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On the Nature of the Lactic Dehydrogenase–Oxidized Coenzyme–Pyruvate Complex*

Giovanni Di Sabato

ABSTRACT: Complexes of the type chicken heart lactic dehydrogenase–oxidized coenzyme–pyruvate have been obtained with DPN and with a number of DPN analogs. These complexes were stabilized by reduction with $NaBH_4$ and isolated on a Sephadex column. The complexes showed a marked decrease in enzymatic activity which was, at least in part, restored upon dilution or treatment with DPNH. *p*-Hydroxymercuric benzoate partially prevented the formation of the complexes and reacted with them more slowly than with the free enzyme. After treatment with $NaBH_4$, the coenzyme–substrate portion of the complexes could be dissociated from the enzyme by precipitating the latter with heat or acids. The enzyme–coenzyme–substrate complex could also be dissociated by treating it with snake venom phosphodiesterase. The behavior of the coenzyme–substrate complex on column and thin-layer chromatography indicates the existence of a covalent bond between the coenzyme and the substrate. The coenzyme is in 1,4-dihydro form, as shown by its behavior with phenazine methosulfate. Also the substrate is in reduced form. The coenzyme–substrate complex obtained after precipitation of the enzyme has a structure different from that of the adducts of DPN and pyruvate obtained in the absence of enzyme. A tentative structure of the coenzyme–substrate complex, consistent with the available evidence, is given.

The formation of inactive, "abortive" ternary complexes of the type LDH[1]–DPN–pyruvate has been studied by Fromm (1961). In this and in other papers (Fromm, 1963; Zewe and Fromm, 1965), it was postulated that the inhibition of the enzymatic activity of LDH's by high concentrations of pyruvate was due to the formation of these complexes.

The formation of "abortive" complexes has been further investigated by Vestling and Künsch (1968) and by Gutfreund *et al.* (1968). Kaplan *et al.* (1968) and Everse *et al.* (1970) have discussed their physiological significance. Griffin and Criddle (1970) have shown that the formation of the ternary complex LDH–DPN–pyruvate involves the monomeric unit of the enzyme.

The isolation of complexes of the type CHLDH–DPN–pyruvate and CHLDH–DPN–pyruvate ($NaBH_4$) has been described by Di Sabato (1968b). In the present paper these observations have been extended and some details of the structure of the coenzyme–substrate portion of these complexes are presented.

Materials and Methods

The chemicals used in this work were obtained from the following sources; Boehringer, Mannhein Co. (DPN, DPNH, TPN), PL Biochemicals Co. (APDPN, DeAPDPN, PADPN, DePADPN, THDPN), Sigma Chemical Co. (NMN, PHMB, PMS, 2,4-dinitrophenylhydrazine), Nutritional Biochemical Co. (sodium pyruvate and sodium borohydride). [2-^{14}C]Pyruvate, [^{14}C]DPN (DPN labeled on the carboxamide group), [^{3}H]DPNAdH (DPN labeled on the adenine ring) and [$^{3}H_4$]-$NaBH_4$ were obtained from New England Nuclear. [4-^{3}H]DPN (DPN labeled in position 4 of the nicotinamide ring) was obtained from Amersham/Searle.

CHLDH was a gift of Dr. N. O. Kaplan. It was prepared according to Pesce *et al.* (1964). Rabbit muscle LDH, DPNase from *Neurospora crassa*, and phosphodiesterase from snake venom were purchased from Worthington Biochemcial Co.

In a typical experiment, suitable amounts of enzyme, coenzyme, and substrate (Di Sabato, 1968b) were incubated in 0.1 M sodium phosphate buffer, pH 6.90, for about 1 hr at room temperature. In the early stages of this work, the incubation time of the sample was about 12–14 hr. These

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[1] The following abbreviations were used in this paper: LDH, lactic dehydrogenase; CHLDH, chicken heart lactic dehydrogenase; APDPN, 3-acetylpyridine analog of DPN; DeAPDPN, desamino-3-acetylpyridine-DPN; PADPN, 3-pyridinecarbaldehyde analog of DPN; DePADPN, desamino-3-pyridinecarbaldehyde-DPN; THDPN, thionicotinamide-DPN; PHMB, *p*-hydroxymercuric benzoate; PMS, phenazine methosulfate. The composition of the complex followed by ($NaBH_4$) indicates that the complex has been treated with $NaBH_4$.

