Adducts of Diphosphopyridine Nucleotide and Carbonyl Compounds*

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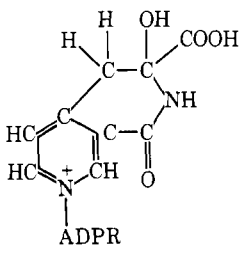
ABSTRACT: The mechanism of the reaction of diphosphopyridine nucleotide (DPN) and pyruvate has been investigated.

The primary product (DPNH-pyruvate) was formed by the addition of pyruvate at position 4 of the nicotinamide ring of DPN. New evidence has been provided for the cyclic structure of this adduct. Similar adducts of other carbonyl compounds and compounds related to DPN have been obtained. Most of the adducts have been isolated and characterized. It has been shown that a stoichiometric reaction occurs between DPNH-pyruvate and DPN to form DPN-pyruvate and DPNH. The transfer of hydride ion from DPNH-pyruvate takes place directly. Other DPNH-carbonyl compound adducts can be oxidized by transferring one hydride ion to suitable acceptors. The chemical and spectral characteristics of the oxidized adducts have been studied. The spectrum of the oxidized adducts is pH dependent with absorption maxima at about 360 and 420 mμ. DPNH-pyruvate can also split to DPN and pyruvate or autoxidize to DPN-pyruvate. The rates of hydride transfer from DPNH, 3-acetylpyridine analog of DPNH, reduced nicotinamide mononucleotide, α-DPNH, TPNH, DPNH-pyruvate, 4-*H*-fluoropyruvate-*N*-benzylnicotinamide, and DPNH-fluoropyruvate to 3-acetyl-*N*-benzylpyridinium chloride have been studied. The factors affecting the rate of hydride transfer have been discussed.

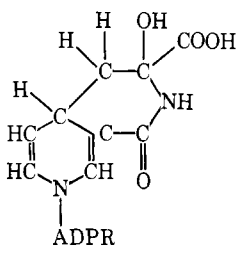
Nucleophilic reactions at the pyridine ring of DPN have received wide interest (Sund *et al.*, 1964; Bruice and Benkovic, 1966). In particular, the addition of carbonyl compounds to DPN and related compounds has been studied by several authors. Huff (1947) has obtained an adduct of acetone and *N*-methylnicotinamide. Burton and Kaplan (1954) obtained adducts of a number of carbonyl compounds with DPN, but failed to obtain any adduct with pyruvate. It was also shown by Burton and Kaplan (1954) and by Burton *et al.* (1957) that these compounds were at the reduction level of DPNH. More recently, Dolin and Jacobson (1964) have obtained both an oxidized and a reduced adduct of DPN and acetone. Ludowieg *et al.* (1964) have described a reduced adduct of acetone and *N*-propylnicotinamide. Lee *et al.* (1966) have briefly described an adduct of DPN and pyruvate that, in view of its absorption maximum at 415 mμ, is probably oxidized. It has also been communicated in a preliminary form (Di Sabato, 1968a) that incubation of DPN and pyruvate at moderately alkaline pH leads to the formation of a reduced adduct (DPNH-pyruvate), an oxidized adduct (DPN-pyruvate), and DPNH. (See Scheme I for formulas.) These compounds, together with unreacted material and breakdown products of DPN, have been separated on a DEAE-cellulose column (Di Sabato, 1968a).

The present paper deals with a further study of some of the properties of the adducts and of the mechanism of formation of DPN-pyruvate and DPNH from DPNH-pyruvate. Some factors affecting the transfer of hydride ion between pyridine compounds are investigated.

SCHEME I



DPN-pyruvate



DPNH-pyruvate

Materials and Methods

The chemicals used in this work were obtained from the following sources: Boehringer, Mannhein Corp. (DPN, TPN, DPNH, TPNH); P-L Biochemicals Inc. (APDPN,[1] DeDPN); Sigma Chemical Co. (NMN, α-DPN, α-DPNH, 2,4-dinitrophenylhydrazine, phenazine methosulfate, sodium fluoropyruvate, glycoaldehyde, glyceraldehyde, sodium α-ketovalerate, sodium α-ketobutyrate); Aldrich Chemical Co. (nicotinamide, butyraldehyde, valeraldehyde); Nutritional Biochemical Corp. (sodium pyruvate and lithium hydroxypyruvate). [2-^{14}C]Sodium pyruvate and [4-^{3}H]DPN (DPN labeled in position 4 of the nicotinamide ring) were obtained from New England Nuclear; [^{14}C]DPN (DPN labeled in the amide group) was purchased from Amersham–Searle.

Published methods were used for the preparation of *N*-benzylnicotinamide chloride (Karrer and Stare, 1937) and

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[1] The following abbreviations were used: DeDPN, desamino-DPN; NMN, nicotinamide mononucleotide; APDPN, 3-acetylpyridine-DPN.

