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Intermediates in the Catalytic Action of Lipoyl Dehydrogenase (Diaphorase)

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Lipoyl dehydrogenase was first discovered in bacteria and shown to be involved in the oxidation of keto acids (Gunsalus, 1954; Reed, 1957). A similar involvement has been shown in mammalian tissues, particularly in the pig-heart α -oxoglutarate-dehydrogenase complex (Sanadi, Langley & White, 1959). Massey (1958, 1960*a*) demonstrated that the lipoyl dehydrogenase of pig-heart muscle is a flavo-protein identical with the flavoprotein which was isolated by Straub (1939) and studied by Savage (1957). This conclusion has been confirmed by Searls & Sanadi (1959), but has also been dismissed (Ziegler, Green & Doeg, 1959). A likely explanation of the inability of Ziegler *et al.* to observe lipoyl-dehydrogenase activity in their diaphorase preparation has, however, been found in the sensitivity of this enzyme to trace metals (Veeger & Massey, 1960), which can abolish the activity towards lipid acid although at the same time it greatly stimulates the activity towards 2:6-dichlorophenol-indophenol, a dye commonly used to assay diaphorase activity. Further studies of this enzyme have shown that two, rather than one, molecules of pyridine nucleotide take part in the reaction:

Lipoic acids + reduced diphosphopyridine nucleotide + H^+ $\rightleftharpoons$ reduced lipoic acids + diphosphopyridine nucleotide

and that a protein dithiol group is formed as an intermediate (Massey & Veeger, 1960).

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Savage (1957) had reported that on the addition of reduced diphosphopyridine nucleotide to diaphorase there was incomplete bleaching of the 450 m μ band of the flavin and an increase in absorption at wavelengths greater than 500 m μ . This observation has been confirmed by Searls & Sanadi (1959). The formation of a red colour with many flavoproteins on the addition of substrates has been reported: e.g. old yellow enzyme (Haas, 1937); reduced diphosphopyridine nucleotide peroxidase (Dolin, 1956, 1957); acyl-coenzyme A dehydrogenases and snake-venom L-amino acid oxidase (Beinert, 1957); diaphorase (Savage, 1957); dihydro-orotic dehydrogenase (Friedmann & Vennesland, 1958); D-amino acid oxidase (Massey & Palmer, 1960). The cause of such a red colour has been the subject of much discussion. In general, the formation of a red colour has been taken to indicate the existence of the enzyme flavin in a half-reduced (or semiquinone or free radical) form, by analogy with the studies on free flavins (Kuhn & Wagner-Jauregg, 1934; Michaelis, Schubert & Smythe, 1936; Beinert, 1956). With reduced diphosphopyridine nucleotide peroxidase, however,

Dolin has presented much evidence that makes it difficult to accept this interpretation of the spectral changes observed with substrates and dithionite. At the moment no generalizations can be made;

each enzyme must be considered separately as a particular case. In this paper results are presented which suggest that with lipoyl dehydrogenase the red colour produced by substrate is due to the formation of a flavin semiquinone. The role of this form in the catalytic action of the enzyme has been investigated; its formation by reduced lipoic acids and its reoxidation by oxidized lipoic acids are the rate-determining steps in the reaction catalysed by this enzyme. A preliminary account of this work has been presented to the Symposium on Free Radicals in Biological Systems at Stanford, California.

EXPERIMENTAL

Materials

Chemicals. Reduced diphosphopyridine nucleotide (DPNH), diphosphopyridine nucleotide (DPN) and *p*-chloromercuriphenyl sulphonate were obtained from the Sigma Chemical Co. (St Louis, Mo., U.S.A.). DL- α -Lipoic acid was a gift from Dr I. C. Gunsalus. DL-Lipoylglycylglycine, dihydrolipoylglycine and dihydrolipoamide were gifts from Dr L. J. Reed. The bovine-serum albumin was the crystalline product of The Armour Laboratories (Eastbourne). Sodium dithionite (not less than 85%) and ethylenediaminetetra-acetate, disodium salt (EDTA), were from British Drug Houses Ltd. Solutions of the latter compound were neutralized with NaOH in making up standard concentrations. All other chemicals used were of analytical-reagent quality. All the experiments described in this paper were performed in the presence of sodium phosphate buffers, at pH 6.3, pH 7.0, pH 7.2 or pH 7.6. Stock 0.3M-buffers of these pH values were prepared by mixing 0.3M-solutions of Na_2HPO_4 and NaH_2PO_4 (22:78 for pH 6.3; 61:39 for pH 7.0; 71:29 for pH 7.2; 86:5:13:5 for pH 7.6, all v/v). All solutions were prepared with glass-distilled water.

Enzyme preparations. Lipoyl dehydrogenase was made by a slight modification of the method described by Massey (1960a). The present method gives larger yields of enzyme, chiefly because of an early ethanolic heat-treatment, which appears to dissociate lipoyl dehydrogenase from the α -oxoglutarate-dehydrogenase complex. With the method previously described, in which all purification steps were carried out at 4°, one-third to one-half of the lipoyl dehydrogenase was discarded in the first $(\text{NH}_4)_2\text{SO}_4$ fractionation (0–0.35 saturation) because it was still associated with the oxoglutarate-dehydrogenase complex (cf. Massey, 1960b).