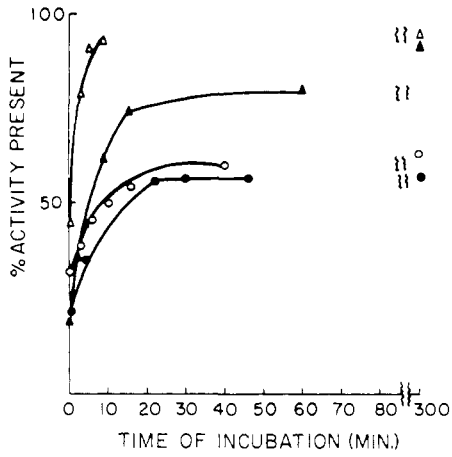


FIGURE 1: Effect of dilution and of DPNH on the reactivation of the CHLDH-DPN-pyruvate complex. The complex was formed in 0.1 M sodium phosphate buffer, pH 6.90. Reactivation was achieved by the following methods: tenfold dilution of the complex in 0.1 M sodium phosphate buffer, pH 6.90, alone (●) or in the presence of 1.6×10^{-4} M DPNH (▲), tenfold dilution of the complex in 0.1 M sodium glycinate buffer, pH 10.00, alone (○) or in the presence of 1.6×10^{-4} M DPNH (△). The concentration of the complex was about 4.3×10^{-6} M (before dilution). At suitable times, an aliquot was withdrawn from the incubation mixture and the enzymatic activity was measured; temperature of incubation, 25°.

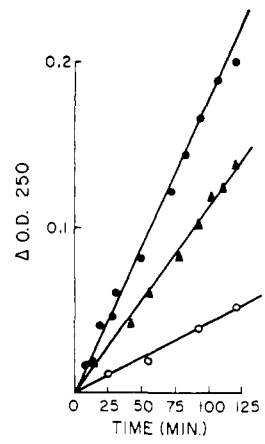


FIGURE 2: Binding of PHMB to CHLDH and CHLDH-APDPN-pyruvate complexes: (●) binding of PHMB to CHLDH (3.5×10^{-6} M); (▲) binding of PHMB to CHLDH-APDPN-pyruvate (3.0×10^{-6} M); (○) binding of PHMB to CHLDH-APDPN-pyruvate ($NaBH_4$) (3.1×10^{-6} M). PHMB was 7×10^{-5} M in all cases. The reactions were run in 0.1 M sodium phosphate buffer, pH 6.90 at 25°.

variations did not seem to affect the composition of the complexes. When required, 0.040 ml of 1 M $NaBH_4$ was added per ml of incubation mixture at the end of the incubation period. $NaBH_4$ was added in two aliquots 20–30 min apart. The mixture was incubated for an additional 30 min after the last addition of $NaBH_4$. The mixture was then passed through a Sephadex G-25 column (28 × 1.6 cm) eluted with a suitable buffer (0.1 M sodium phosphate, pH 6.90–0.15 M ammonium carbonate-bicarbonate, pH 10.20, or 0.1 M Tris·HCl, pH 8.80). The complex was eluted from the column at about 22–30 ml of effluent, depending on the type of buffer used. The amount of substrate present in the enzyme–coenzyme–substrate complexes was determined by using [2-^{14}C]pyruvate and by referring its specific radioactivity to the amount of enzyme present. The latter was estimated by applying an ϵ_M of 1.9×10^5 at 280 mμ for CHLDH (Pesce *et al.*, 1964). The amount of coenzyme present in the complexes was estimated by ribose determination (Lampen, 1953) and/or by the ratio of absorption at 260 and 280 mμ.

The cleavage of the complex by snake venom phosphodiesterase (about 0.005 mg of enzyme/ml of incubation mixture) was carried out in 0.1 M Tris·HCl buffer, pH 8.80, containing 0.025 M $MgCl_2$ at 36°. The incubation time was for 2–3 hr. Published procedures were used for the isolation of the complexes using DEAE-cellulose columns (Di Sabato, 1968a), the reaction with PHMB (Di Sabato and Kaplan, 1963), the separation of compounds on thin-layer chromatography (Di Sabato, 1970), the splitting of DPN by DPNase (Kaplan, 1955), the reduction of DPN with ethanol and alcohol dehydrogenase (Racker, 1955), the measurement of the enzymatic activity of LDH (Di Sabato, 1965), the formation of the 2,4-dinitrophenylhydrazone of pyruvic acid (Silverstein and Boyer, 1964), and the colorimetric determination of 2,4-dinitrophenylhydrazones (Lu, 1939). Oxidation of the complexes was carried out with about 0.05% PMS, in 2–4 ml of sample, in the dark. Reduction of the complexes with sodium dithionite was carried out using 4% $Na_2S_2O_4$ in 2.6% sodium carbonate for about 2 hr at room temperature.

The standardization of [$^3H_4$]$NaBH_4$ was carried out by reducing [^{14}C]pyruvate with [$^3H_4$]$NaBH_4$ and by calculating the $^3H/^{14}C$ ratio of the resulting [^3H,^{14}C]lacetate. The radioactive lactate was isolated on a column of Dowex 1-X8 (Cl^- form) eluted with a concentration gradient of HCl, from 0 to 0.12 N.

