THE REACTIVITY OF THE BOUND DPN OF MUSCLE TRIOSE PHOSPHATE DEHYDROGENASE*

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It has been reported that kidney and liver particulate preparations contain bound pyridine nucleotides which may be removed by a variety of methods[4,5]. If the pyridine nucleotides are bound to various enzymes, the question arises as to the availability of these bound nucleotides to dehydrogenases and to enzymes which split pyridine nucleotides. In order to eliminate the influence of certain complicating factors such as diffusion and permeability, it appeared advantageous to study the availability of enzyme-bound pyridine nucleotides in a model system containing only one soluble crystalline enzyme which forms a stable complex with DPN***. Three such model systems are known. Mammalian TPD***, when crystallized from rabbit muscle, firmly binds two moles of DPN per mole of enzyme[6]. The bound DPN can be removed by charcoal[6] or ultracentrifugation[7], or can be converted to reduced diphosphopyridine nucleotide (DPNH). Since TPD–DPNH as well as TPD–DPN have low dissociation constants[8], the availability of enzyme-bound DPNH also can be studied with this system. Two other enzymes which are known to form stable complexes with pyridine nucleotides are yeast TPD[9] and horse liver alcohol dehydrogenase[10].

It also appeared at the outset of this work that comparisons of the rates of splitting of different bonds of bound DPN would provide information concerning the mode of attachment of DPN to the enzyme. In addition, since crystalline preparations of TPD can be obtained in an active or inactive form (TPD_a or TPD_i)—depending on the state of oxidation of sulfhydryl groups—the effect of sulfhydryl groups on the availability of enzyme-bound DPN may be assessed.

In this report it is shown that free DPN is more readily available to enzymic attack than is DPN–TPD_i, which in turn is more readily available than DPN–TPD_a. In fact, the latter appears to be completely unavailable for reduction by other dehydrogenases. However, DPNH–TPD_a is as readily available to other enzymes as

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*** The following abbreviations will be used: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; DPNH and TPNH, the reduced forms; Tris, tris(hydroxymethyl)-aminomethane; DPNase, diphosphpoyridine nucleotidase; EDTA, ethylenediaminetetraacetic acid tetrasodium salt; ATP, adenosine triphosphate; TPD, triose phosphate dehydrogenase; DPN–TPD_a, DPN bound to active TPD; DPNH–TPD_a, DPNH bound to active TPD; DPN–TPD_i, DPN bound to inactive TPD; HDP, hexose diphosphate.

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free DPNH. The observation by CORI *et al.*[11] that DPNH–TPD is as rapidly oxidized by lactic dehydrogenase as free DPNH has been confirmed and extended to include other enzymes.

METHODS

Enzyme preparations

DPNase was prepared according to the method of KAPLAN *et al.*[12] from extracts of zinc-deficient mats of wild type *Neurospora crassa*.

Adenosine deaminase was prepared essentially according to KAPLAN *et al.*[13]. The preparations obtained by use of this procedure contained varying amounts of opaque material which limited the amount of enzyme that could be assayed in a spectrophotometer. Two modifications were introduced in order to obtain a clear colorless solution. The insertion of these additional steps in the procedure of KAPLAN *et al.* led to a product 5 times purer than that reported by those authors. The first modification, inserted after the first alcohol precipitation, was to filter a solution of the enzyme through a Buchner funnel containing a pad made up of equal quantities of activated charcoal and permutit, the weight of each being 2/3 the amount of takadiastase taken as starting material. The second modification, inserted after the acetone precipitation, was to treat a solution of the enzyme, containing 9–10 mg protein/ml, with an equal volume of Alumina Cγ (13.5 mg/ml). After 10 minutes of gentle stirring, the suspension was centrifuged and the supernatant was precipitated with alcohol at —12° C in accord with the directions of KAPLAN *et al.* This fina product assayed 700–850 u/mg protein. The enzyme was lyophilized and stored as a powder at —18° C. No loss of activity was observed after one year of storage.