The modifications of the previous preparation were:

(i) The extract of the pig-heart particles made with 1% (w/v) $(\text{NH}_4)_2\text{SO}_4$ was heated at 45° for 15 min. with continuous stirring in the presence of 3% (v/v) ethanol and 0.06 mM-EDTA. After cooling, the precipitate which had formed was centrifuged off in a Servall refrigerated centrifuge at 10 000 *g* for 20 min. The supernatant was treated as previously described and after fractionation on a calcium phosphate gel-cellulose column had 60–80% of the specific activity of the final preparation of Massey (1960a). (ii) The product from the calcium phosphate gel-cellulose column was further purified by heating at 68–70° for 5 min. in the presence of 0.06M-sodium phosphate, pH 7.0, 0.1M-

$(\text{NH}_4)_2\text{SO}_4$ and 0.1 mM-EDTA, protein concentration 2–6 mg./ml. The white precipitate formed was centrifuged off in a refrigerated centrifuge at 25 000 *g* for 10 min. and the yellow supernatant was brought to 0.55 saturation with $(\text{NH}_4)_2\text{SO}_4$ (34 g./100 ml. of supernatant). Any precipitate which formed when the solution was allowed to stand for 15 min. at 0° was centrifuged off and discarded. The supernatant was brought to 0.7 saturation by adding 10 g. of $(\text{NH}_4)_2\text{SO}_4$ /100 ml. of 0.55-saturated supernatant. After 15 min. at 0° the yellow precipitate of enzyme was centrifuged off and dissolved in a suitable buffer, e.g. 0.03M-phosphate, pH 7.0. The product was identical with the enzyme previously described by Massey (1960a), with an overall yield from the first extract of 60–85%.

Methods

Enzyme assays. All assays were performed spectrophotometrically at 340 m μ in the thermostatically controlled cell chamber of a Beckman model DU spectrophotometer. Apart from the experiment reported in Fig. 7, all assays were carried out at $25 \pm 0.2^\circ$. Optical cells of 1 cm. light path were used throughout and the total volume in each assay was 3 ml. Each cell contained 150 μ moles of phosphate buffer of the appropriate pH value, 3 μ moles of EDTA and 2 mg. of bovine-serum albumin. When studying the oxidation of DPNH by oxidized lipoic acids the buffer used was at pH 6.3; reaction was started by adding successively 0.6–4.0 μ moles of DL-lipoic acid or 0.3–4.0 μ moles of DL-lipoylglycylglycine, 0.15 μ mole of DPNH, 0.2 μ mole of DPN and an appropriate amount of enzyme (an amount such that the extinction change/min. was less than 0.1; the amounts used in a particular experiment are given in the legends to the Figures in the Results section). When studying the reduction of DPN by reduced lipoic acids the buffer used was at pH 7.6; reaction was begun by the successive addition of 0.15–2.0 μ moles of DPN, 0.15–2.0 μ moles of dihydrolipoylglycine or dihydrolipoamide and an appropriate amount of enzyme (as described above). Details of the amounts of substrates and enzyme are given in the Results section. Solutions of dihydrolipoic acid derivatives were prepared as soon as possible before use; 0.3 mM-EDTA was present in such solutions to prevent metal-catalysed oxidation.

Determination of anaerobic spectra. The influence of various substrates and reagents on the absorption spectrum was studied in specially constructed tubes in which a glass tube of 1 cm. internal diameter, furnished with two projecting side arms and topped by a B14 standard ground-glass socket, was fused to an optical cell of 1 cm. light path (Beckman cat. no. 2097). After placing the enzyme and reagents in the appropriate compartments the cell was closed with a B14 standard ground-glass cone fitted with a stopcock. All ground-glass joints were greased and the tube was evacuated on a water pump, filled with O₂-free N₂, evacuated again and the whole cycle was repeated at least four times, the final step being an evacuation.

Preparation of enzyme for stopped-flow experiments. Purified enzyme (Massey, 1960a) was dialysed overnight against 0.05M-phosphate buffer, pH 6.3 or pH 7.6. Before use, dialysis tubing was soaked inside and out with 1 mM-EDTA for at least 10 min. and then washed with glass-distilled water. After dialysis, the enzyme was adjusted to the required concentration (see description of individual experiments) by dilution with the appropriate 0.05M-

buffer. Substrate solutions were prepared by dilution from stock solutions and were made up so that they contained a final concentration of 0.05M-phosphate buffer. Unless otherwise noted a small amount (0.3 mM) of EDTA was also present in all experiments. When the reoxidation by oxidized lipoic acids was studied, the enzyme semiquinone was prepared anaerobically as described in the previous section (except that the final gas phase was O_2 -free N_2) by tipping from the side arm the required amount of DPNH. The partially reduced enzyme was then immediately transferred with a hypodermic syringe to the stopped-flow apparatus.

Stopped-flow determinations. These were carried out with the apparatus of Gibson (1954) modified by the use of a 2 cm. light-path observation tube as described by Gibson & Antonini (1960), and by the use of a Hilger D246 monochromator as a source of light. With this equipment extinction changes are recorded photographically from 3 msec. after mixing of the reagents for as long as is required. The concentrations of the solutions were adjusted so that a change in extinction from 0.01 to 0.06 in the 2 cm. light path was observed.

Expression of results

Enzyme concentration and turnover numbers. It is convenient to distinguish between experiments where the amount of enzyme was small compared with the amounts of substrates, and the stopped-flow experiments, where the amounts of enzyme and substrates were of similar orders. The first are called 'catalytic experiments' or 'experiments with catalytic quantities of enzyme' and the others are called 'stopped-flow experiments'.