Mass spectrophotometry was carried out in an LKB gas chromatograph mass spectrometer Model 9000. A Zeiss spectrophotometer Model PMQ II, was used for spectrophotometric work. Scintillation mixture prepared according to Bray (1960) was used for measuring radioactivity in a three-channel Packard liquid scintillation spectrometer, Model 3003. The instrument was calibrated for overflow of isotopes into each channel when more than one isotope was used.

Results

Pyruvate alone (*i.e.*, in the absence of coenzyme) did not bind to the enzyme even after treatment of a mixture of enzyme and pyruvate with $NaBH_4$. However, it did bind to the enzyme when coenzymes or coenzyme analogs were present. Therefore, in general, the finding of pyruvate bound to the enzyme after incubation with coenzyme or coenzyme analog and passage through a Sephadex column, was considered evidence for the formation of a complex of the type enzyme–coenzyme–substrate (Di Sabato, 1968b).

When a mixture of CHLDH, pyruvate, and DPN or DPN analog (APDPN, DeAPDPN, PADPN, DePADPN, or THDPN) was treated with $NaBH_4$ and passed through a Sephadex column, 2–4 molecules of pyruvate and nucleotide were bound per molecule of enzyme. Essentially no complex was formed with NMN or TPN. When a mixture of CHLDH and DPN or DPN analog was treated with $NaBH_4$, some reduced coenzyme was bound to the enzyme emerging from the Sephadex column. This finding (due to the relatively high binding constant of reduced coenzymes to LDH's) should not be confused with the formation of the LDH–coenzyme–substrate complex.

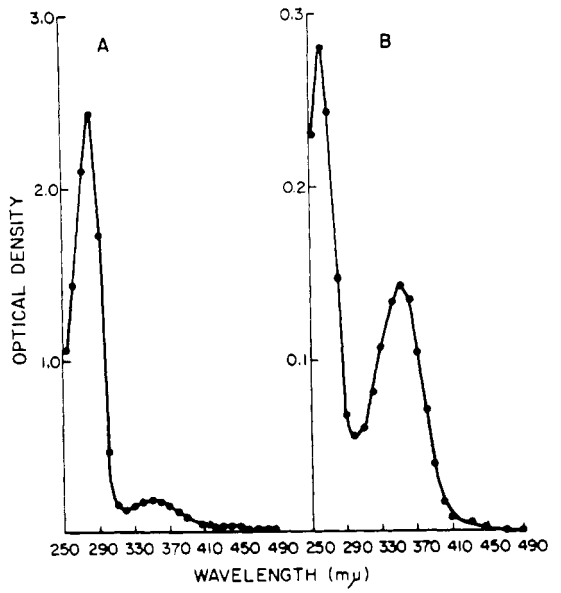


FIGURE 3: Spectra of CHLDH–APDPN–pyruvate ($NaBH_4$): (A) before precipitation of the enzyme; (B) after precipitation of the enzyme with boiling. The spectra were taken in 0.15 M ammonium carbonate–bicarbonate buffer, pH 10.20.

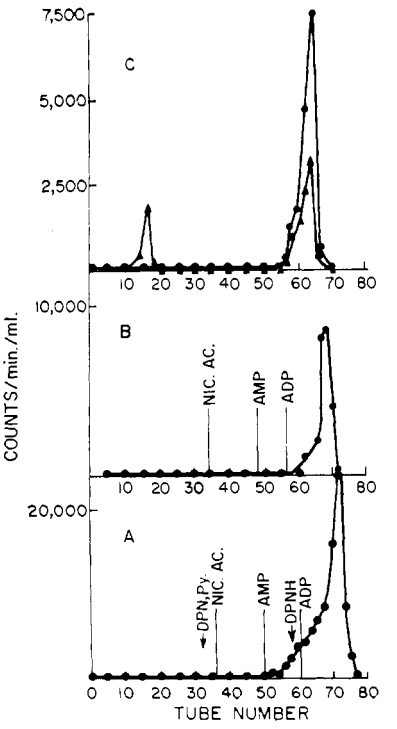


FIGURE 4: Chromatographic pattern on a DEAE-cellulose column of the radioactivity of DPN–pyruvate ($NaBH_4$) labeled in different positions of the molecule: (A) DPN–[^{14}C]pyruvate ($NaBH_4$); markers, nicotinic acid, AMP, and ADP. The approximate positions of elution of pyruvate, DPN, and DPNH are also indicated. (B) [^{14}C]DPN–pyruvate ($NaBH_4$): markers, as above. (C) [4-^{3}H]-DPN–[^{14}C]pyruvate ($NaBH_4$), (●) ^{14}C radioactivity, (▲) tritium radioactivity. The CHLDH–coenzyme–substrate ($NaBH_4$) complex was obtained as usual. The enzyme was precipitated by boiling. The DEAE-cellulose column was eluted with a concentration gradient between 0.0015 and 0.15 M ammonium carbonate–bicarbonate buffer, pH 10.20.

