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KINETIC STUDIES ON THE DIPHOSPHOPYRIDINE NUCLEOTIDE CYTOCHROME *c* REDUCTASE FROM HEART*

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A soluble, highly purified DPNH**-cytochrome *c* reductase has been obtained by MAHLER *et al.*[1] from heart muscle sarcosomes. The overall stoichiometry of the reaction catalyzed by the flavoprotein was found to be:

$$\text{DPNH} + 2\ \text{cytochrome}^{3+} \rightleftharpoons \text{DPN}^{+} + 2\ \text{cytochrome}^{2+} + \text{H}^{+} \qquad (1)$$

The reaction therefore involves the participation of three substrate molecules, two of which are proteins. Numerous problems are posed by this reaction, some of which may be approached by kinetic analysis. A preliminary survey of the kinetics has been reported[2]. The kinetics of the overall reaction are here subjected to a more extended experimental analysis. The objects were to determine the values of the kinetic parameters involved in the reaction, to discuss their nature, and to determine their behavior as a function of pH. The results so obtained form the basis for a discussion of the possible mechanism of the reaction.

MATERIALS AND METHODS

Preparation of the enzyme

The enzyme was prepared from a dilute alcohol extract of pig heart sarcosomes by a slight modification of method of MAHLER *et al.*[1]. Removal of the contaminating heme pigments was facilitated by washing the sarcosomes with cold 0.05 *M* sodium acetate buffer at pH 5.4 and then several times with cold distilled water. The optical density ratios D_{280}/D_{440} and D_{410}/D_{440} of the enzyme at the last stage of purification were 5.1 and 0.95. The values of these ratios obtained by MAHLER *et al.*[1] were 7.0 and 0.85. Although electrophoretic and sedimentation studies indicated that the

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** The following abbreviations are used: DPN^{+} and DPNH, unreduced and reduced forms of diphosphopyridine nucleotide, respectively; tris, tris(hydroxymethyl)aminomethane; cyt, cytochrome *c*.

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enzyme was not homogeneous, identical kinetic results have been obtained from purified material prepared at different times.

Cytochrome c

Commercial preparations of cytochrome *c* (0.34 % iron) contained inhibitory impurities as well as both the reduced and oxidized forms of the cytochrome. Use of such material resulted in a non-linear dependence of reaction velocity on enzyme concentration. The cytochrome was therefore purified by ion exchange on Amberlite IRC 50 (grade XE-64) according to the method of MARGOLIASH[3]. The reduced component was reoxidized by dissolving the lyophylized material in water, acidifying to pH 2 and quickly neutralizing to pH 7. The cytochrome solution was then dialyzed several hours against water before use. The optical density ratios at 550 mμ (reduced) to 280 mμ (oxidized) and 550 mμ (reduced) to 550 mμ (oxidized) were 1.23 and 2.9 respectively. These ratios are the same as those obtained by MARGOLIASH for oxidized cytochrome *c* containing 0.43 % iron. The purified cytochrome in the standard assay system gave a linear relation between activity and enzyme concentration over a 200 fold range in enzyme concentration.

Deuterium-labelled pyridine nucleotide

DPNH was obtained from Sigma Chemical Company and was approximately 90 % pure. This material was used directly in the kinetic studies. The deuterated isomer of DPNH was prepared by the following exchange reaction. Fifty mg of DPNH in 20 ml of D_2O were incubated anaerobically for 4 hours at 25° with 3 mg of the purified heart enzyme in 0.01 *M* tris acetate buffer at pH 8.2. The solution was then placed in a boiling water bath for 3 minutes, cooled, centrifuged and lyophylized. The resulting reduced DPN contained at least 0.65 moles of deuterium* per mole of nucleotide. DRYSDALE AND COHN[4] have shown that the labelling is stereospecific and occurs in the so-called β-position, that is, the position opposite to that obtained in the reduction of DPN by deuterium-labelled ethanol with alcohol dehydrogenase[5].

Buffer

Tris acetate, 0.01 *M*, was used as the buffer up to pH 8.5. Above this pH, the buffer was 0.002 *M* in tris acetate and 0.008 *M* in sodium acetate. Experiments at pH 8.1 showed that replacing the tris cation by sodium cation had no effect on the overall velocity.