The enzyme concentration was calculated assuming that the molar-extinction coefficient of lipoyl dehydrogenase at 455 $m\mu$ is the same as that of flavinadenine dinucleotide at 450 $m\mu$, i.e. 1.13×10^4 cm.²/m-mole (Whitby, 1953). $E_{340\ m\mu}$ DPNH was taken as 6.22×10^3 cm.²/m-mole (Kornberg & Horecker, 1953).

Turnover numbers from the catalytic experiments are expressed as moles of substrate oxidized or reduced/min./mole of enzyme [as shown by Savage (1957), there is 1 mole of flavinadenine dinucleotide/mole of enzyme]. The extinction changes in stopped-flow experiments were expressed as first-order velocity constants in units of min.⁻¹, which are then directly comparable with turnover numbers as defined above.

RESULTS

Effect of substrates on the absorption spectrum.

Fig. 1 shows the effect on the visible spectrum of diaphorase produced by the anaerobic addition of either DPNH or dihydrolipoamide. A similar change was produced on the addition of dihydrolipoylglycine. The strong fluorescence of the untreated enzyme disappeared almost completely under these conditions. Fig. 2 shows the effect of the addition of DL- α -lipoic acid after the addition of DPNH. It is evident that the oxidized substrate causes a return of the spectrum almost to that of the oxidized enzyme. The extinction changes at 530 $m\mu$ produced by anaerobic titration of the oxidized enzyme with DPNH are plotted in Fig. 3,

which shows that a stoichiometric reaction has occurred with maximal extinction change on the addition of 1 mole of DPNH/mole of enzyme. These results were obtained with anaerobic spectrophotometer cells, and the readings were taken 30 sec.

Fig. 1. Effect of DPNH or dihydrolipoamide on the absorption spectrum (1 cm. light path) of pure diaphorase in 0.05M-phosphate, pH 7.6, at 25°; enzyme concentration 1.2 mg./ml. —, Oxidized enzyme; x—x, after addition of 1.9 moles of DPNH/mole of enzyme; ○—○, after addition of 18 moles of dihydrolipoamide/mole of enzyme. Additions of reducing substrates were made, and spectra were determined under anaerobic conditions (see Experimental section), in two separate experiments. The total volume in each was 3 ml.; the spectra shown are corrected for the slight volume changes after addition of DPNH or dihydrolipoamide.

Fig. 2. Effect on the anaerobic absorption spectrum of diaphorase (2.0 mg./ml.) of DPNH followed by lipoic acid. Temperature 25°; 0.05M-phosphate buffer, pH 6.3. x, Original oxidized enzyme; ○, after addition of 3.0 moles of DPNH/mole of enzyme; △, after further addition of 40 moles of DL- α -lipoic acid/mole of enzyme.

Fig. 3. Increase in $E_{530\text{ m}\mu}$ produced by the anaerobic addition of DPNH to diaphorase in 0.05M-phosphate, pH 7.6, at 25°. Each point represents the result obtained in a separate experiment, in which DPNH was added to a solution of diaphorase (1.2 mg./ml.). Increase in $E_{530\text{ m}\mu}$ was determined immediately after mixing (30 sec.) with a Beckman DU spectrophotometer. Actual increases in $E_{530\text{ m}\mu}$ ranged from 0.017 with the lowest amount of DPNH to 0.053 with the larger amounts of DPNH. The molar ratio of DPNH to enzyme was calculated assuming that $E_{455\text{ m}\mu}$ of oxidized enzyme was $1.13 \times 10^4 \text{ cm}^2/\text{m-mole}$ and the $\Delta E_{530\text{ m}\mu}$ was calculated on the same assumption.

Fig. 4. Increase in $E_{530\text{ m}\mu}$ produced by the addition of DL-dihydrolipoamide to diaphorase in 0.05M-phosphate, pH 7.6, at 25°. Results were obtained by the stopped-flow method. Final concentration of enzyme in the reaction chamber was $12.3\text{ }\mu\text{M}$, with the molar ratios of DL-dihydrolipoamide shown. ●, Increase in extinction during the rapid phase of the reaction; ▲, total increase in extinction (rapid + slow phases).

Fig. 5. Effect of DPNH, O_2 and dithionite on $E_{455\text{ m}\mu}$ and $E_{530\text{ m}\mu}$ of diaphorase (2.58 mg./ml.) in 0.05M-phosphate, pH 7.6, at 25° (total volume 3 ml.). Changes were followed with time in two separate anaerobic cells. In the first experiment (x) 4.4 moles of DPNH/mole of enzyme were added anaerobically. At 22 min. air was admitted and the tube inverted once to bring into contact with air. At 34 min. the tube was inverted once again to mix with air. At 53 min. 10.8 moles of dithionite/mole of enzyme were introduced into a side arm of the cell and anaerobic conditions were re-established. At 60 min. the dithionite was mixed with the enzyme. In the other experiment (●), the same amount of enzyme, which had no previous treatment with DPNH and O_2 , was mixed anaerobically with 5.4 moles of dithionite/mole of enzyme. For convenience this reaction is shown to begin at 60 min. In both experiments the dithionite solution (10 mM) was made up freshly with cold de-aerated 0.05M-phosphate, pH 7.6 (containing 0.6 mM-EDTA) immediately before use.