When the inactive CHLDH–DPN–pyruvate complex was incubated in dilute solution alone or with DPNH, a time-dependent recovery of enzymatic activity was observed (Figure 1). The recovery was faster in the presence of DPNH and at higher pH.

Di Sabato and Kaplan (1963) have shown that PHMB inactivates LDH's. On the other hand, the loss in enzymatic activity of the LDH–oxidized coenzyme–pyruvate complexes suggested that coenzyme and substrate may be bound to the enzyme at the same site as PHMB. In order to clarify this point, CHLDH which had been treated with PHMB (about 4 moles of PHMB bound/mole of enzyme) was incubated with APDPN and pyruvate and then reduced with $NaBH_4$. After passage through Sephadex, about 0.8 mole of pyruvate was bound per mole of enzyme. The enzyme untreated with PHMB binds about 2.7 moles of substrate. Furthermore, as shown in Figure 2, PHMB reacted more slowly with the CHLDH–APDPN–pyruvate complex than with the free enzyme. The binding of PHMB to the CHLDH–APDPN–pyruvate ($NaBH_4$) complex was even slower. This is in harmony with the observation that $NaBH_4$ stabilizes the complex.

Isolation of the Coenzyme–Substrate ($NaBH_4$) Complex. Dissociation of the CHLDH–coenzyme–pyruvate ($NaBH_4$) complexes could be achieved by one of the following methods: (a) precipitation of the protein by boiling for 5–10 min in 0.15 M ammonium carbonate–bicarbonate buffer, pH 10.20; (b) precipitation of the protein by treatment with 10% HCl or 10% $HClO_4$ (final concentrations); (c) treatment of the complex with snake venom phosphodiesterase. These experiments were designed in order to answer the following questions. (1) Are the coenzyme and the substrate covalently bound after dissociation of the LDH–coenzyme–substrate ($NaBH_4$) complexes? (2) If so, what are the chemical groups that are involved? (3) What is the oxidation–reduction state of the coenzyme? (4) What is the oxidation–reduction state of the substrate?

Figure 3A shows the spectrum of the CHLDH–APDPN–pyruvate ($NaBH_4$) complex isolated on a Sephadex column. The absorption maxima at 280 and 350 mμ are due to the protein, and to the coenzyme, respectively. Figure 3B shows the spectrum of the same sample after precipitation of the enzyme by boiling. The protein absorption had disappeared and the material left in solution showed absorption maxima at 260 and 350 mμ. Similar results (except for differences in the position of the absorption maximum above 300 mμ) were obtained with other coenzyme analogs and with DPN. Experiments in which [^{14}C]pyruvate or [^{14}C]DPN were used, showed that at least 80–90% of the pyruvate and coenzyme were present in the supernatant, after precipitation of the enzyme.

Figure 4 shows the elution patterns from a DEAE-cellulose column of the radioactivity of the DPN–pyruvate ($NaBH_4$) complex obtained after precipitation of the enzyme by boiling. Figure 4A refers to the DPN–[^{14}C]pyruvate ($NaBH_4$) complex, Figure 4B to the [^{14}C]DPN–pyruvate ($NaBH_4$) complex and Figure 4C to the [4-^{3}H]DPN–[^{14}C]pyruvate ($NaBH_4$) complex. Unlabeled nicotinic acid, AMP, and ADP were used as markers for the position of the radioactivity. In Figure 4A the approximate positions at which pyruvate, DPN, and DPNH would be eluted from the column are also indicated. The elution patterns of radioactivity shown in Figure 4 clearly indicate that the coenzyme and the substrate portion of the complex are eluted at the same position, which is different from the position of elution of DPN, pyruvate, or DPNH.

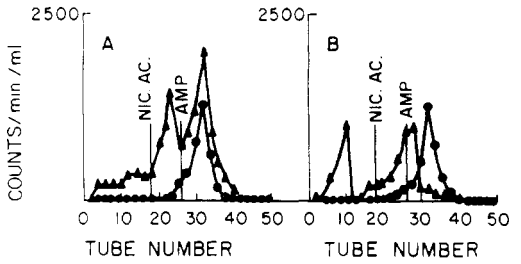


FIGURE 5: Chromatographic pattern on a DEAE-cellulose column of the radioactivity of the CHLDH–DPN–pyruvate ($NaBH_4$) complex labeled in different positions and treated with phosphodiesterase from snake venom: (A) CHLDH–[4-^{3}H]DPN–[^{14}C]pyruvate ($NaBH_4$); (B) CHLDH–[^{3}H]DPNAdH–[^{14}C]pyruvate ($NaBH_4$). Nicotinic acid and AMP were used as markers: (●) ^{14}C radioactivity; (▲) tritium radioactivity; elution, as indicated in the legend to Figure 4.