Reaction velocity

Reaction rates were measured by following the appearance of the reduced cytochrome band at 550 mμ. For initial velocity measurements a Beckman DU spectrophotometer with photomultiplier tube was coupled to a Brown recorder with which 80 to 100 % transmission could be expanded to full scale. At low cytochrome concentrations, 1.5 to $20 \cdot 10^{-6}$ *M*, cuvettes of 10 cm optical path were used for greater sensitivity. Using this apparatus and a 10 cm cuvette, the complete reduction of $5 \cdot 10^{-7}$ *M* cytochrome *c* solution corresponds to deflection of full scale on the recorder (11 inches). The pen speed was one second per full scale deflection and the paper moved at the rate of 4 inches per minute. Measurements were confined to the first 10 % of the reaction and thus initial velocities were reproducible within 5 %. Since the 550 mμ absorption band of reduced cytochrome is very narrow, severe limitations are placed upon the slit width of the monochromator if true initial velocities are to be obtained. It was necessary that the slit width be maintained between 0.03 and 0.09 mm for all the experiments and under the above conditions the recorder traces were a linear function of time.

Purified enzyme was stored at —20° for several weeks with no appreciable loss in activity. Since repeated freezing and thawing leads to inactivation, the stock solution was divided and frozen in several small samples. Dilute working solutions were made up fresh in buffer at pH 8.0 and kept at 0° for the duration of a series of measurements. Small samples of the enzyme were then added to the complete reaction mixture and mixed. The time between addition of the enzyme and the beginning of usable record was of the order of 30 seconds. The total elapsed time for each experiment was 1.5 to 3 minutes.

The activity of the working dilution of enzyme decreases somewhat during the course of a series of experiments. This was corrected when necessary by frequent activity tests under standard assay conditions (see below).

Standard assay

One ml of solution, 0.4 m*M* in DPNH, 0.085 m*M* in cytochrome *c* and 0.01 *M* in tris acetate at pH 8.0 and 25° was placed in a 1.2 ml cuvette of 1 cm optical path. Under these conditions, addition of 0.02 μg of purified enzyme, based upon biuret analysis[6] against a serum albumin standard, gives an optical density change at 550 mμ of 0.0002 per second. This corresponds to a turnover number of 8800 moles of cytochrome reduced per mole of enzyme per minute.

* Dr. GEORGE R. DRYSDALE kindly analyzed the samples for deuterium content.

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Denaturation

At 25° and the concentration of enzyme used in these experiments, irreversible denaturation above pH 8.7 and below pH 7.2 is quite rapid. Neither versene nor albumin prevent this denaturation. At 14°, however, and under the same conditions this denaturation is slow enough so that the measurements of initial velocity are not affected. Most of the experiments reported in this paper, therefore, were carried out at 14 ± 0.5°.

RESULTS

Initial velocity

Fig. 1 shows LINEWEAVER-BURK plots[7] of data which have been obtained at pH 9.1 and 14° in 0.01 *M* acetate. These plots of the reciprocal initial velocity *versus* the reciprocal of the DPNH concentration are linear at each concentration of cytochrome *c* as shown in Fig. 1A. The same data when plotted *versus* the reciprocal of the cytochrome concentration are shown in Fig. 1B and a linear plot is again obtained. In both plots, the common point of intersection of the lines is on the abscissa. In plots of a similar nature, it has been found that the LINEWEAVER-BURK plot is linear over a 50 to 100 fold DPNH concentration range at any cytochrome concentration.