after mixing. It is shown in a later section, however, that these extinction changes occur within milliseconds after mixing. Fig. 4 shows a similar plot of the extinction increases at 530 $m\mu$, produced by the addition of graded amounts of DL-dihydrolipoamide, measured with the stopped-flow apparatus. On the addition of the compound there is a distinct biphasic increase in absorption, a very rapid reaction (half-time < 20 msec.) followed by a much slower reaction (half-time about 3 sec.). This probably reflects the differences in reaction rates of the natural and unnatural optical forms of the dihydrolipoamide with the enzyme. The extinction changes occurring in the fast phase of the reaction are half those of the total change (fast plus slow) when the total amount of DL-dihydrolipoamide is < 1 mole/mole of enzyme. Above this range the proportion of the rapidly produced change increases relative to that of the slow change; at a molar ratio of DL-dihydrolipoamide:enzyme of 6, 90% of the total change occurs during the rapid phase.

Effect of oxygen and dithionite on the absorption spectrum. Fig. 5 shows the changes in extinction at 455 and 530 $m\mu$ in an experiment designed to determine the effect of O_2 and dithionite on the red form of the enzyme produced by DPNH. In this experiment DPNH was added anaerobically and the decrease in extinction at 455 $m\mu$ and the increase in extinction at 530 $m\mu$ were followed with time. After the immediate changes noted very little further change occurred, and during this time the spectrum was recorded. After 22 min. air was admitted and the contents of the tube were mixed gently once in order to bring them into contact with the air. A small increase in extinction at 455 $m\mu$ and a small decrease at 530 $m\mu$ occurred; further changes were very slow until the tube was again inverted once after 34 min. A gradual increase in extinction at 455 $m\mu$ accompanied by a corresponding decrease at 530 $m\mu$ occurred until the absorption spectrum of the original oxidized enzyme was regained. Freshly prepared dithionite was then added to a side arm of the tube and the contents were made anaerobic as described in the Experimental section. At 60 min. the dithionite was tipped into the enzyme + DPN solution (during the oxidation with air the 340 $m\mu$ absorption due to the added DPNH disappeared). There was a fairly rapid decrease in extinction at 455 $m\mu$ and a corresponding increase at 530 $m\mu$ to close to the values originally obtained on the addition of DPNH. This was followed by a slow decrease in both the 455 $m\mu$ and the 530 $m\mu$ extinction values, extending over a period of 1 hr. under these conditions. Fig. 5 also shows, for comparison, the effect of adding dithionite alone. It is clear that the red colour also appears as an

intermediate under these conditions but, whereas the total changes in extinction are the same as when DPNH is present, the rate of change is faster, suggesting that the small molar excess of DPNH over enzyme present may protect the flavin from complete reduction. The spectra recorded at various stages in this experiment are given in Fig. 6. Ferricyanide also causes a rapid return of the spectrum to that of the oxidized form when added to the red form made with DPNH. This oxidation reaction is being studied by the stopped-flow technique; the results will be reported separately.

Effect of p-chloromercuriphenyl sulphonate on the red intermediate. When the red intermediate is produced by the addition of DPNH to lipoyl dehydrogenase in an anaerobic experiment, the subsequent addition of the sulphydryl reagent causes a further bleaching of the visible colour, giving a spectrum similar to that of the fully reduced enzyme obtained by the addition of dithionite. The major part of the extinction change (which results in the complete loss of the red colour) occurs within the first 10 min.; this is followed by a further, slower change. The results of such an experiment are shown in Fig. 7. With larger molar ratios of DPNH to enzyme than those used in obtaining Fig. 7 the full spectral changes were completed very rapidly (within 3–5 min.). These experiments must be done at low temperatures, otherwise turbidity develops rapidly. If p-chloromercuriphenyl sulphonate is added before DPNH, no reduction of the enzyme flavin occurs, as judged by the lack of changes in extinction either at 455 $m\mu$ or 530 $m\mu$. The possible significance of these observations is considered in the Discussion.

Fig. 6. Spectra recorded at various times in the experiment described in Fig. 5. ●, Oxidized enzyme, before any additions; ×, after addition of DPNH, read between 7 and 21 min.; ▲, after addition of dithionite to ×, read after 130 min.

Fig. 7. Effect of *p*-chloromercuriphenyl sulphonate on the absorption spectrum of diaphorase after the addition of DPNH. Enzyme concentration, 1.44 mg./ml. ($21.2 \mu\text{M}$) in 0.05 M-phosphate, pH 7.2, temperature $5 \pm 1^\circ$. —, Oxidized enzyme; ●—●, after the addition of 2.9 moles of DPNH/mole of enzyme (spectrum recorded 5–20 min. after addition of DPNH: no significant changes were observed in this time); ▲—▲, after the further addition of 8.2 moles of *p*-chloromercuriphenyl sulphonate/mole of enzyme (spectrum recorded 20–30 min. after the addition); ■—■, same as ▲—▲, but 15 hr. later.