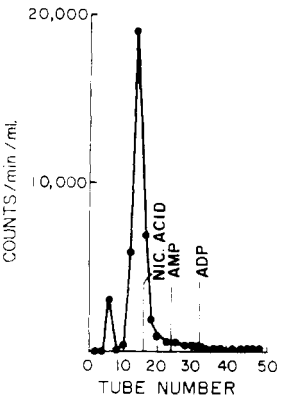


FIGURE 6: Chromatographic pattern on a DEAE-cellulose column of the radioactivity of the DPN–[^{14}C]pyruvate ($NaBH_4$) complex after treatment with PMS. The CHLDH–DPN–[^{14}C]pyruvate ($NaBH_4$) complex was obtained as usual. The enzyme was precipitated by boiling: markers, nicotinic acid, AMP, ADP; elution, as indicated in the legend to Figure 4.

In other experiments, the CHLDH–[^{14}C]DPN–pyruvate ($NaBH_4$) complex was incubated for about 1 hr in 10% $HClO_4$. The protein was spun down and the supernatant was neutralized with NaOH. The CHLDH–DPN–[^{14}C]-pyruvate ($NaBH_4$) complex was treated in the same way. The two supernatants [containing the acid-decomposition product of [^{14}C]DPN–pyruvate ($NaBH_4$) and of DPN–[^{14}C]pyruvate ($NaBH_4$), respectively] were passed through two DEAE-cellulose columns. The radioactivity was eluted in the same volume of buffer from both columns. The fraction from each column containing the radioactivity was concentrated and analyzed on thin-layer chromatography. Table I shows that the acid-decomposition product of [^{14}C]DPN–pyruvate ($NaBH_4$) and of DPN–[^{14}C]pyruvate ($NaBH_4$) had the same R_F values in all the solvents used. These complexes were well separated from [^{14}C]pyruvate (particularly in solvents D and E), [^{14}C]lactate (particularly in solvent D), and [^{14}C]-DPN (particularly in solvent F).

Experiments with Phosphodiesterase. Treatment of the LDH–coenzyme–substrate ($NaBH_4$) complex with snake venom phosphodiesterase restored the enzymatic activity of the complex, showing that, once the pyrophosphate bond is cleaved, neither of the moieties resulting from the action of phosphodiesterase on the coenzyme remains bound to LDH.

The CHLDH–[4-^{3}H]DPN–[^{14}C]pyruvate ($NaBH_4$) complex was treated with phosphodiesterase and passed through a DEAE-cellulose column. The elution pattern of Figure 5A shows that the ^{14}C was eluted from the column together with the tritium. The complex of [^{14}C]pyruvate and DPN tritium-labeled in the adenine moiety CHLDH–DPNAd^{3}H–[^{14}C]-pyruvate ($NaBH_4$) was also treated with phosphodiesterase. The elution patterns of Figure 5B show that the ^{14}C was eluted from the column clearly separated from the tritium. These data show that pyruvate is bound to the nicotinamide moiety of DPN. The first fraction of tritium radioactivity shown in Figure 5A (tubes 18–26) is probably reduced NMN, derived from the cleavage by phosphodiesterase of some DPNH bound to the enzyme. No effort was made to identify the first fraction of tritium radioactivity (tubes 2–12) shown in Figure 5B.

Experiments with PMS. The complex DPN–pyruvate ($NaBH_4$) obtained after precipitation of the protein by boiling, was treated with PMS. A decrease in optical density at wavelengths above 300 mμ was observed. No new absorption maxima at 370 or 420 mμ appeared.

The DPN–[^{14}C]pyruvate ($NaBH_4$) complex was oxidized with PMS and then passed through a DEAE-cellulose column. Figure 6 shows that the bulk of the radioactivity was eluted from the column at the same position as authentic pyruvate (*cf.* Figure 6 with Figure 4A).[2] Analogous results were obtained on a Dowex 1-X8 (Cl^- form) column, eluted with a concentration gradient of HCl from 0 to 0.12 N. However, only a small fraction of the material was precipitated as the 2,4-dinitrophenylhydrazone. This is probably due to the well-known instability of [^{14}C]pyruvate (Silverstein and Boyer, 1964).

In other experiments, the [^{14}C]DPN–pyruvate ($NaBH_4$) complex, obtained after precipitation of the protein by boiling, was oxidized with PMS and chromatographed on a DEAE-cellulose column. The radioactivity was eluted from the column at the same position as authentic DPN (not shown).

TABLE I: R_F Values on Thin-Layer Chromatography of the Acid-Decomposition Products of [^{14}C]Pyruvate–DPN ($NaBH_4$) and Pyruvate–[^{14}C]DPN ($NaBH_4$), and of [^{14}C]-Pyruvate, [^{14}C]Lactate, and [^{14}C]DPN.