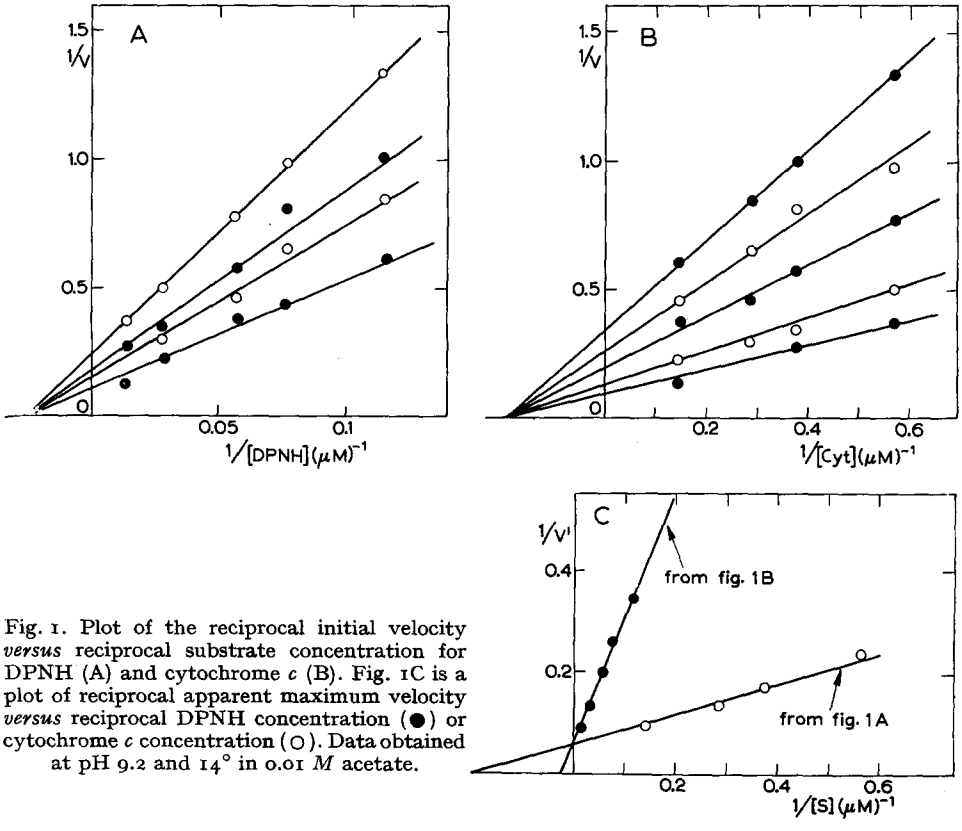

Fig. 1. Plot of the reciprocal initial velocity *versus* reciprocal substrate concentration for DPNH (A) and cytochrome *c* (B). Fig. 1C is a plot of reciprocal apparent maximum velocity *versus* reciprocal DPNH concentration (●) or cytochrome *c* concentration (○). Data obtained at pH 9.2 and 14° in 0.01 *M* acetate.

However, plots of the reciprocal initial velocities *versus* reciprocal cytochrome concentration over a wide range of cytochrome concentration show a marked downward deviation at concentrations of 2 to 3 times the Michaelis constant at that particular

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pH. For this case all the kinetic parameters have been obtained from extrapolations of linear plots at low concentrations of cytochrome *c* as shown in Fig. 1B.

When the apparent maximum velocities, V', as determined from the intercepts of the curves on the ordinate in Figs. 1A and 1B are plotted *versus* the reciprocal concentration of cytochrome or DPNH, Fig. 1C is obtained. Thus the same maximum velocity is obtained from both sets of data.

In Fig. 1, the initial velocity of the reaction at constant pH is described by the simple empirical equation

$$v = V \Big/ \left[1 + \frac{K_{\mathrm{DPNH}}}{[\mathrm{DPNH}]}\right] \left[1 + \frac{K_{\mathrm{cyt}}}{[\mathrm{cyt}]}\right] \qquad (2)$$

where V is the maximum velocity and K_{DPNH} and K_{cyt} are the Michaelis constants for DPNH and cytochrome.

Kinetic characteristics of the reaction

Some reaction mechanisms which yield rate equation (2) have been considered[8,9,10] for the case

$$\mathrm{A} + \mathrm{B} \rightleftharpoons \mathrm{C} + \mathrm{D}.$$

Several variations in the intermediate reaction sequences give relations of the same form. The chief point of interest in this respect is that kinetically the reaction may be described as a "two substrate" case although the overall stoichiometry as expressed in equation (1) shows that three substrate molecules are involved. It would seem, therefore, that the two cytochrome molecules must react in sequence. MAHLER *et al.* have made a similar interpretation on the basis of the linearity of a LINEWEAVER-BURK plot *versus* reciprocal concentrations of cytochrome *c*.

Deuterium effects and the rate-limiting step

It has been found that the reductase catalyzes a stereospecific exchange of deuterium for hydrogen in the β-position[5] of the para carbon in the nicotinamide ring of DPNH in the absence of added cytochrome[4]. This finding was utilized to prepare specifically labelled deutero DPNH for use as a substrate. Hydrogen is reversibly transferred from DPNH to an acceptor group on the enzyme, presumably the flavin nucleotide, where it exchanges with hydrogen or deuterium ions of the medium. The Michaelis constant for DPNH was found to be unaffected by the presence of deuterium in the active position in the nicotinamide ring of the nucleotide. The maximum velocity of the reaction at a series of pH values however was decreased by a factor of 2.3, which suggests that the rate-limiting step in the overall reaction is the dehydrogenation of DPNH.