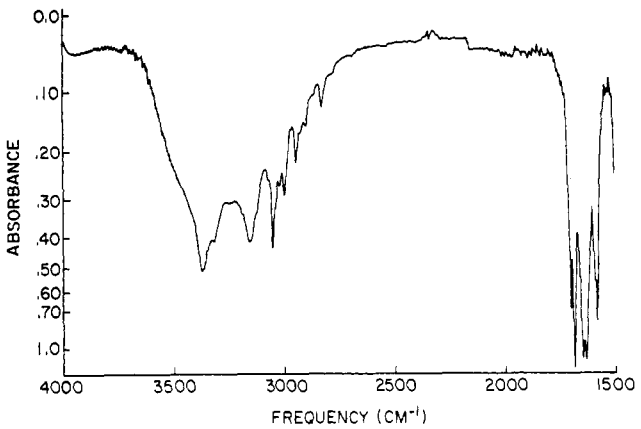


FIGURE 1: Infrared spectrum of 4-*H*-fluoropyruvate-*N*-benzylnicotinamide in KBr pellet.

3-acetyl-*N*-benzylpyridinium chloride (Anderson and Berkelhamer, 1958).

4-*H*-Fluoropyruvate-*N*-benzylnicotinamide was prepared by reacting equimolar amounts of fluoropyruvate and *N*-benzylnicotinamide chloride in 0.05 M sodium carbonate–bicarbonate buffer, pH 10.20. The pH was maintained between pH 10.0 and 10.5 by addition of NaOH. After a short time, 4-*H*-fluoropyruvate-*N*-benzylnicotinamide started to precipitate out of solution. When the reaction was complete, the compound was collected by filtration, then crystallized from ethanol–ether, mp 170°.

The 2,4-dinitrophenylhydrazone of pyruvic acid was obtained according to Lu (1939).

In a typical experiment, DPN (2×10^{-2} M) was incubated with pyruvate (2×10^{-2} M) in 0.1 M sodium carbonate–bicarbonate buffer, pH 10.20, for 24–48 hr. The products of the reaction were separated on columns of DEAE-cellulose. Details of this procedure have been given elsewhere (Di Sabato, 1968a).

Thin-layer chromatography for the identification of the 2,4-dinitrophenylhydrazone of pyruvic acid was carried out on precoated sheets of silica gel F-254 on aluminum (E. Merck AG, Darmstadt, Germany). The solvents used were acetone–water (50:50), butanol–acetic acid–water (50:35:15), propanol–ammonia (60:40), and pyridine–acetic acid–water (50:35:15).

Kinetic analyses were carried out by incubating the indicated amounts of reagents at 36°. Aliquots of the incubation mixture were withdrawn at suitable intervals and read at an appropriate wavelength in a Zeiss spectrophotometer, Model PMQ II. The molar excess of 3-acetyl-*N*-benzylpyridinium chloride was sufficient to maintain pseudo-first-order conditions over most of the reaction. First-order rate constants were calculated from the slopes of plots of $\log (OD_\infty - OD_t)$ *vs.* time. Second-order rate constants were obtained from the slopes of the first-order rate constants *vs.* the concentration of 3-acetyl-*N*-benzylpyridinium chloride.

A mixture prepared according to Bray (1960) was used for measuring radioactivity in a Packard Tri-Carb liquid scintillation spectrometer, Model 3003.

Infrared spectra were taken in a Perkin-Elmer, Model 337 infrared spectrophotometer using KBr pellets.

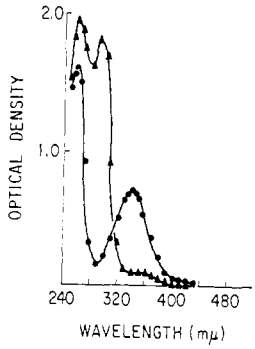


FIGURE 2: Spectra of DPNH-pyruvate in ammonium carbonate–bicarbonate buffer, pH 10.20 (●), and in 1.0 N HCl (▲).

Results

Chemical and Spectral Properties of the Adducts. As discussed in another publication (Di Sabato, 1968a), the products of the reaction of DPN and pyruvate were DPNH-pyruvate, DPN-pyruvate, and DPNH. In the same paper evidence was presented for attributing to DPNH-pyruvate and DPN-pyruvate the structures shown in Scheme I and for recognizing DPNH as one of the products of the reaction.

In order to obtain further evidence for the structure of DPNH-pyruvate, some properties of the model compound 4-*H*-fluoropyruvate-*N*-benzylnicotinamide were studied. The elemental analysis of 4-*H*-fluoropyruvate-*N*-benzylnicotinamide gave the following results.[2] *Anal.* ($C_{16}H_{15}N_2O_4F$) Calcd: C, 60.37; H, 4.76; N, 8.80. Found: C, 59.51; H, 4.52; N, 9.05. These data are compatible with a structure similar to that shown in Scheme I. Part of the infrared spectrum of 4-*H*-fluoropyruvate-*N*-benzylnicotinamide is shown in Figure 1. The band at about 3400 cm^{-1} is probably due to the hydroxyl stretching frequency. Rossotti and Rossotti (1958) found a band in this region in a series of compounds similar to 4-*H*-fluoropyruvate-*N*-benzylnicotinamide.