Compound	R_F in Solvent[a]					
	A	B	C	D	E	F
[^{14}C]Pyruvate–DPN ($NaBH_4$)	0.06	0.67	0.89	0.17	0.44	0.67
Pyruvate–[^{14}C]DPN ($NaBH_4$)	0.06	0.67	0.89	0.17	0.44	0.67
[^{14}C]Pyruvate		0.73	0.94	0.25	0.56	0.75
[^{14}C]Lactate		0.67	0.89	0.28	0.50	0.61
[^{14}C]DPN		0.72	0.89	0.06		0.47

[a] Composition of the solvents: A, petroleum ether–pyridine (64:36): B, ethanol–ammonia–water (88:4:8); C, acetone–water (50:50); D, butanol–acetic acid–water (75:15:10); E, propanol–ammonia (64:36); F, pyridine–acetic acid–water (50:35:15).

[2] Occasionally, in these experiments, a peak of radioactivity (not shown) appeared at approximately the position of ADP. Probably it is a product of partial oxidation of the complex.

The nature of this material was established by the fact that it was cleaved by DPNase and reduced by ethanol and catalytic amounts of alcohol dehydrogenase. Analogous results were obtained when APDPN or PADPN were used in the formation of the complexes. These experiments show that oxidation of the DPN–pyruvate ($NaBH_4$) complex with PMS leads to its decomposition into DPN and probably pyruvate.

The [4-^{3}H,^{14}C]DPN–pyruvate ($NaBH_4$) complex (prepared from [4-^{3}H]DPN and [^{14}C]DPN), was obtained after precipitation of the enzyme by boiling. The complex was treated with PMS and then passed through a DEAE-cellulose column. The $^3H/^{14}C$ ratio of the DPN emerging from the column was about one-half of that of the complex before oxidation. The remainder of the tritium radioactivity emerged from the column at the breakthrough volume. These experiments clearly show that the DPN of the DPN–pyruvate ($NaBH_4$) complex is reduced at position 4 of the nicotinamide ring.

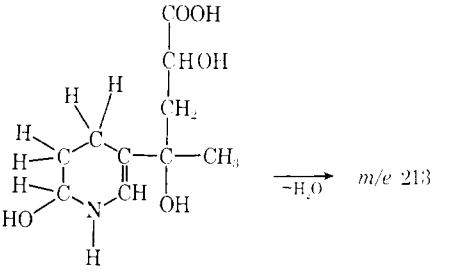
Oxidation–Reduction State of the Substrate. In order to establish the oxidation–reduction state of the substrate of the CHLDH–DPN–pyruvate ($NaBH_4$) complex, tritium-labeled $NaBH_4$ ([$^3H_4$]$NaBH_4$) (the standardization of which was carried out as described under Materials and Methods) was used for the reduction of the CHLDH–DPN–[^{14}C]-pyruvate. The enzyme was precipitated by boiling and the coenzyme–substrate complex was isolated on a DEAE-cellulose column. Determination of the $^3H/^{14}C$ ratio showed that the complex had about 1.3–1.5 atoms of tritium per molecule of pyruvate. In control experiments, [^{14}C]DPN was reduced with [$^3H_4$]$NaBH_4$. The resulting DPNH, isolated on a DEAE-cellulose column, had 0.4–0.5 atom of tritium per molecule of coenzyme. No attempt was made to investigate whether the failure to find 1 atom of tritium per molecule of DPNH was due to an exchange with the solvent or, more probably, to isotope discrimination during the reduction of DPN with [$^3H_4$]$NaBH_4$.

No 2,4-dinitrophenylhydrazone formation could be detected when 2,4-dinitrophenylhydrazine was added to a solution of the APDPN–pyruvate ($NaBH_4$) complex. This indicates the absence of carbonyl groups in the complex.[3]

It has been shown that sodium dithionite reduces DPN or DPN analogs to the corresponding 1,4-dihydro compounds (Von Euler *et al.*, 1936; Yarmolinsky and Colowick, 1956). When sodium dithionite was used, instead of $NaBH_4$, to reduce the CHLDH–DPN–pyruvate complex the enzyme emerged from the Sephadex column with some reduced coenzyme bound to it, but with only negligible amounts of substrate. These results show that sodium dithionite, unlike $NaBH_4$, cannot reduce (and, therefore, stabilize) the enzyme–coenzyme–substrate complex.