Snake venom pyrophosphatase was prepared according to a modification[14] of the procedure of HURST AND BUTLER[15]. The starting material, lyophilized venom of *Crotalus adamanteus*, was purchased from the Ross Allen Institute. The purified enzyme was stored as a lyophilized powder at —18° C, and in this state exhibited no loss of activity after one year's storage.

DPN kinase was prepared from pigeon liver[16] and pyridine nucleotide transhydrogenase was isolated from *Pseudomonas fluorescens*[17]. DPN *iso*citric dehydrogenase was obtained from yeast[18], and lactic dehydrogenase was prepared from rabbit muscle[19]. TPN *iso*citric dehydrogenase was obtained as a phosphate or Tris extract of an acetone powder of washed, minced pig heart[20]; aldolase was crystallized from rabbit muscle[21]. Horse liver and yeast alcohol dehydrogenases were crystallized by the methods of BONNICHSEN[22] and RACKER, respectively[23].

TPD was crystallized from rabbit muscle[6] in the presence[24] or absence of 0.002 *M* EDTA. Preparations of TPD obtained in the absence of EDTA required activation by sulfhydryl reagents whereas TPD crystallized in the presence of EDTA exhibited full activity in the absence of sulfhydryl reagents.

In this paper DPN–TPD_a refers to a dialyzed solution of DPN–TPD which had been recrystallized 5 times in the presence of EDTA. Dialysis was for 8–12 hours against 200 volumes of 0.002 *M* EDTA pH 7.4, and then for 6–12 hours against 200 volumes of distilled water. DPN–TPD_i refers to a dialyzed solution of DPN–TPD which had been recrystallized five times in the absence of EDTA. Dialysis was for 8–12 hours against 200 volumes of 0.002 *M* phosphate or Tris buffer pH 7.5, and then for 6–12 hours against 200 volumes of 0.0005 *M* buffer.

DPNH–TPD_a was prepared from DPN–TPD_a. A cell containing DPN–TPD_a ($3.5 \cdot 10^{-4}$ *M* bound DPN), Tris ($9 \cdot 10^{-2}$ *M*), arsenate ($9 \cdot 10^{-3}$ *M*) and glyceraldehyde ($2 \cdot 10^{-2}$ *M*) was allowed to stand at room temperature for 15 minutes following the addition of the glyceraldehyde. The pH of the reaction mixture was 8.4. The DPNH–TPD_a complex was precipitated with ammonium sulfate at pH 8.5 and 0.88 saturation. The amorphous precipitate was dissolved in 4 volumes of 0.05 *M* Tris pH 8.5 and either used immediately or subjected before use to another precipitation followed by dialysis for 2 hours against 200 volumes of 0.1 *M* Tris adjusted to pH and then for 3 hours against 200 volumes of 0.005 *M* Tris pH 8.5. The results obtained with bound DPNH did not vary with the preparative method. Sixty % of the enzyme activity present in the original DPN–TPD_a was recovered after the first ammonium sulfate precipitation. The same percentage of original DPN was recovered as ammonium sulfate precipitable DPNH. An essential requirement in this preparation was maintenance of the pH at 8.4 to 8.5 in order to prevent the TPD catalyzed conversion of DPNH at lower pH values to a compound which exhibits considerable light absorption at 290 mμ[25].

Coenzyme and substrates

The free DPN and DPNH with which bound nucleotides were compared were obtained by heating the bound nucleotides in a boiling water bath for 1.5 minutes. This denatured the TPD and liberated the free nucleotide. For measurements based on the alkaline fluorescence of DPN, the

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coagulated TPD was not removed since it dissolved in the hot alkali used to develop fluorescence. When the rate of reduction or oxidation of free DPN or DPNH by various dehydrogenases was measured spectrophotometrically, the coagulated protein was removed by centrifugation and the supernatant used.