Fig. 8. Effect of DL-lipoylglycylglycine on the rate of oxidation of DPNH by catalytic quantities of enzyme (x), and on the rate of FADH' oxidation (●), measured by the stopped-flow technique. Catalytic rates were calculated as turnover numbers (moles of DPNH oxidized/min./mole of enzyme); the rates of FADH' oxidation were calculated as the first-order rate constants (min.^{-1}) for the extinction change at $530 \text{ m}\mu$. In both experiments the buffer was 0.05 M-phosphate, pH 6.3; temperature 25° . In the 'catalytic' experiments the conditions were as given in the Experimental section; reaction was begun by the addition of 0.11–0.33 $\mu\text{g.}$ of enzyme. In the stopped-flow experiments FADH' was made anaerobically by the addition of 0.75 mole of DPNH/mole of enzyme and transferred immediately to the stopped-flow apparatus, where it was mixed with the concentrations of lipoylglycylglycine shown. Final concentration of enzyme in the reaction chamber was $15.3 \mu\text{M}$.

Rate of formation of the red intermediate by reduced *d* phosphopyridine nucleotide. When DPNH is mixed with lipoyl dehydrogenase the red colour is produced so rapidly that it is impossible, with the present apparatus, to estimate accurately the rate of formation, except at concentrations of DPNH about the same as those of the enzyme. Thus in stopped-flow experiments at pH 6.3 and 25° when enzyme at a final concentration of $21.5 \mu\text{M}$ was mixed with a final concentration of $20 \mu\text{M}$ -DPNH, the first-order rate constant for the increase in extinction at $530 \text{ m}\mu$ was 8400 min.^{-1} . Increasing the concentration of DPNH fivefold resulted in such a rapid formation of the red colour that no quantitative measurement of its rate could be made. It was confirmed, however, that the full extinction change at $530 \text{ m}\mu$ (expected to follow

Fig. 9. (A) Effect of DL-lipoic acid on the rate of catalytic oxidation of DPNH (x), and on the rate of FADH' oxidation (●), measured by the stopped-flow method. In both experiments the buffer was 0.05 M-phosphate, pH 6.3; temperature $22 \pm 1^\circ$. Rates are expressed as in Fig. 8. In the catalytic experiments the conditions were as given in the Experimental section; reaction was begun by the addition of 4.4–6.6 $\mu\text{g.}$ of enzyme. Stopped-flow experiments were performed as described in Fig. 8; final concentration of enzyme in the reaction chamber, $27 \mu\text{M}$; ratio of DPNH to enzyme used to perform the FADH' enzyme, 3.0 moles/mole. (B) Results with lipoylglycylglycine (shown on a more convenient scale in Fig. 8) are given for comparison.

mixing with DPNH) had taken place, and so must have been more than 90% complete in less than 3 msec. The minimum turnover number is then greater than $50\,000\text{ min.}^{-1}$.

Rates of reoxidation of the red intermediate by oxidized lipoic acids. Previous work by Sanadi, Langley & Searls (1959) and by Massey (1960a) had shown that the rates of catalytic oxidation of DPNH by various lipoic acid derivatives varied considerably with the derivative used. In the present work it has been found that there is a close correlation between the rates of the enzyme-catalysed oxidation of DPNH by the lipoyl derivatives used and the rates of reoxidation of the red intermediate (produced by DPNH addition) by the same lipoyl derivatives. This phenomenon is illustrated in Figs. 8 and 9 for DL-lipoylglycylglycine and DL- α -lipoic acid. It may be seen that lipoylglycylglycine reacts about 50 times as rapidly as lipoic acid in the catalytic oxidation of DPNH; similarly, the former compound is about 50 times as fast as the latter compound in re-converting the red intermediate into the oxidized enzyme. In these Figures and following ones, the turnover numbers for the catalytic oxidation of DPNH in the reaction $\text{DPNH} + \text{H}^+ + \text{oxidized lipoic derivative} \rightleftharpoons \text{DPN} + \text{reduced lipoic derivative}$ are for a two-electron transfer; as will be considered in the Discussion, the numerically similar or identical turnover numbers from the stopped-flow experiments probably represent only a one-electron transfer due to the interconversion of flavinadenine dinucleotide (FAD) and its semiquinone ($\text{FADH}^\cdot$).

Kinetics of the reduction of diphosphopyridine nucleotide by dihydrolipoamide. When plots according to the method of Lineweaver & Burk (1934) were made of the effect of DPN concentration on its rate of reduction by dihydrolipoamide, the results shown in Fig. 10 were obtained at different concentrations of dihydrolipoamide. Such kinetics are consistent with the general mechanism given by Alberty (1953) for a two-substrate system in which the enzyme exists in two different states (oxidized and reduced) and where the substrates combine (and react) with particular forms of the enzyme:

where E represents oxidized enzyme, E' reduced enzyme, A a reducing substrate, B its oxidized form, C an oxidizing substrate, D its reduced form and k_1 – k_8 the rate constants of the reactions shown. With this mechanism the limiting rate at infinite

concentration of one substrate at any particular concentration of the other may be obtained by Lineweaver & Burk extrapolation; such extrapolated values for infinite concentrations of dihydrolipoamide are shown in Fig. 10 as a function of DPN concentration. In Fig. 11 the extrapolated limiting rates for infinite DPN concentration are plotted as a function of dihydrolipoamide concentration. Under the conditions of salt concentration, pH and temperature given in the legend to Fig. 10, K_m for DL-dihydrolipoamide is 0.3 mM, K_m for DPN is 0.2 mM, and $V_{\max}$ (the limiting rate with infinite concentrations of both substrates) is 3.33×10^4 moles of DPN reduced/min./mole of enzyme.