Mass Spectrometry of the Acid-Cleavage Product of APDPN–Pyruvate ($NaBH_4$). In order to gain more information on the structure of the coenzyme–substrate ($NaBH_4$) complex, CHLDH–APDPN–[^{14}C]pyruvate ($NaBH_4$) was incubated with 10% HCl and the products were isolated on a Dowex 1-X8 (Cl^- form) column eluted with a concentration gradient of HCl from 0 to 0.12 N. The fraction containing the radioactivity was taken to dryness and the mass spectrum of the residue obtained. Major peaks appeared at *m/e* 213, 199, and 185. Other peaks appeared at *m/e* above 213. The ion with *m/e* 213 probably results from the loss of the elements of water from the compound shown in Chart I. The structure of this compound was derived on the basis of: (a) the finding by Kim and Chaykin (1968) that 1-(2,6-dicholorobenzyl)-6-hydroxy-1,4,5,6-tetrahydronicotinamide is the primary acid-decomposition product of 1-(2,6-dichlorobenzyl)-1,4-dihydronicotinamide; (b) the observation that in 1 N HCl, DPNH is split at the nicotinamide–ribose bond (Burton and Kaplan, 1963). [We found in separate experiments that this split occurs also with the coenzyme–substrate ($NaBH_4$) complexes.] Loss of the elements of water could occur either by dehydration of the substrate moiety (*cf.* Budzikiewicz *et al.*, 1967) or by dehydration of the ring (Kim and Chaykin, 1968). The presence of the ion at *m/e* 199 can be explained with the loss of CH_2 from the *m/e* 213 ion. The loss of a second CH_2 from the *m/e* 199 ion or of NHCH from the *m/e* 213 ion can explain the presence of the peak at *m/e* 185. Similar fragmentations have been found for piperdine (Budzikiewicz *et al.*, 1967; Cornu and Massot, 1966). The peaks at *m/e* above 213 may represent other products resulting from the treatment of the complex with acid.

CHART I



Experiments with Rabbit Muscle Lactic Dehydrogenase. Some of the experiments described in the previous paragraphs were carried out also with rabbit muscle LDH. The results were essentially the same as those obtained with CHLDH, except for the fact that smaller amounts of coenzyme and substrate were bound to the rabbit muscle enzyme (*cf.* Everse *et al.*, 1970).

Discussion

The experiments described in this paper give some new information on the structure and significance of the "abortive ternary complexes" described by various authors (Griffin and Criddle, 1970, and references therein).

The fact that the isolated CHLDH–oxidized coenzyme–pyruvate complexes show a loss in enzymatic activity, lends support to the hypothesis that the inhibition of LDH's by high concentrations of pyruvate is due to the formation of "abortive ternary complexes." Our data, presented in this paper (Figure 1) and elsewhere (Di Sabato, 1968b), shows that the enzymatic activity of the enzyme–oxidized coenzyme–pyruvate complexes can be restored by addition of DPNH. Therefore, it is possible that the pyruvate/DPNH ratio has some importance in the regulation of the activity of LDH's *in vivo*. It is possible that, when the amount of DPNH present in the cells is low, LDH is inhibited by the formation of the

[3] It should be noted that in the method by Lu (1939), the 2,4-dinitrophenylhydrazone of pyruvic acid is extracted in the organic phase (ethyl acetate). However, in our experiments, the radioactivity of the APDPN–[^{14}C]pyruvate ($NaBH_4$) complex remained in the aqueous phase. This may be due to the fact that the substrate is covalently bound to the coenzyme.

TABLE II: Some Properties of the Coenzyme–Pyruvate Adducts Obtained in the Absence of Enzyme (Di Sabato, 1968a) and of the Coenzyme–Pyruvate ($NaBH_4$) Complexes (This Paper).

	Absorption Maximum of Primary Product (>300 mμ) (the Coenzyme is DPN) (mμ)	Absorption Maximum of Product of Oxidation with PMP (>300 mμ) (the Coenzyme is DPN) (mμ)	% of Initial ^3H Present in Compound Obtained with [4-^{3}H]DPN after PMS Treatment	Effect of PMS on Stability of Primary Product	Formation of Primary Product with APDPN
Coenzyme–pyruvate (no enzyme)	340	370, 420 (pH dependent)	*Ca.* 0	No obvious effect	–
Coenzyme–pyruvate ($NaBH_4$)	330	No absorption maxima at 370 and 420	*Ca.* 50	Dissociates	+

enzyme–DPN–pyruvate complex. This would channel pyruvate toward the Krebs' cycle with consequent production of DPNH which would reactivate LDH by dissociating the ternary complex (*cf.* Kaplan *et al.*, 1968; Everse *et al.*, 1970).

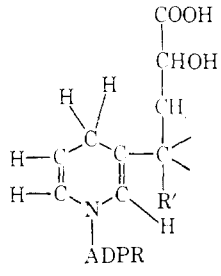
The chromatographic behavior on DEAE-cellulose of the coenzyme–substrate ($NaBH_4$) complexes indicates the presence of a covalent bond between coenzyme and substrate. This is also shown by the identical behavior on thin-layer chromatography of the acid-decomposition product of [^{14}C]DPN–pyruvate ($NaBH_4$) and DPN–[^{14}C]pyruvate ($NaBH_4$) (Table I).