The spectral properties of DPNH-pyruvate and DPN-pyruvate were investigated in some detail.

Figure 2 shows the spectrum of DPNH-pyruvate at pH 10.20 (absorption maxima at 258 and 340 mμ) and of its acid-decomposition product in 1 N HCl (absorption maxima at 258 and 290 mμ).

The spectrum of DPN-pyruvate was pH dependent. Figure 3 shows spectra of DPN-pyruvate at pH 8.30, 9.61, 10.08, and 10.84. It appears that, as a result of raising the pH, the absorption maximum shifted from 370 to about 420 mμ. The p*K* for the spectral shift was 9.60. A clear isosbestic point (at about 398 mμ) was good evidence that the shift in absorption maximum represented a change among only two interconvertible species. In 1 N HCl the absorption maximum shifted from 370 to 363 mμ (not shown).

Other oxidized adducts were obtained by treating the corresponding reduced adducts with phenazine methosulfate. These compounds showed spectra and pH-dependent spectral variations similar to those shown in Figure 3. The p*K* values of the different compounds were, however, different. They are

[2] Elemental analyses were carried out by Galbraith Laboratories, Inc., Knoxville, Tenn.

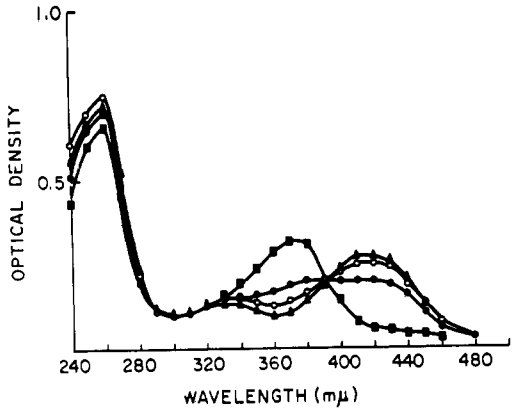


FIGURE 3: Spectra of DPN-pyruvate at different pH values: (■) 0.1 M Tris-HCl, pH 8.30; (●) 0.1 M sodium glycinate, pH 9.61; (○) 0.1 M sodium glycinate, pH 10.08; (▲) 0.1 M ammonium carbonate–bicarbonate buffer, pH 10.84.

shown in Table I. The absorption maxima are also recorded. The compounds derived from DPN also had a peak at 260 mμ.

Formation of Reduced Adducts Other Than DPNH-Pyruvate. A large number of reduced adducts of carbonyl compounds other than pyruvate and pyridine derivatives other than DPN were obtained. Thus, fluoropyruvate, hydroxypyruvate, α-ketovalerate, α-ketocaproate, acetone, dihydroxyacetone, butyraldehyde, valeraldehyde, glycolaldehyde, and glyceraldehyde were used as carbonyl compounds. TPN, DeDPN, NMN, α-DPN, APDPN, *N*-benzylnicotinamide chloride, and 3-acetyl-*N*-benzylpyridinium chloride were used as pyridine derivatives. 4-*H*-Fluoropyruvate-*N*-benzylnicotinamide had an absorption maximum at 360 mμ in 0.1 M carbonate–bicarbonate buffer, pH 10.20. Its acid-decomposition product (in 1 N HCl) had an absorption maximum at about 305 mμ.

The yields of the different adducts varied considerably. For instance, when 1×10^{-2} M DPN was incubated with 4×10^{-2} M pyruvate, DPNH-pyruvate was obtained in about 60% yield; DeDPNH-pyruvate, TPHN-pyruvate, NMNH-pyruvate, and α-DPNH-pyruvate were obtained, under comparable conditions, in yields varying between 8 and 20%. These yields can be related to the equilibrium constants for the reaction (G. Di Sabato, in preparation). Thus, preliminary data show that the equilibrium constant for the formation of DPNH-pyruvate is about 86 M^{-1}, while it is about 36 M^{-1}, 38 M^{-1}, 34 M^{-1}, and 25 M^{-1}, respectively, for the adducts mentioned above. Only relatively small differences in the velocity of formation of these adducts were found. The equilibrium constant for the formation of APDPNH-pyruvate is about 7 M^{-1}. Obviously, the unfavorable equilibrium derives from the fact that APDPN cannot form a six-membered ring with the carbonyl group of pyruvate. The oxidizability of the adducts may also affect their yields. For instance, DPNH-α ketocaproate was isolated only in very small amounts, perhaps because easily oxidizable.