Fig. 10. Effect of DPN and dihydrolipoamide concentrations on the rates of the reaction dihydrolipoamide + DPN $\rightarrow$ lipoamide + DPNH + H^+ . Buffer, 0.05 M-phosphate, pH 7.6; temperature 25°. x, with 0.1 mM-DL-dihydrolipoamide; ●, with 0.133 mM-DL-dihydrolipoamide; ▲, with 0.2 mM-DL-dihydrolipoamide; ■, with 0.333 mM-DL-dihydrolipoamide. The continuous line without experimental points represents the values obtained by extrapolation to infinite DL-dihydrolipoamide concentration. Reaction was begun by the addition of 0.22 $\mu\text{g.}$ of enzyme.

Fig. 11. Effect of DL-dihydrolipoamide concentration on the $V_{\max, \text{DPN}}$ values obtained for the experiment described in Fig. 10.

Rates of red-intermediate formation by reduced lipoic acids. The rates at which the red intermediate is formed by reduced lipoic acids have been determined by the stopped-flow technique under conditions of temperature, pH and salt concentra-

Fig. 12. Comparison of the maximum rates (∞ [DPN]) in the reaction dihydrolipoamide + DPN $\rightarrow$ lipoamide + DPNH + H^+ ($\bullet$), and the rates of FADH $^+$ formation (Δ). The latter were measured by the stopped-flow method; the former are the values shown in Fig. 11. In the stopped-flow experiments (Δ), performed at 25° in 0.05 M-phosphate, pH 7.6, the final concentration of enzyme in the reaction chamber was either 21.5 μ M or 12.3 μ M; concentrations of DL-dihydrolipoamide were as shown.

Fig. 13. Effect of DL-dihydrolipoyl-glycine concentration on the rate of the enzyme-catalysed reaction dihydrolipoyl-glycine + DPN $\rightarrow$ DL-lipoyl-glycine + DPNH + H^+ ($\times$), and the rate of FADH $^+$ formation ($\bullet$) by dihydrolipoyl-glycine. Buffer, 0.05 M-phosphate, pH 7.6; temperature 25°. In the catalytic experiments the dihydrolipoyl-glycine concentration was varied as shown; the DPN concentration used was 0.67 mM. No significant variation in the rates was observed on lowering the DPN concentration to 67 μ M. In the stopped-flow experiments the final concentration of enzyme in the reaction chamber was 21.5 μ M; concentrations of DL-dihydrolipoyl-glycine were as shown.

tion identical with those of Fig. 10. Results with dihydrolipoamide expressed as turnover numbers are given in Fig. 12, which also shows for comparison the extrapolated turnover numbers (at infinite DPN concentration) obtained from the catalytic experiments described in Figs. 10 and 11. It is evident that the rate of formation of the red intermediate by dihydrolipoamide is the same as that of the overall catalytic reduction of DPN by the compound. A comparison of the time course of the formation of the red colour with a theoretical model is shown in Fig. 14.

Fig. 13 shows a comparison of the turnover numbers of the catalytic reduction of DPN by another reduced lipoyl derivative, dihydrolipoyl-glycine, and the turnover numbers obtained by the stopped-flow technique. The results for dihydrolipoamide from Fig. 12 are also included in the figure to illustrate the much greater rates of reaction obtained with the latter substrate. It should be noted that the turnover numbers from the catalytic experiments with dihydrolipoyl-glycine are actual experimental values, not extrapolated ones as with dihydrolipoamide. When an analysis of the kinetics of the reduction of DPN by dihydrolipoyl-glycine similar to that for dihydrolipoamide (shown in Figs. 10 and 11) was attempted, the changes in rate on varying the DPN concentration at any fixed concentration of dihydrolipoyl-glycine were within the limits of experimental error of the measurements.

Rates of reoxidation of the red intermediate by diphosphopyridine nucleotide. When the red intermediate was produced anaerobically by the addition of DL-dihydrolipoamide it was found that the rate of reoxidation by DPN was so fast that it could not be estimated by the stopped-flow technique. Thus in a typical experiment the red intermediate was made anaerobically by the addition of 0.1 mM-dihydrolipoamide to 43 μ M-enzyme at pH 7.6, transferred immediately to the stopped-flow apparatus and mixed with 0.4 mM-DPN at 25°. (Note that the concentrations of all reactants are halved on mixing.) The reoxidation under these conditions was complete by the time of first observation (3 msec.), indicating that the disappearance of red colour occurred at a rate giving a turnover number considerably in excess of 5×10^4 min. $^{-1}$.

DISCUSSION

It is known from the work of Kuhn & Wagner-Jauregg (1934), Michaelis *et al.* (1936) and Beinert (1956) that free flavins are reduced by two single-electron reduction steps in which the oxidized flavin is yellow, the half-reduced (or semiquinone or free-radical) form is red and the fully reduced form is nearly colourless. As mentioned in the intro-

