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Reactions of DPN$^+$ and pyruvate

A number of carbonyl adducts of DPN have been obtained by BURTON AND KAPLAN[1]. However these authors have failed to show formation of any adduct when using pyruvate. An oxidized adduct of pyruvate and DPN$^+$ has been obtained by LEE *et al.*[2]. Owing to the possible importance of pyruvate–DPN$^+$ adducts as models for the study of the mechanism of action of lactate dehydrogenases (EC 1.1.1.27), we have undertaken the study reported in this note. The new findings reported are: formation of two adducts (one oxidized and one reduced) of pyruvate and DPN$^+$, and formation of DPNH by incubation of pyruvate and DPN$^+$ at moderately alkaline pH's. These compounds have been separated on DEAE-cellulose. Experiments designed to elucidate the mechanism of the reaction between DPN$^+$ and pyruvate are also reported.

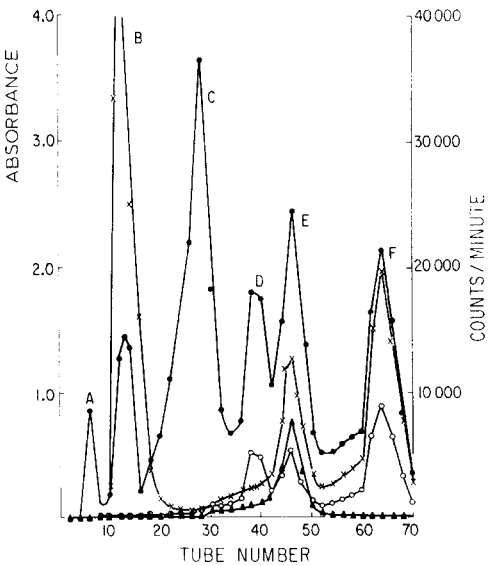

Fig. 1. Elution pattern of the products of the reaction between DPN$^+$ and pyruvate from DEAE-cellulose. DPN$^+$: $2.0 \cdot 10^{-3}$ M, [1-^{14}C]pyruvate: $2.0 \cdot 10^{-3}$ M in 0.1 M sodium carbonate–bicarbonate buffer (pH 10.20). Elution with ammonium–carbonate bicarbonate buffer (pH 10.20), 0.0015–0.07 M. ●—●, absorbance at 260 mμ; ○—○, absorbance at 340 mμ; ▲—▲, absorbance at 420 mμ; ×—×, radioactivity.

Fig. 1 shows the chromatographic separation over a DEAE-cellulose column of the products of the reaction between $2.0 \cdot 10^{-3}$ M DPN$^+$ and $2.0 \cdot 10^{-3}$ M [1-^{14}C]pyruvate in 0.1 M sodium carbonate–bicarbonate buffer (pH 10.20) at 21° for 24–28 h. The column was eluted with a linear concentration gradient of ammonium carbonate–bicarbonate buffer (pH 10.20) between 0.0015 and 0.07 M.

The most important characteristics of the chromatographic fractions are shown

TABLE I

PROPERTIES OF THE CHROMATOGRAPHIC FRACTIONS OF THE PRODUCTS OF THE REACTION BETWEEN [1-^{14}C]PYRUVATE AND DPN$^+$

Enzymic oxidation carried out with pyruvate and catalytic amounts of lactate dehydrogenase. Chemical oxidation carried out with phenazine methosulfate. Pyruvate/coenzyme: molar ratio. Radioactivity precipitated: precipitation with 2,4-dinitrophenylhydrazine.

	A	*B*	*C*	*D*	*E*	*F*
$\lambda_{max\ 1}$ (mμ)	259	259	259	259	259	258
$\lambda_{max\ 2}$ (mμ)				338	420	340
Enzymic oxidation (%)				80	3	3
Chemical oxidation (%)				73	6	60
$\lambda_{max\ 1}$, pH 7.0 (mμ)				259	259	258
$\lambda_{max\ 2}$, pH 7.0 (mμ)				338	370	340
$\lambda_{max\ 1}$, 1 M HCl (mμ)				259	259	258
$\lambda_{max\ 2}$, 1 M HCl (mμ)					363	290
Cyanide reaction (%)	<0.1	85	<0.1	<10	60	<10
Pyruvate/coenzyme					0.8	1.0
Radioactivity precipitated (%)		85			2	15
Holman reaction	100	100	1	100	8	8

in Table I. The fractions are indicated with letters, according to their order of elution from the column.

Fractions A, B and C were represented essentially by nicotinamide (derived from the splitting of DPN at the nicotinamide–ribose bond), unreacted DPN$^+$ and adenosine diphosphate ribose (also derived from the splitting of DPN at the nicotinamide–ribose bond), respectively. These fractions were obtained with DPN$^+$ alone as well as with DPN$^+$ *plus* pyruvate. In the experiments with DPN$^+$ *plus* pyruvate, Fraction B also contained some unreacted pyruvate.