The isolation of the reduced adducts of pyridine derivatives and carbonyl compounds made possible the calculation of their molar extinction coefficients. These are recorded in Table II together with the methods used for their determination.

Compounds having no carbonyl group or carbonyl compounds having no active hydrogen (*e.g.*, pyruvonitrile, mesoxalate) did not form adducts.

Mechanism of Formation of DPNH. Figure 4 shows chromatograms obtained by taking samples of a mixture composed of 0.02 M DPN and 0.02 M pyruvate in 0.1 M sodium carbonate–bicarbonate buffer, pH 10.20, and passing them through a DEAE-cellulose column. Fractions D, E, and F represent DPNH, DPN-pyruvate, and DPNH-pyruvate, respectively. These assignments have been made on the basis of the properties of these compounds and of the position at which they are eluted from the DEAE column (Di Sabato, 1968a). In Figure

TABLE I: p*K* Values and λ_{max} of Absorbance for the Tautomeric Forms of the Oxidized Adducts of Carbonyl Compounds and Nicotinamide Derivatives.

Adduct	p*K*	$\lambda_{max,1}$[b]	$\lambda_{max,2}$[b]
DPN-fluoropyruvate	7.35	385	410
Fluoropyruvate-*N*-benzylnicotinamide	7.40	376	415
DPN-pyruvate	9.60	370	420
DPN-acetone[a]	9.70	363	403
NMN-acetone[a]	9.5	357	396

[a] Values taken from Dolin and Jacobson (1964). [b] mμ.

TABLE II: Molar Extinction Coefficients of Adducts of Some Pyridinium Compounds and Pyruvate or Fluoropyruvate.[a]

Pyridinium Compound	Carbonyl Compound	$\epsilon_M \times 10^{-3}$ at $\lambda_{max,1}$	$\epsilon_M \times 10^{-3}$ at $\lambda_{max,2}$	Method of Determination[b]
DPN	Pyruvate	14.8 (260)	6.5 (340)	A
DPN	Pyruvate	16.2 (260)	6.8 (340)	B
DPN	Fluoropyruvate	15.4 (260)	7.1 (340)	C
TPN	Pyruvate	17.8 (260)	6.8 (340)	A
DeDPN	Pyruvate	13.6 (250)	6.5 (340)	A
DeDPN	Pyruvate	15.3 (250)	7.3 (340)	B
NMN	Pyruvate		6.0 (340)	A
NMN	Pyruvate		5.8 (340)	B
APDPN	Fluoropyruvate		5.5 (340)	D
N-Benzylnicotinamide	Fluoropyruvate		3.9 (360)	E

[a] Optical densities were measured in sodium or ammonium carbonate–bicarbonate buffer, pH 10.00–10.20. λ_{max} are given in parentheses. [b] (A) Determination of the ribose and/or phosphate content of the adduct. (B) Determination of the specific radioactivity of the [^{14}C]pyruvate adduct. (C) Determination of the specific radioactivity of the [^{14}C]DPN adduct. (D) This adduct was not isolated. The ϵ_M value was determined by saturating a solution of APDPN with fluoropyruvate. (E) Determination of the specific radioactivity of the *N*-benzyl[^{14}C]nicotinamide adduct.

TABLE III: Mechanism of Formation of DPNH and DPN-pyruvate.[a,b]

	Reactants and Specific Radioactivity	Products and Specific Radioactivity
	Expt A	
1st incubation	[^{14}C]DPN + pyruvate	[^{14}C]DPNH-pyruvate
2nd incubation	[^{14}C]DPNH-pyruvate (84×10^3), + DPN	DPNH + [^{14}C]-DPN-pyruvate, (78×10^3)
	Expt B	
1st incubation	DPN + pyruvate	DPNH-pyruvate
2nd incubation	DPNH-pyruvate + [^{14}C]DPN (32×10^3)	[^{14}C]DPNH (31×10^3) + DPN-pyruvate

[a] Specific radioactivities are expressed in cpm/μmole of compound. For further details, see text. [b] Experimental conditions were: 0.02 M DPN (or [^{14}C]DPN), 0.02 M pyruvate in 0.1 M sodium carbonate–bicarbonate buffer, pH 10.20; incubation time, about 24 hr at 21°; separation of products on DEAE-cellulose eluted with a linear gradient of ammonium carbonate–bicarbonate buffer, pH 10.20.