When the overall reaction was carried out with DPNH and cytochrome *c* in a medium of D_2O, the initial velocities were approximately the same as those obtained in H_2O.

The pH-dependence of the maximum velocity

Extrapolated maximum velocities have been determined in 0.01 M acetate buffers at 14° as a function of pH and are plotted in Fig. 2. It has been shown[11–14] that the shape of such curves may be quantitatively described by equations derived for mechanisms which assume ionizing groups in the enzyme-substrate complex. In the case

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of the reductase the shape of the curve in Fig. 2 indicated that more than two ionizable groups were involved in the enzyme-substrate complex*. Mathematical analysis showed that the curve could be represented by an equation which involves three ionization constants and which is of the form

$$V = \frac{V''}{[1 + (H^+)/K_{aES} + K_{bES}/(H^+)]\ [1 + (H^+)/K'_{aES}]} \qquad (3)$$

where V'' is the so-called pH-independent maximum velocity and K_{aES}, K_{bES}, and K'_{aES} represent ionization constants. The solid line drawn through the experimental points in Fig. 2 is constructed from equation (3) with $pK_{aES} = 7.6$, $pK_{bES} = 8.5$ and $pK'_{aES} = 7.6$. Although more than one set of pK values may represent a curve which is correct within experimental error, the permissable variation is small and the above pK values are probably correct to within 0.2 units.

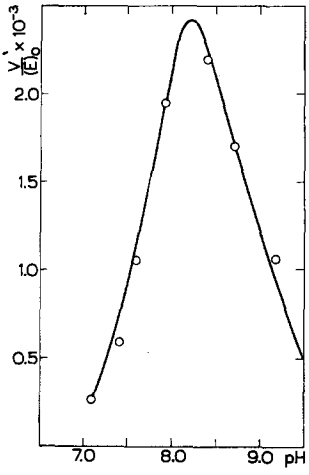

Fig. 2. Plot of the pH-dependence of the maximum velocity in 0.01 M acetate. Solid curve drawn from equation (2) and ionization constants shown in Table I.

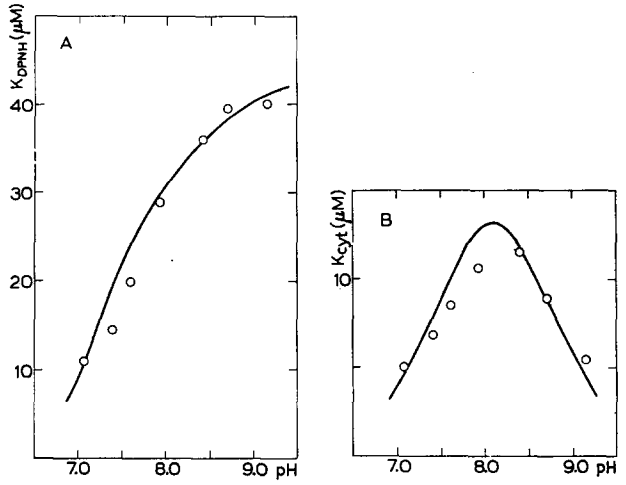

Fig. 3. Plots of the pH-dependence of the Michaelis constant for DPNH (A) and cytochrome c (B). Data obtained at 14° in 0.01 M acetate. Solid curves drawn from ratios of equation (5) to equation (2) and equation (6) to equation (2).

The pH-dependence of the Michaelis constants and maximum velocity-Michaelis constant ratio

The variation of the Michaelis constants of DPNH and cytochrome c as a function of pH are shown in Fig. 3. For a "one substrate" case, for example fumarase[15], the Michaelis constant is found to be described by equations involving both the ionization constants of the enzyme-substrate complex and those of the ionizing groups in the enzyme alone. This theory may be extended to the "two substrate" case of the reductase and the solid line drawn in Fig. 3 will be discussed later in these terms.

