by oxamate, ability to form abortive ternary complexes [*25*]). Furthermore, the inhibition constants of the reduced DPN–pyruvate adduct are identical for the L-lactate dehydrogenases from chicken and the D-lactate dehydrogenases from *Limulus* and *Nereis*. This identity in inhibition constants may indicate that the reduced DPN–pyruvate adduct resembles the geometric positions of DPNH and pyruvate in the transition state of both types of enzymes equally well. Since the specificity for the hydrogen of DPNH is brought about by a specific orientation of the substrate with respect to the coenzyme, the question arises as to whether the stereospecificity of the products could also be the result of a specific geometric position of the pyruvate with respect to the nicotinamide ring. In an attempt to investigate this possibility we built space-filling models of the reduced DPN–pyruvate adduct, as well as models of pyruvate and DPNH. When we subsequently fitted the models of DPNH and pyruvate together in a manner shown in Fig. 14C, it became apparent that a closer fit between the pyruvate and the nicotinamide ring could be achieved by a slight rotation of the carbonyl group of the pyruvate, along the C_2–C_3 bond, either in a clockwise or in a counterclockwise direction. The two positions are illustrated in the upper part of Fig. 15. In both cases the carbonyl group is in direct contact with the A-hydrogen of the dihydropyridine ring, which is marked X. The lower part of Fig. 15 shows the orientation of the products after the hydride ion has been transferred from DPNH to the pyruvate molecule. In the case where the carbonyl group was rotated counterclockwise, the products are DPN^+ and L-lactate, whereas in the other case, D-lactate is formed.

If we assume that the structures presented in Fig. 15 resemble the transition states of the reactions catalyzed by L-lactate and D-lactate dehydrogenase, respectively, it becomes apparent why both enzymes are equally inhibited by the reduced DPN-pyruvate adduct. The structure of this adduct may resemble the two transition states to approximately the same extent, since its structure appears to be an intermediate to the two transition states.

The Mechanism of Action of Dehydrogenases

A possible mechanism for the reduction of the pyruvate molecule by DPNH is presented in Fig. 14D. The spatial relationship between the reduced coenzyme and

the substrate, as suggested by the data in this paper, is taken into consideration. The mechanism assumes the involvement of a proton-donating group on the enzyme. After the reduction of the pyruvate, it is assumed that this group takes up a proton from the surrounding medium to return it to its original state. Evidence for the presence of such groups at or near the active site of LDH has been accumulating over the years; they include imidazol (*29–34*), the ϵ-amino group of a lysine (*29, 30, 32*), a sulfhydryl group (*35–39*), a tryptophan residue (*40, 41*), and a tyrosine residue (*42*). Schwert concluded recently that the pH dependence of the LDH kinetics clearly indicates that a group with a pK value of approximately 7 may function as the source as well as the acceptor of the proton (*43*). Schwert also postulated (*43*) that a group with a pK of 9.3 (presumably a lysine residue) may be involved in the binding of the pyruvate carboxyl

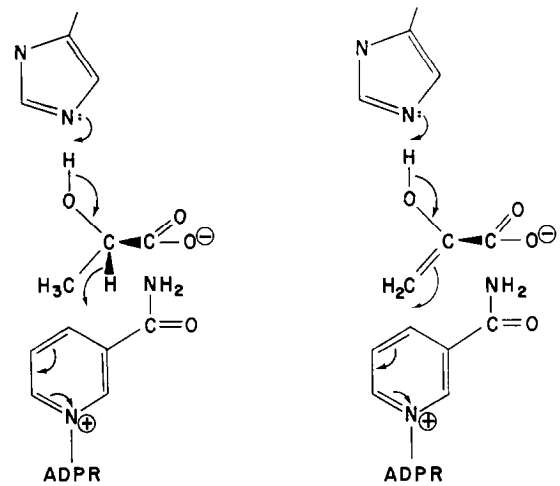


FIG. 16. A comparison of the proposed reaction mechanisms for the LDH-catalyzed oxidation of lactate and the LDH-catalyzed formation of the DPN–pyruvate binary complex. The imidazole residue is postulated to be the proton-donating group on the lactate dehydrogenase.

group. These assumptions are supported by kinetic evidence as well as by titration experiments (*29, 30, 32*). The function of the cysteyl, tyrosyl, and tryptophanyl residues is not clear at the present time.

The interpretation of the data presented in this paper strongly favors a mechanism which involves a direct transfer of the hydride between the coenzyme and the substrate, as was originally proposed by Vennesland (*44*) and Westheimer (*45, 46*). In fact, the proposed mechanism may explain how alcohol dehydrogenase can stereospecifically distinguish the two hydrogen atoms on the carbinol group of ethanol, as was also observed by these authors (*47*).