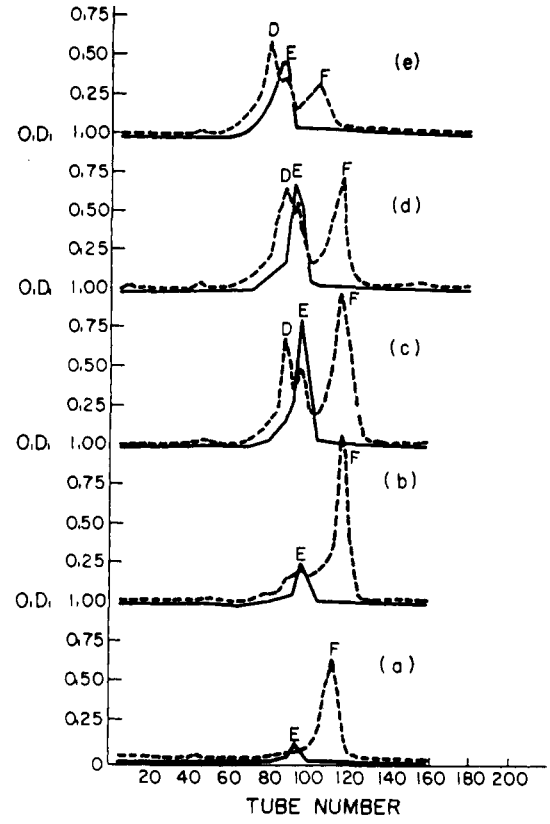


FIGURE 4: Chromatograms on DEAE-cellulose of the products formed at different times of incubation of DPN and pyruvate: (a) 3 hr, (b) 7 hr, (c) 24 hr, (d) 72 hr, (e) 144 hr; (-----) optical density at 340 mμ, (——) optical density at 420 mμ. The incubation was carried out in 0.1 M sodium carbonate–bicarbonate buffer, pH 10.20, at about 21°. The column was eluted with a linear concentration gradient of ammonium carbonate–bicarbonate buffer, pH 10.20.

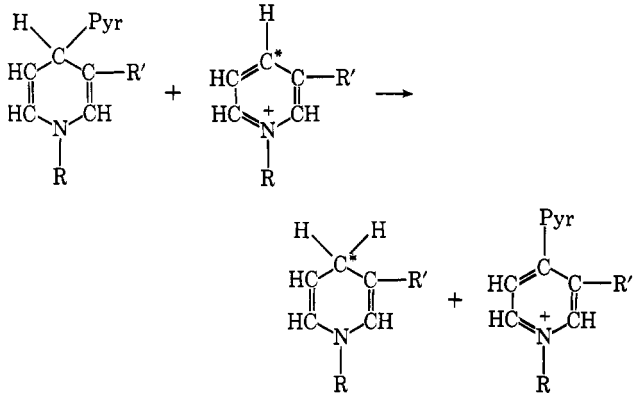
4 only the optical densities at 340 and 420 mμ are shown. Therefore, compounds such as DPN, nicotinamide, and ADPR do not appear. The chromatograms obtained at different times clearly show that substantial amounts of DPNH-pyruvate were formed during the first period of the reaction (3–7 hr), while very little DPNH or DPN-pyruvate was formed. Subsequently, the amount of DPNH-pyruvate remained approximately constant or it decreased while the amount of DPNH and DPN-pyruvate increased, so that, at about 72-hr incubation, approximately equal amounts of DPNH, DPN-pyruvate, and DPNH-pyruvate were present in the mixture. These changes were also observed in the unresolved mixture where the absorbance at 340 mμ appeared before the absorbance at 420 mμ. A possible interpretation of these facts is that DPNH-pyruvate was formed first in the reaction of DPN and pyruvate and that DPNH and DPN-pyruvate were formed in a subsequent reaction of DPNH-pyruvate with unreacted DPN.

In order to test this possibility, the following two sets of experiments were carried out (Table III). In experiment A, pyruvate was incubated with [^{14}C]DPN. At about 24-hr incubation, the mixture was passed through a DEAE-cellulose column and [^{14}C]DPNH-pyruvate was isolated. This adduct was concentrated, and then incubated in carbonate–bicarbonate buffer, pH 10.20, with nonlabeled DPN. At about 24-hr incubation, the mixture was passed again through a DEAE-cellulose column. The compounds isolated were nonlabeled DPNH, [^{14}C]DPN-pyruvate, and small amounts of [^{14}C]-DPNH-pyruvate. In another experiment (expt B of Table III), nonlabeled DPN was incubated with pyruvate. The incubation mixture was passed through a DEAE-cellulose column and the nonradioactive DPNH-pyruvate was isolated and incubated with [^{14}C]DPN. At about 24-hr incubation, the mixture was chromatographed a second time on DEAE-cellulose. The products were [^{14}C]DPNH, nonlabeled DPN-pyruvate, and small amounts of nonlabeled DPNH-pyruvate. In both sets of experiments the specific radioactivities of the reactants and of the products were essentially the same. The results of these experiments clearly show that DPNH and DPN-pyruvate were formed from DPNH-pyruvate, probably by transfer of a hydride from DPNH-pyruvate to DPN, as illustrated in Scheme II. Further evidence for this mechanism will be given later.