Fig. 4 shows the pH-dependence of the maximum velocity-Michaelis constant

* If only two ionizable groups are essential for enzymic activity, the dependence of the maximum velocity upon pH yields a curve which is symmetrical and bell-shaped[15]. Inspection of Fig. 2 shows that this curve is not symmetrical. For example, although the maximum of the curve is at pH 8.2, the maximum velocity at pH 9.2 is approximately twice as great as the maximum velocity at pH 7.2. Thus at least three ionizable groups must be postulated to account for the pH-dependence shown in Fig. 2.

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ratio for DPNH and cytochrome *c*. As will be seen in the discussion of the possible mechanisms for the reaction, these plots are quite significant.

The effect of temperature

All experimental results cited in the above sections, except those with deutero DPNH, have been obtained at 14°. The pH range for kinetic experiments at 25° is restricted somewhat by rapid and irreversible denaturation of the enzyme. Between the pH values 7.2 to 8.5 the pH-dependence of the maximum velocity and Michaelis constant exactly parallels those values obtained at 14°. The maximum velocities at all pH values at 14° were approximately 50% less than those obtained at 25°. On the other hand, the Michaelis constants for both substrates at the lower temperature were decreased by only 25%.

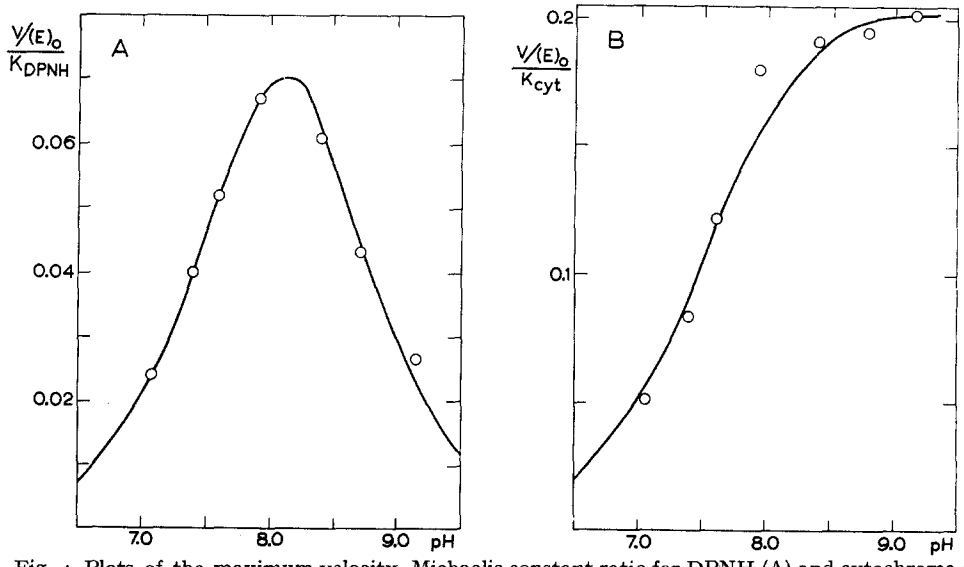

Fig. 4. Plots of the maximum velocity–Michaelis constant ratio for DPNH (A) and cytochrome *c* (B). Data obtained at 14° in 0.01 *M* acetate. Solid curves drawn from equations (5) and (6) and ionization constants listed in Table I.

INTERPRETATION

Possible mechanisms for the reaction

It is quite clear from equation (2) that although the reaction at constant pH involves three substrate molecules, the mechanism need only be described in terms of two. Several possible "three substrate" cases have been considered. A characteristic reaction mechanism which involves a complex of the enzyme with two cytochrome molecules at the same time is that the steady state equations derived for the initial velocity contain a term in cytochrome concentration squared. In "three substrate" cases where the cytochrome molecules react in sequence, such square terms are also encountered. However such equations reduce to the form given by equation (2) by assuming that the rate of reduction of the second molecule of cytochrome is not a limiting step in the overall reaction. This would presumably require the occurrence of a one electron intermediate which could be provided by a semiquinone of the flavin moiety. Another possibility is the participation of the bound iron atoms in the

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reductase[16]. Under these assumptions, the mechanism may be considered exactly analogous to the "two substrate" case and all other mechanisms considered are of this form. Thus, the following reaction mechanism has been used:

$$\begin{gathered} E + DPNH \rightleftharpoons E\text{-}DPNH \\ E\text{-}DPNH + cyt^{3+} \rightleftharpoons E\text{-}DPNH\text{-}cyt^{3+} \rightleftharpoons E'\text{-}DPNH + cyt^{2+} \end{gathered} \quad (4)$$

where E'-DPNH is capable of reducing another molecule of cytochrome *c*. It must be remembered, however, that the conclusions to be drawn on the basis of this mechanism are true for almost all* other "two substrate" mechanisms which obey equation (2).