duction, several flavoproteins have been reported to form a red colour on the addition of reducing substrate. From the work of Dolin (1956) on DPNH peroxidase it is clear, however, that the mere formation of red colour on the addition of reducing substrate is not necessarily an indication of semiquinone formation. With diaphorase the evidence is nevertheless strongly in favour of the view that the red colour represents a stage in reduction between that of oxidized and reduced forms of flavinadenine dinucleotide. Thus it is produced by the addition of DPNH or reduced lipoic acids and disappears with return of the yellow colour and fluorescence of the oxidized enzyme on the addition of DPN, oxidized lipoic acids, potassium ferricyanide and oxygen. Furthermore, it is produced as an intermediate in the reduction of the enzyme by dithionite, where the final form of the flavin is that of full reduction. Thus by analogy with the previous work on free flavins it should represent the semiquinone or free radical form of the flavinadenine dinucleotide (FADH[•]). Whether a free radical in the conventional sense exists in the protein is still a matter for experiment. The stability of the red colour in the absence of any oxidizing agent is remarkable. The effect of *p*-chloromercuriphenyl sulphonate in discharging the red colour and at the same time causing a general bleaching of the visible absorption (Fig. 7) seems to suggest that the red colour is stabilized by interaction with a protein sulphhydryl group. Recent work (Massey & Veeger, 1960) has indicated that in the catalytic action of the enzyme a protein disulphide bridge is converted into a dithiol grouping. As discussed in the last-named paper, a likely sequence of events is that one of the nascent sulphhydryl groups of the protein transfers a hydrogen atom and an electron to flavinadenine dinucleotide to convert the latter into FADH[•]; this sulphur atom would thus be left as a free radical. Possibly the stability of the enzyme-FADH[•] is due to a limited overlap of the electron clouds of the FADH[•] and the S[•]; this would be disturbed by a mercaptide-forming substance such as *p*-chloromercuriphenyl sulphonate. If this situation existed it is possible that very little free radical might be detected.

A feature of the results is the stoichiometry of the formation of red colour and the amount of reducing substrate added. The titration data for both DPNH and dihydrolipoamide show that the full change in extinction at 530 m μ is produced by 1 mole of DPNH or 1 mole of the natural isomer of dihydrolipoamide/mole of FAD. If the red colour is due to the conversion of FAD into FADH[•], as appears likely, it is necessary to account for the fate of the other electron involved in these reactions, and the idea that a protein disulphide-

dithiol reaction takes part in the electron exchange provides a reasonable explanation for the phenomenon.

A comparison of the rates of catalytic action under a variety of conditions and the rates of formation and reoxidation of the red intermediate (Figs. 8-14) shows that the red form is a true intermediate in the enzyme action. In these figures the rate constants for the overall catalysis (a two-electron change) are the same as those for the formation or oxidation of the red intermediate. If the intermediate is a semiquinone, then this one-electron change occurs at the same rate as the two-electron-change enzyme catalysis. Thus the rate determinations are consistent with the titration data of Figs. 3 and 4, indicating that the second electron is donated or accepted by another group in the protein. Further, the rate of conversion of FAD into FADH[•] appears to be the rate-determining step in the catalytic reduction of DPN by dihydrolipoic acids, and the reoxidation of FADH[•] by oxidized lipoic acids the rate-determining step in the catalytic oxidation of DPNH. This conclusion is supported by the extremely high rates of FADH[•] formation by DPNH and the reoxidation of FADH[•] by DPN which could be observed, but which were too fast to measure by the stopped-flow method. This conclusion is also supported strongly by a consideration of the kinetics of the enzyme-mediated reaction between DPN and dihydrolipoamide. The results presented in Figs. 10 and 11 may be treated in terms of the scheme given by Alberty (1953), which is reproduced in the Results section. In this particular case dihydrolipoamide is A in the scheme and DPN is C. Then the concentration of dihydrolipoamide required to allow the

Fig. 14. Comparison of the observed changes in extinction at 530 m μ , on mixing diaphorase and DL-dihydrolipoylglycine, with the calculated values. Concentration of diaphorase 12.3 μ M after mixing; concentrations of DL-dihydrolipoylglycine: (A) 75 μ M, (B) 50 μ M, (C) 35 μ M; 0.05M-phosphate buffer, pH 7.6, at 25°; 2 cm. light path. Lines were calculated as described in the text.

reaction to proceed at half-maximum speed (with infinite DPN concentration) may be written

$$K_{\text{dihydrolipoamide}} = [k_7(k_2 + k_3)]/[k_1(k_3 + k_7)],$$

and the corresponding quantity for DPN is

$$K_{\text{DPN}} = [k_3(k_6 + k_7)]/[k_5(k_3 + k_7)].$$

The maximum rate, with infinite concentrations of both substrates, is $V_{\text{max.}} = k_3 k_7 / (k_3 + k_7)$. Now, the value of $V_{\text{max.}}$ in the experiments of Fig. 10 with catalytic quantities of enzyme is

$$3.33 \times 10^4 \text{ min.}^{-1};$$

this result agrees within experimental error with the rate obtained in stopped-flow experiments on the formation of FADH' from FAD by dihydrolipoamide, when extrapolated to infinite substrate concentration (Fig. 12). This maximum rate may, since no DPN was present in the stopped-flow experiments and k_7 does not come into consideration, be equated with k_3 . Then k_3 (stopped flow) = $k_3 k_7 / (k_3 + k_7)$ (catalytic), requiring that $k_7 \gg k_3$, in agreement with the finding that the rate of re-oxidation of FADH' by DPN is too rapid to measure by the stopped-flow method. Further, when no DPN is present, $K_{\text{dihydrolipoamide}}$ becomes $(k_2 + k_3)/k_1$, whereas in the presence of DPN this expression must be multiplied by $k_7/(k_3 + k_7)$ to give the formula already quoted. It follows that the agreement between the catalytic and stopped-flow measurements of $K_{\text{dihydrolipoamide}}$ shown in Fig. 12 is again consistent with a value of $k_7 \gg k_3$.