It has been established that only the keto forms of the oxidized substrates are the active species in the lactate, malate, and alcohol dehydrogenase systems (*44, 48*). Our proposed mechanism correlates with these findings. It has also been found that the enol form of pyruvate may be required for the formation of the abortive ternary complex (*27, 49*), and a mechanism for the formation of the abortive complex has been proposed (*27*). The relationship in the mechanisms of lactate oxidation and abortive complex formation becomes apparent from a comparison of their proposed mechanisms as illustrated in Fig. 16. Part A shows the oxidation of L-lactate by DPN^+,

whereas part B illustrates the formation of the abortive complex between enzyme, DPN^+, and enol-pyruvate.

The specific inhibition of the malate, alcohol, and glutamate dehydrogenases by the reduced adducts, as well as the ability of these enzymes to form abortive ternary complexes (*1*), suggest that similar mechanisms such as that for LDH may be operative in other dehydrogenases.

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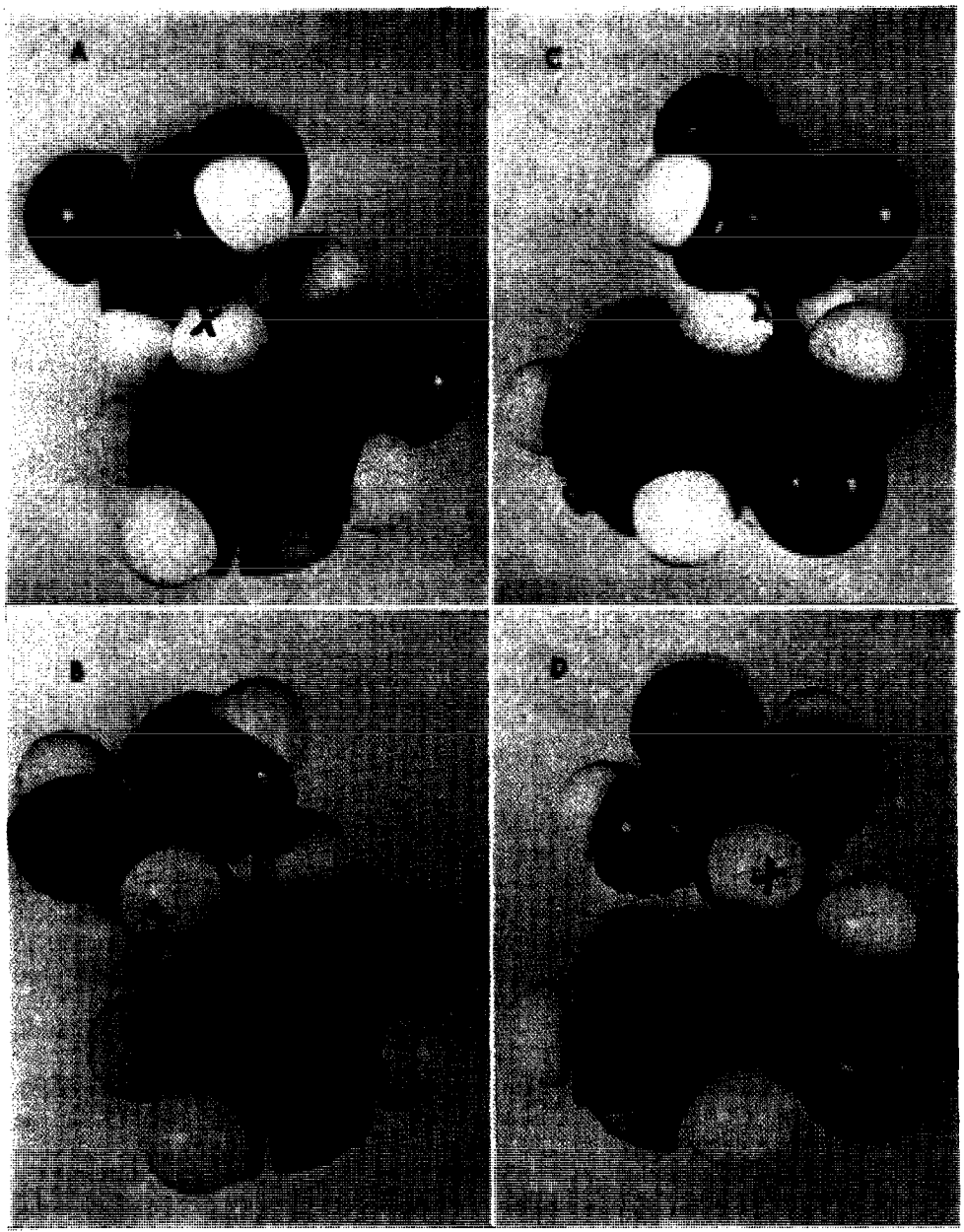

FIG. 15. Part A: The orientation of the pyruvate molecule around the nicotinamide moiety of DPNH in the transition state of lactate dehydrogenase, leading to the stereospecific reduction of pyruvate to L-lactate. The products (DPN^+ and L-lactate) are shown in Part B. Part C: The orientation of pyruvate around the nicotinamide moiety, leading to the reduction of pyruvate to D-lactate. The products are shown in Part D. The A-hydrogen of the dihydropyridine ring is marked X. For simplicity reasons only the nicotinamide part of the DPNH molecule is shown. Photograph taken by W. A. Moller.