Michaelis constant

The independence of K_{DPNH} of cytochrome concentration and K_{cyt} of DPNH concentration shown in Fig. 1 allows the denominator of equation (2) to be factored. Normally, a more complicated form of the equation would be expected**. It may be shown from the steady state equations derived for the mechanism that this condition requires K_{DPNH} to become identical with the dissociation constant defined by the relation

$$K = [E]\,[DPNH]/[E\text{-}DPNH]$$

However, the same statement is not necessarily true for K_{cyt}.

This case is very similar to yeast alcohol dehydrogenase where from a similar independence of the Michaelis constant one would predict that the Michaelis constants for DPNH and DPN were also dissociation constants. It has been shown by binding studies of DPNH and DPN by the enzyme that this is the case[17]. For cytochrome *c* reductase, however, difficulties arising from the autooxidizability of the reductase and uncertainties concerning its purity must be surmounted before binding equilibrium data can be obtained.

The pH-dependence of the kinetic parameters

Equation (3), has been derived for the mechanism in equation (4) and implies the existence of three ionizable groups in the enzyme-substrate complex which must be in a certain ionized form for the enzymic reaction to take place. Since DPNH has no ionizable groups in the pH range investigated, the groups necessary for DPNH oxidation must be associated with the enzymically active site. On the other hand, cytochrome *c* does contain groups which ionize in the pH range 7 to 9 [18], and it is not possible to distinguish from kinetic analysis alone whether the ionizing group necessary

* There is an exception to this statement when there is an ordered sequence of events such that an enzyme-cytochrome complex must be formed before DPNH may react with the enzyme. In view of the fact that DPNH reacts with the enzyme in the absence of cytochrome (*i.e.* autooxidizability, isotopic exchange), this case is considered unlikely.

** For many "two substrate" cases, the resultant form of the equation equivalent to equation (2) which expresses the initial velocity as a function of the two substrate concentrations is[8]

$$v = V \Big/ \left(1 + \frac{K_A}{[A]} + \frac{K_B}{[B]} + \frac{K_{AB}}{[A]\,[B]}\right)$$

where A and B are the two substrates and K_A, K_B and K_{AB} are Michaelis constants. When plots similar to Fig. 1A and 1B are made, the Michaelis constant K_{AB} can be determined from the intersection of the curves since it may be shown that [A]', the negative concentration of A which corresponds to the point of intersection of the curves, equals K_{AB}/K_B, and [B]', the negative concentration of B which corresponds to the point of intersection, equals K_{AB}/K_A. Since K_A and K_B are obtained in the usual manner from plots similar to Fig. 1C, K_{AB} may be easily derived. For the cytochrome *c* reductase, these points of intersection fall directly on the abscissa and thus $[A]' = K_A = K_{AB}/K_B$ and $[B]' = K_B = K_{AB}/K_A$. Therefore $K_{AB} = K_A K_B$ and the denominator may be factored as in equation (2).

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for the reduction of cytochrome c is associated either with the cytochrome or with the enzyme*.

Equations which describe the pH-dependence of the Michaelis constant and maximum velocity-Michaelis constant ratio have been derived assuming that two of the ionizable groups are associated with the oxidation of DPNH. The relationships for the maximum velocity-Michaelis constant ratio are shown in equations (5) and (6)

$$V/K_{\mathrm{DPNH}} = V''/K'_{\mathrm{DPNH}} \left[\frac{1}{(1 + \mathrm{H}^+/K_{a\mathrm{E}} + K_{b\mathrm{E}}/(\mathrm{H}^+)} \right] \tag{5}$$

where V''/K'_{DPNH} is the ratio of the so-called pH-independent kinetic parameters and $K_{a\mathrm{E}}$ and $K_{b\mathrm{E}}$ are the first and second ionization constants of the groups in the enzymic site with no DPNH bound; and

$$V/K_{\mathrm{cyt}} = V''/K'_{\mathrm{cyt}} \left[\frac{1}{1 + (\mathrm{H}^+)/K'_a} \right] \tag{6}$$

where V''/K'_{cyt} is again the ratio of the pH-independent kinetic parameters and K'_a is the ionization constant of the ionizable group involved. Fig. 4 shows the pH-dependence of the maximum velocity-Michaelis constant ratio and the solid curve drawn through the experimental points has been constructed from equation (5) and (6).