Experiments with Tritium-Labeled Compounds. In some experiments, the reaction of DPN and pyruvate was carried out in tritiated water. Some tritium was found in DPNH and DPNH-pyruvate. DPN-pyruvate did not contain isotope. Although no effort was made to elucidate the mechanism of this incorporation, it is felt that it represents an exchange of the hydrogen of DPNH-pyruvate with the solvent. Similar exchange has been described by San Pietro (1955) for the adduct of DPN and cyanide. This finding also confirms the reduced state of DPNH-pyruvate and the oxidized state of DPN-pyruvate.

In order to gain more information on the mechanism of the reaction described in the preceding paragraph, some experiments were carried out with DPN labeled with tritium in

SCHEME II



position 4 of the nicotinamide ring ([4-^3H]DPN). As shown in Table IV, when [4-^3H]DPN was incubated with pyruvate (1st incubation), label was found in DPNH and DPNH-pyruvate confirming the reduced state of DPNH-pyruvate. The specific radioactivities of these two compounds were about 130 and 70%, respectively, of the specific radioactivity of [4-^3H]DPN. The [4-^3H]DPNH-pyruvate obtained was then incubated with unlabeled DPN (2nd incubation). [4-^3H]DPNH with specific radioactivity about one-half that of [4-^3H]DPNH-pyruvate was obtained. No appreciable amounts of label were found in DPN-pyruvate, confirming the oxidized state of this adduct. While these results confirm the reaction illustrated in Scheme II, they also show that the transfer of hydrogen between DPNH-pyruvate and DPN takes place directly.

Other Reactions of DPNH-pyruvate. In control experiments, the isolated [^{14}C]DPNH-pyruvate (obtained from the incubation of DPN with [^{14}C]pyruvate) was concentrated and then incubated *alone* in 0.1 M sodium carbonate–bicarbonate buffer, pH 10.20, for different lengths of time. The incubation mixture was then passed through a DEAE-cellulose column. Chromatograms obtained at 3 hr and 30 min, 48 hr, and 120 hr of incubation are shown in Figure 5. Fractions A, B, and C are

TABLE IV: Transfer of Tritium from [4-^3H]DPNH-pyruvate.

	Reactants and Specific Radioactivity	Products and Specific Radioactivity
1st incubation	[4-^3H]DPN (120×10^3) + pyruvate	[4-^3H]DPNH (157×10^3) + [4-^3H]DPNH-pyruvate (84×10^3) + DPN-pyruvate
2nd incubation	[4-^3H]DPNH-pyruvate (84×10^3) + DPN	[4-^3H]DPNH (44×10^3) + DPN-pyruvate

[a] Specific radioactivities are expressed in cpm/μmole of compound. For further details, see text. [b] Same experimental conditions as in Table III.

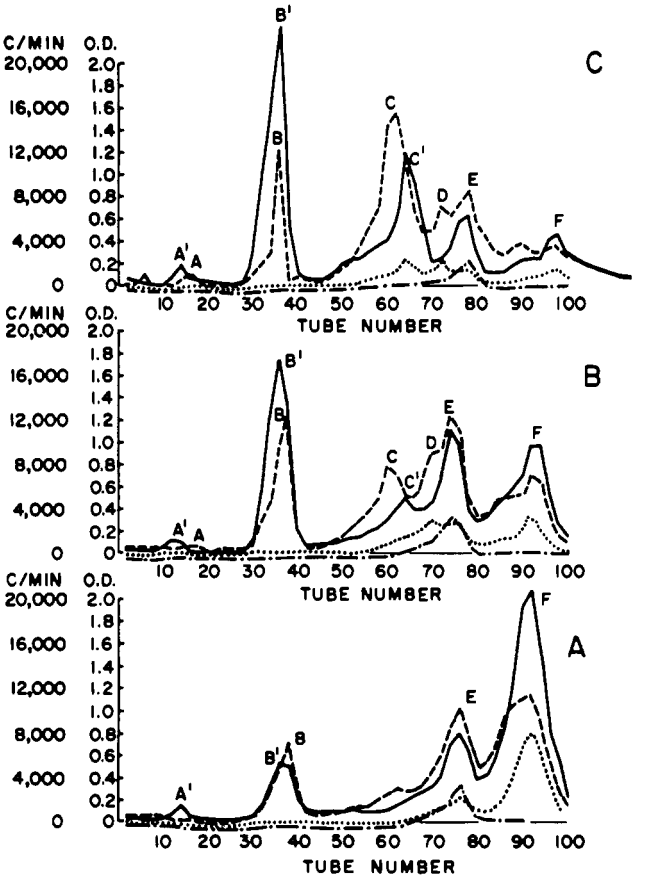


FIGURE 5: Chromatograms on DEAE-cellulose of the products formed at different times of incubation of [^{14}C]DPNH-pyruvate: (A) 3 hr and 30 min, (B) 48 hr, (C) 120 hr; (---) optical density at 260 mμ, (· · ·) optical density at 340 mμ; (·-·-·-·) optical density at 420 mμ; (——) radioactivity. Same experimental conditions as shown in the legend to Figure 4.