The following fractions (D, E and F) were obtained only when pyruvate was present together with DPN$^+$. Fraction D was represented by material having absorption peaks at the same wavelengths (259 and 338 mμ) as DPNH with molar extinction coefficients similar to those of DPNH. About 80% of the absorbance at 340 mμ was quenched upon addition of pyruvate *plus* catalytic amounts of lactate dehydrogenase. The disappearance of the absorbance peak at 340 mμ in 1.0 M HCl was as expected with DPNH. The reaction with cyanide was essentially negative. When the reaction was carried out in tritiated water, nonexchangeable tritium was present in this fraction. It seems, therefore, that the bulk of the material present in this fraction was DPNH. The mechanism of formation of DPNH from DPN$^+$ and pyruvate at alkaline pH is under investigation. A mechanism for the non-enzymatic formation of DPNH from dithionite and DPN$^+$ in water has been postulated by YARMOLINSKI AND COLOWICK[3].

Fraction E was composed of material having peaks of absorption at 259 and 420 mμ. The peak at 420 mμ disappeared, and a new peak appeared at 370 or 363 mμ

at pH 7.0 or in 1.0 M HCl, respectively. These spectral characteristics are very similar to those described by BURTON, SANPIETRO AND KAPLAN[4] for the carbonyl adducts of DPN in the oxidized state. The material of Fraction E was not oxidized enzymatically (with pyruvate and lactate dehydrogenase) or chemically (with phenazine methosulfate). This fraction also contained a peak of radioactivity which coincided with the absorbance peaks. It was calculated that the molar ratio between the radioactive material and the 259–420 mμ absorbing material was about 0.8. The radioactivity was not precipitated with 2,4-dinitrophenylhydrazine, and the Holman reaction (for the determination of amides) was essentially negative. From all these data it seems that Fraction E was composed essentially of an adduct of pyruvate and DPN in the oxidized state. The facts that the radioactivity of this fraction was not precipitated with 2,4-dinitrophenylhydrazine and that the Holman reaction was essentially negative suggest a cyclization between the carbonyl group of the substrate and the amide group of the coenzyme. Our data do not give any indication as to the site of addition of pyruvate to the pyridine ring of the coenzyme. However, by analogy with the reactions of other carbonyl compounds which seem to add at position 4 of the pyridine ring of the coenzyme, we favor this position over position 2.

Fraction F was composed of material having absorption peaks at 258 and 340 mμ. In 1 M HCl, the peak at 340 mμ disappeared and a new peak appeared at 290 mμ. These spectral characteristics are very similar to those described by BURTON AND KAPLAN[5] for reduced adduct of dihydroxiacetone and DPN. A substantial part of the material in this fraction was oxidized chemically. Analogous to findings with Fraction E, the peak of radioactivity present in Fraction F coincided with the absorbance peaks; the molar ratio radioactive material:coenzyme was about 1.0, and only small amounts of radioactivity were precipitated with 2,4-dinitrophenylhydrazine. The Holman reaction was essentially negative in this fraction, too. It seems that all these data suggest that the material of this fraction is an adduct of pyruvate and DPN in the reduced state. It also seems reasonable to postulate for this adduct a cyclization between the carbonyl group of pyruvate and the amido group of the coenzyme on the basis of the lack of precipitation of radioactive material with 2,4-dinitrophenylhydrazine and the negativity of the Holman reaction.

Coenzyme analogs were also tested for their ability to react with pyruvate. When desamino-DPN was used, the products were: reduced coenzyme, an oxidized and a reduced adduct, as with DPN. On the other hand, when 3-acetylpyridine-DPN was used, only very small amounts of reduced coenzyme and oxidized adduct were formed, while no formation of reduced adduct was detected. This seems to be consistent with the possibility of a cyclization between the carbonyl group of pyruvate and the amido group of the coenzyme, insofar as no amido group is present in the molecule of 3-acetylpyridine–DPN.

In order to obtain more information on the mechanism of formation of the products of the reaction between DPN^+ and pyruvate at alkaline pH, the effect of several buffers on the velocity of the appearance of the absorbance at 340 mμ and 420 mμ was tested. These experiments clearly show that the formation of the products of the reaction is catalyzed by anions. Ammonia, carbonate and hydroxyl ions catalyze the formation of adducts with rate constants of $4.3 \cdot 10^{-3}\ M^{-1} \cdot min^{-1}$, $5.4 \cdot 10^{-3}\ M^{-1} \cdot min^{-1}$ and $52\ M^{-1} \cdot min^{-1}$, respectively. When pyruvate was incubated in carbonate-bicarbonate buffer (pH 9–10), in tritiated water, the isotope was found in pyruvate. It is

possible, therefore, that the formation of products in the reaction of DPN^+ and pyruvate involves the enolization of pyruvate. The rate of isotope incorporation was catalyzed by anions, similar to the formation of products of the reactions between pyruvate and DPN^+. Experiments designed to elucidate the possible implication of these findings from the enzymological point of view are under way. At any rate, it seems rather significant that DPNH is a product of the reaction between DPN^+ and pyruvate. To the best of our knowledge, this is the first time that DPNH has been obtained in a non-enzymatic reaction between oxidized coenzyme and oxidized substrate.

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