The pH-dependence of the Michaelis constants may be obtained from the ratio of equation (3) to equation (5) and equation (3) to equation (6). As can be seen from these ratios, the Michaelis constants are rather complicated functions of the hydrogen ion concentration.

This pH-dependence is shown in Fig. 3 and the solid curves drawn have been constructed from the ratios V to V/K_{DPNH} and V to V/K_{cyt}. The ionization constants and the pH-independent parameters used to construct Figs. 2–4 are listed in Table I.

TABLE I

THE pK VALUES OF IONIZING GROUPS NECESSARY FOR ENZYMIC ACTIVITY AND VALUES OF THE pH-INDEPENDENT KINETIC PARAMETERS AT 14° IN 0.01 M ACETATE

	E	*E-DPNH-cyt*
pK_a	7.7	7.6
pK_b	8.5	8.5
pK'_a§	7.4	7.6
$V''/(\mathrm{E})_0$	4000 min^{-1}	
K'_{DPNH}	$40 \cdot 10^{-6}$ M	
K'_{cyt}	$20 \cdot 10^{-6}$ M	

§ This group may either be associated with the enzyme or the cytochrome. See text.

* There have been enzyme reactions where the ionization of the substrate has been considered[15]. However, it has been assumed that the inactive form of the substrate is an exceedingly poor competitive inhibitor for the enzymic site. Under such conditions, the substrate ionization will have no effect on the maximum velocity and "total" effect upon the Michaelis constant. Obviously, there are other cases which must be considered. If the Michaelis constant of the active form and the dissociation constant of the inactive form of the substrate are equal, the effect of substrate ionization will be just the opposite of the first case considered, that is, the Michaelis constant will not be affected at all, and the substrate ionization would have a "total" effect on the maximum velocity. If the inactive form of the substrate is much more strongly bound to the enzyme than the active form both the maximum velocity and Michaelis constant would be affected. It is important to note, however, that the plot of the maximum velocity-Michaelis constant ratio is not affected by these different alternatives, and furthermore would have the same shape whether the ionizing group were in the enzyme or in the substrate. From these considerations, it is quite apparent from Figs. 2–4 that if the ionizing group being considered is associated with the cytochrome, the Michaelis and dissociation constants of the active and inactive forms of the cytochrome must be equal.

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The fit of the calculated curves to the experimental points seems to justify the assumption of three groups which are ionizable in the pH range 7 to 9 and which are essential for enzymic activity. It has been shown that two of these groups are located in the enzymic site and are essential for the oxidation of DPNH. Furthermore, there is a third ionizing group only one conjugate form of which permits the reaction to proceed.

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SUMMARY

1. Initial velocities for the reaction catalyzed by DPNH-cytochrome *c* reductase from pig heart sarcosomes have been determined at 14° as a function of DPNH, cytochrome *c* and hydrogen ion concentration.

2. Maximum velocities and Michaelis constants have been calculated from the data over a pH range of 7 to 9.2.

3. At constant pH, the kinetic results follow the rate equation

$$v = V \Big/ \left[1 + \frac{K_{\mathrm{DPNH}}}{[\mathrm{DPNH}]}\right] \left[1 + \frac{K_{\mathrm{cyt}}}{[\mathrm{cyt}]}\right]$$

which indicates that the enzymic reaction may be treated as a "two substrate" case even though the overall stoichiometry of the reaction requires three substrate molecules.

4. The pH-dependence of the maximum velocity indicates that three ionizing groups are involved in the enzymic reaction. Furthermore, the pH-dependence of the maximum velocity-Michaelis constant ratio shows that two of the three groups are in the enzymically active site and are associated with the oxidation of DPNH. The third ionizing group is involved in the reduction of cytochrome *c*. It is impossible to determine kinetically whether this third group is associated with the enzyme or with the cytochrome itself.

5. Ionization constants for the groups involved and the so-called pH-independent kinetic parameters have been calculated.

6. The β-deuterium labelled reduced DPN has been made by the stereospecific exchange reaction catalyzed by the enzyme. Use of this material as a substrate shows that the Michaelis constant for DPNH in unaffected but that the maximum velocity is decreased approximately 2.3 fold.

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