As a further test of the validity of the above analysis of the kinetics, the theoretical time course of FADH' formation was calculated from the kinetic constants obtained from Figs. 10 and 11 and the extinction coefficient $E_{530 \text{ m}\mu}$ for FADH' formation given by the titration experiment with DL-dihydrolipoamide shown in Fig. 4. In Fig. 14 the theoretical time course of extinction changes at 530 m μ for three different concentrations of dihydrolipoamide is compared with the values actually observed in stopped-flow experiments. This figure provides a striking demonstration of the role of the red intermediate in the overall catalytic reaction, since the curves were calculated without disposable constants.

The conversion of the oxidized enzyme into the semiquinone in the stopped-flow experiments may be written:

$$dy/dt = k_2 x - k_1 y(a + y),$$

$$dx/dt = k_1 y(a + y) - (k_2 + k_3) x,$$

where y is the concentration of oxidized enzyme, a is the difference between the concentration of substrate and of enzyme, x is the concentration of enzyme-substrate complex and k_1 , k_2 and k_3 have the same meanings as in the analysis of the preceding paragraphs. To obtain numerical values, k_3 is

taken to be equal to $V_{\text{max.}}$, and a minimum value for k_2 of 1300 sec.⁻¹ is derived from catalytic studies of the reaction

With this value of k_2 and the value of $K_{\text{dihydrolipoamide}}$ from Fig. 12, $k_1 = 1.24 \times 10^7 \text{ M}^{-1} \text{ sec.}^{-1}$. Numerical calculation with these values yields the excellent agreement of Fig. 14 for the time course of the appearance of the semiquinone, thus supporting further the arguments based on the initial rates of colour formation at 530 m μ . Although the value assigned to k_2 is a lower limit rather than an estimate of its actual value, this in no way affects the meaning of the agreement of Fig. 14, because increasing the value of k_2 [with corresponding adjustment of k_1 to maintain $(k_2 + k_3)/k_1$ equal to $K_{\text{dihydrolipoamide}}$ influences the course of colour formation significantly only in the first 2–3 msec., i.e. before observation by the stopped-flow method can begin.

Beinert (1957) has presented evidence that changes involving coloured intermediates of flavoprotein enzymes can take place fast enough to admit of their participation in the enzyme catalytic mechanisms. We believe that the experiments reported here constitute a convincing demonstration that these coloured compounds not only can but do function as intermediates in catalysis.

SUMMARY

1. The anaerobic addition of either reduced diphosphopyridine nucleotide or dihydrolipoic acid derivatives to diaphorase results in the production of a red colour, characterized by an increase in extinction in the 500–650 m μ region of the spectrum, and changes in other regions of the visible spectrum.

2. The effect of diphosphopyridine nucleotide, oxidized lipoic acids, potassium ferricyanide, oxygen and dithionite on the red form of the enzyme indicate that it represents an intermediate oxidation level of the enzyme-bound flavinadenine dinucleotide between the fully oxidized and fully reduced forms. By analogy with previous work, this form has been ascribed to the semiquinone or free radical state of the flavin (FADH').

3. The effect of *p*-chloromercuriphenyl sulphate on the red form is described; the results indicate that the semiquinone form is stabilized by interaction with a protein sulphhydryl group, possibly a sulphur radical.

4. The rates of formation of the red intermediate and of its reoxidation under a variety of conditions have been studied by the stopped-flow method. Comparison of the rates obtained with catalytic rates estimated under the same conditions shows

that the rate of formation of FADH' by dihydro-lipoic acids is the rate-determining step in the catalytic reduction of diphosphopyridine nucleotide by dihydrolipoic acids. Similarly, the rate of reoxidation of FADH' by oxidized lipoic acids is the rate-determining step in the catalytic oxidation of reduced diphosphopyridine nucleotide by oxidized lipoic acids. The results of the rate experiments and titration experiments show that in the overall two-electron oxidation-reduction reaction catalysed by the enzyme the flavinadenine dinucleotide is reduced to FADH' and reoxidized only once per catalytic cycle. The relation of this finding to the reaction mechanism of the enzyme is discussed.

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The Hydrolysis of Dipeptides by Different Regions of Rat Small Intestine

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Recent studies of the small intestine have shown that its absorptive capacity for different nutrients varies according to the region. In the rat, Fisher & Parsons (1950b), showed that glucose is most rapidly absorbed in the duodenum and this is also reported to be the most active area for the absorption of iron (Brown & Justus, 1958). On the other hand, the distal half of the small intestine in the rat seems to be the site of maximal absorption of vitamin B₁₂ (Coates & Holdsworth, 1958), of ¹³¹I-

labelled fat (Benson, Chandler, Vansteehuyse & Gagnon, 1956) and of water (Barry, Matthews & Smyth, 1959). Kremens, Linner & Nelson (1954) have shown, using dogs, that the distal half of the small intestine is more important than the proximal half in maintaining fat balance.

Information on the site of absorption of protein breakdown products, i.e. peptides and amino acids, is scanty. Neil (1959) has reported that, in the rat, the rates of absorption of L-cystine and L-cysteine are highest in the duodenum, decrease in the jejunum and are lowest in the ileum; Korelitz & Frank (1959) have shown that translocation of

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