The experiments with phosphodiesterase show that the pyruvate molecule is bound to the pyridine part of the coenzyme. Consistent with this idea is the observation that the absorption maximum at wavelengths above 300 mμ of the complexes is different from that of the corresponding reduced coenzyme (*e.g.*, 330 mμ, instead of 340 mμ for the complex with DPN; 350 mμ instead of 360 mμ for the complex with APDPN) (Di Sabato, 1968b). Binding of a compound at a site other than the pyridine ring of the coenzyme would probably not cause a difference in the position of the absorption maximum.

The observation that PMS quenches the optical density of the coenzyme–substrate ($NaBH_4$) complex at wavelengths above 300 mμ strongly suggests the presence of reduced coenzyme. The fact that treatment with PMS causes a decrease of the radioactivity of the [4-^{3}H]DPN–pyruvate ($NaBH_4$) complex, indicates that the coenzyme is in the 1,4-dihydro form.

The observation that the DPN–pyruvate complex reduced with [^{3}H$_4$]$NaBH_4$ has a higher specific radioactivity than DPN reduced in the same way, indicates also that the substrate is present in reduced form in these complexes. Consistent with this idea are the lack of reaction of the APDPN–pyruvate ($NaBH_4$) complex with 2,4-dinitrophenylhydrazine and the observation that sodium dithionite is ineffective in forming complexes.

CHART II



The chemical and spectral characteristics of adducts of DPN (and related compounds) and carbonyl compounds (particularly pyruvate) formed in the absence of enzyme have been investigated recently (Di Sabato, 1968a, 1970). Some of the characteristics of these adducts and of the coenzyme–substrate ($NaBH_4$) complexes are summarized in Table II. Clearly, the complexes of the type DPN–pyruvate ($NaBH_4$) are different from the adduct of the type DPNH–pyruvate. In particular, position 4 of the nicotinamide ring of the coenzyme can be ruled out as the site of reaction of pyruvate in the former type of complexes.

A probable structure of the complexes of the type coenzyme–pyruvate ($NaBH_4$) is shown in Chart II. A probable mechanism for the formation of this type of complex is a reaction of the enolic form of pyruvate (Griffin and Criddle, 1970; Coulson and Rabin, 1969) with the C=O group in position 3 of APDPN or PADPN to give the relatively unstable ternary complex. Upon treatment with $NaBH_4$, pyruvic acid is reduced to lactic acid. This stabilizes the complex. While the proposed reaction between the enolic form of pyruvate and the carbonyl group of APDPN or PADPN is a normal aldol condensation, it is more difficult to visualize the same reaction with the C=O of the carboxamide group of DPN. A possible interpretation of the mechanism of this reaction is the formation of a hydrogen bond between the side chain of an amino acid residue of the enzyme and the C=O group of DPN. This should facilitate the attack by the enol of pyruvate.

The proposed mechanism of formation of the enzyme–oxidized coenzyme–pyruvate complexes also explains why no complexes are formed with enzyme, DPNH, and lactate (Fromm, 1967; Di Sabato, 1968b). Indeed, the absence of active hydrogen(s) in lactate prevents its binding to the coenzyme. Actually, Wiener and Schwert (1958) described the LDH–DPNH–lactate complex. However, this complex, which can be detected only fluorometrically, is probably different from the complexes described in the present paper in the sense that it does not involve a covalent bond of coenzyme and substrate.

The formation of a 1,4,5,6-tetrahydro compound is made improbable by the absence of the absorption maximum at about 290 mμ, characteristic of these compounds (Wallenfels, 1959).

When the complex is oxidized with PMS, it dissociates. As mentioned previously, no conclusive evidence for the identification of pyruvate as one of the products of the oxidation of DPN–pyruvate ($NaBH_4$) has been reached. The formation of pyruvate would imply oxidation by PMS of the lactic acid residue of the coenzyme–substrate ($NaBH_4$) complex, as well as of the coenzyme. Nonenzymatic oxidation of hydroxy acids by PMS has been shown (Kun, 1956).

The mass spectral data are consistent with the structure shown in Chart I. However, a precise interpretation of these data suffers from the uncertainty of the structure of the product(s) of the acid cleavage of the reduced coenzymes and coenzyme analogs. On the other hand, confirmation of the structure by other methods (*e.g.*, elemental analysis, nuclear magnetic resonance) was hampered by the fact that only very minute amounts of the complexes were available.

In conclusion, evidence has been provided here for the first time of the formation of a covalent bond between coenzyme and substrate *dependent on the presence of LDH*. It has also been shown that in the LDH–oxidized coenzyme–pyruvate complexes, the substrate is bound at position 3 of the pyridine ring of the coenzyme. On the other hand, Wallenfels (1959) and Di Sabato (1970) have shown that the chemical characteristics of the group at position 3 of the pyridine ring of coenzymes influence the rate and equilibrium of the hydride transfer. Thus, it is conceivable that the carboxamide group of DPN, by reversibly combining with the substrate or with some chemical group(s) on the enzyme surface, can regulate the rate and the extent of hydride transfer with different dehydrogenases and/or in different physiological conditions.

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