nicotinamide, DPN, and ADPR, respectively. Fraction B′ is [^{14}C]pyruvate. Fractions D, E, and F are DPNH, [^{14}C]DPN-pyruvate, and [^{14}C]DPNH-pyruvate, respectively. Fraction C′ appeared at relatively long times of incubation. This material has not been characterized yet. From its position in the chromatogram it seems to have two negative charges. It may be a dimerization product of the substrate. These chromatograms clearly show that the amount of [^{14}C]DPNH-pyruvate decreased with time, while the amount of DPN and [^{14}C]-pyruvate increased with time. In order to clarify the nature of the reactions involved, the fraction of radioactivity B′ was isolated over a Dowex 1-X8 (Cl^- form) column eluted with a concentration gradient of HCl. About 70% of the radioactivity was eluted in a single fraction. This material was precipitated with 2,4-dinitrophenylhydrazine. The 2,4-dinitrophenylhydrazone of this material had, on thin-layer chromatography with four different solvents, the same mobility as the 2,4-dinitrophenylhydrazone of authentic pyruvic acid. The composition of the solvents used has been mentioned under Materials and Methods. These results indicate that [^{14}C]DPNH-pyruvate splits into DPN and [^{14}C]pyruvate. The relatively low recoveries of [^{14}C]pyruvate may be due to the well-known instability of this compound (Silverstein and Boyer, 1964).

Figure 5A,B also shows that incubation of DPNH-

pyruvate by itself results in the slow production of DPN-pyruvate. For some time the possibility was considered that DPN-pyruvate was formed in an oxidation–reduction reaction by which one molecule of DPNH-pyruvate reduced another molecule of DPNH-pyruvate at the carbon-2 of pyruvate. Thus, one molecule of DPN-pyruvate and one of DPNH-lactate would be formed. If this mechanism were correct, the specific radioactivity of [4-^3H]DPNH-pyruvate should increase with time. This, however, was not found. Similarly, no reduced compounds that could have resulted from the decomposition of the doubly reduced DPNH-pyruvate were found. It was, therefore, concluded that, in the absence of DPN, DPN-pyruvate was probably formed by the autoxidation of DPNH-pyruvate. This same hypothesis has been advanced by Dolin and Jacobson (1964) in order to explain the oxidation of the reduced adduct of DPN and acetone. When 1.5×10^{-2} M DPNH-pyruvate was incubated with 6.0×10^{-2} M DPN in 0.1 M sodium carbonate–bicarbonate buffer, pH 10.20, for 24 hr at 21° about equal amounts of DPN-pyruvate were formed by autoxidation of DPNH-pyruvate and by transfer of hydrogen to DPN, according to Scheme II.

Hydride Transfer between Pyridine Derivatives. The finding of a direct, nonenzymatic hydride transfer between DPNH-pyruvate and DPN prompted us to study this reaction in more detail. In this paragraph, some data will be presented concerning the hydride transfer from various pyridine derivatives to 3-acetyl-*N*-benzylpyridinium chloride. This compound was chosen as acceptor of the hydride ion because, unlike other compounds tested, it does not give very high blanks of optical density. It has been used by Cilento (1960) as hydride acceptor from DPNH.

Separate experiments showed that varying the concentrations of the hydride donor from 1.7×10^{-3} M to 5.7×10^{-3} M did not appreciably affect the rate of oxidation, indicating that the reaction was first order with respect to the reduced compound. In Table V are shown the second-order rate constants for the transfer of hydride ion from DPNH, APDPNH, NMNH, α-DPNH, TPNH, DPNH-pyruvate, 4-*H*-fluoropyruvate-*N*-benzylnicotinamide, and DPNH-fluoropyruvate to 3-acetyl-*N*-benzylpyridium chloride. It appears that DPNH, NMNH, and TPNH were oxidized at approximately the same rate. APDPNH was oxidized at a rate about tenfold slower, 4-*H*-fluoropyruvate-*N*-benzylnicotinamide and DPNH-fluoropyruvate at a rate about fivefold slower than DPNH. α-DPNH and DPNH-pyruvate were oxidized at a rate approximately twofold faster than DPNH. The data also show that the rate of oxidation of DPNH, TPNH, and 4-*H*-fluoropyruvate-*N*-benzylnicotinamide was independent of pH, between pH 6.90 and 8.50, and of the type of buffer used.

TABLE V: Second-Order Rate Constants for the Transfer of Hydride Ion from Reduced Pyridine Derivatives to 3-Acetyl-*N*-benzylpyridinium Chloride.[a]

Reduced Pyridine Derivatives	Concn of 3-Acetyl-*N*-benzyl-pyridinium Chloride (M)	pH	k (M^{-1} min^{-1})
DPNH	0.08–0.32	6.90	3.1×10^{-1}
		8.50	3.0×10^{-1}
APDPNH	0.08–0.32	8.50	3.4×10^{-2}
NMNH	0.08–0.32	8.50	3.5×10^{-1}
α-DPNH	0.08–0.32	8.50	6.4×10^{-1}
TPNH	0.08–0.32	6.90	3.1×10^{-1}
		8.50	3.3×10^{-1}
DPNH-pyruvate	0.07–0.14	6.90	5.5×10^{-1}
4-*H*-Fluoropyruvate benzylnicotinamide	0.08–0.32	7.25	6.2×10^{-2}
		8.50	5.6×10^{-2}
DPNH-fluoropyruvate	0.16–0.32	6.90	6.9×10^{-2}

[a] Reduced compounds: $1.7–5.7 \times 10^{-3}$ M. Buffers: 0.25 M sodium phosphate buffer, pH 6.90; 0.25 M Tris-HCl, pH 7.25; 0.25 M Tris-HCl, pH 8.50; temperature, 36°.

Discussion

The data presented here (Figure 4) clearly show that DPNH-pyruvate is the primary product of the reaction of DPN and pyruvate. DPNH-pyruvate can further react in a number of ways. (1) It can reduce DPN, forming DPNH and DPN-pyruvate. (2) It can split to starting material, forming DPN and pyruvate. (3) It can undergo autoxidation, forming DPN-pyruvate.

The oxidation product of DPNH-pyruvate (DPN-pyruvate) studied in this work has spectral and chemical characteristics similar to those found by Dolin and Jacobson (1964) for the DPN-acetone adduct. Although these authors described the formation of a reduced and oxidized adduct of DPN and acetone, it is the present work that shows for the first time that the oxidized adduct can also be formed by hydride ion transfer from the reduced adduct to DPN. Our data also show that the hydride transfer is direct. The observation that [4-^3H]-DPNH-pyruvate transfers the label to hydride acceptors shows that the adduct is in 1,4-dihydro form. In other words, the pyruvate molecule is attached at position 4 of the nicotinamide ring of the coenzyme. A few other cases of nonenzymatic hydride transfer between pyridine derivatives have been described in the literature (Spiegel and Drysdale, 1960; Drysdale *et al.*, 1961; Cilento, 1960; Ludowieg and Levy, 1964).

Actually, the specific radioactivities of the products of the reactions shown in Table IV are lower than expected. The failure to obtain [4-^3H]DPNH with twice the specific radioactivity of [4-^3H]DPN in the first incubation and [4-^3H]DPNH with the same specific radioactivity as [4-^3H]DPNH-pyruvate in the second incubation is probably due to exchange of the label of [4-^3H]DPNH-pyruvate with the solvent. Failure to obtain the theoretical amount of label in cases in which the hydrogen was transferred directly has been reported (*e.g.*, Spiegel and Drysdale, 1960; Drysdale *et al.*, 1961). However, some of the data presented in Table IV could also be explained with a tritium isotope effect.

Ludowieg *et al.* (1964) obtained a reduced adduct of acetone and *N*-propylnicotinamide. The nuclear magnetic resonance of this adduct showed evidence of a structure similar to that shown in Scheme I. A somewhat different structure was proposed by Dolin and Jacobson (1964) for the adduct of DPN

and acetone. Our elemental analysis and infrared spectrum seem to confirm the structure proposed by Ludowieg *et al.* (1964). The pH dependence of the spectrum of DPN-pyruvate and similar compounds is also consistent with a cyclic structure.

Whether or not the reactions and the compounds discussed above are relevant to the mechanism of action of dehydrogenases remain to be seen. The fact that the transfer of hydride between DPNH-pyruvate and DPN is direct likens this reaction to the enzymatic one. It should also be mentioned that Van Eys *et al.* (1958) postulated a binding of pyruvate to the para position of DPN as part of a proposed mechanism of action of lactic dehydrogenases.

The question also arises concerning the relationship between the adducts described in this paper and the lactic dehydrogenase-DPN-pyruvate complex (Fromm, 1961, 1963; Di Sabato, 1968b; Griffin and Criddle, 1970). Preliminary experiments indicate that pyruvate binds to the coenzyme at different sites in the two types of adducts.

The differences in the rate of oxidation of DPNH, AP-DPNH, DPNH-pyruvate, 4-*H*-fluoropyruvate-*N*-benzylnicotinamide, and DPNH-fluoropyruvate (Table V) can be explained with the inductive effect of the groups at position 3 or 4 of the nicotinamide ring. Wallenfels (1959) found analogous effects with a number of 1,4-dihydropyridine derivatives. The difference in the rate of oxidation between α-DPNH and DPNH may be due to the fact that α-DPN and DPN are present in different conformations in aqueous solutions (Sarma *et al.*, 1968). The observation that NMNH and TPNH are oxidized at the same rate indicates that the portion of the nucleotide outside of the pyridine ring is not important in determining the rate of hydride transfer.

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