Free DPN and DPNH, obtained by heat coagulation of the appropriate TPD complex, did not differ, with respect to reaction rates with other enzymes, from DPN and DPNH obtained from other sources. DPN, of approximately 80% purity, as assayed by reduction with ethanol and alcohol dehydrogenase[26], was obtained from the Sigma Chemical Company. DPNH of 80% purity was prepared and assayed enzymically with alcohol dehydrogenase[27]. TPN was obtained from the Pabst Laboratories and was 95% pure. ATP, 2'-adenylic acid and hexose diphosphate were obtained from Schwartz Laboratories. 5'-adenylic acid was purchased from the Ernst Bischoff Company.

Activity measurements

Neurospora DPNase activity was measured by following the disappearance of alkaline fluorescence from successive aliquots[28]. The activities of takadiastase deaminase, snake venom pyrophosphatase, and DPN kinase were followed by enzymic methods which are described in a separate paper[29]. The various enzymic reactions were stopped by placing aliquots in appropriate buffers which had been heated in boiling water for one minute before and after the addition of the aliquot. An aliquot taken 15 to 30 seconds after the start of the reaction was set arbitrarily as zero reaction. Heating also served to convert bound to free nucleotides which could then be assayed.

In all cases, the bound nucleotide and the free nucleotide control were tested at approximately equal concentrations. In order to correct for differences in rate due to slight differences in concentration, the data obtained were plotted as % of total reaction versus time. With this treatment of the data, as long as the initial DPN concentration is in the range where initial reaction velocity is directly proportional to the substrate concentration (below the Michaelis constant (K_m) for the enzyme), the initial velocity expressed in terms of % of total reaction is fairly independent of the initial substrate concentration. Comparisons of the initial reaction velocities of bound and free DPN were made by reference to the plotted data.

The activities of the DPN-linked dehydrogenases were measured by following the appearance or disappearance of light absorption at 340 mμ. A Beckman Model DU spectrophotometer and 1.2 ml capacity silica cells of one cm light path were used. The protocols for the various dehydrogenases are given in the legend to Table VI.

TPD activity was measured at room temperature in a system containing in 1.05 ml, 0.5–2.0 micrograms of TPD, 30–50 micrograms of aldolase, 65 μmoles Tris pH 8.5, 8.1 μmoles arsenate pH 8.2, 5 μmoles cysteine hydrochloride, 2 μmoles EDTA pH 8.0, 0.72 μmoles DPN and 2.5 μmoles of hexose diphosphate. The reaction was started by the addition of hexose diphosphate. Protein was determined spectrophotometrically[18].

Our best preparation of TPD_a catalyzed, in the presence of cysteine at 28° C, the reduction of 10,600 mols DPN/min/mol enzyme (specific activity = 10,600). Of the TPD_a preparations used, the average specific activity was 6500 in the presence of cysteine and 60% that figure in the absence of reducing agent. The average TPD_i preparation had a specific activity of 2500 in the presence of cysteine and showed no activity in the absence of reducing agent.

Calculation of degree of dissociation of TPD–DPN complexes

In order to determine the availability of TPD-bound DPN to other enzymes, it is essential to estimate the extent to which the observed availability is due to action of free DPN liberated by dissociation of the complex.

The dissociation constant expressed by VELICK *et al.*[8] for TPD–(DPN) complexes, may be written as follows:

$$K' = \frac{[\text{free enzyme sites}]\ [\text{DPN}]}{[\text{bound enzyme sites}]} \qquad (1)$$

From data on charcoal-treated TPD, which can bind three moles of DPN, these authors obtained values for K' of 6.4, 3.7, 0.96 and $0.08 \cdot 10^{-6}$ moles per liter when the number of moles of DPN bound per mole of total enzyme present were 0.58, 1.22, 2.47 and 2.98 respectively. The drift of these values for K' was interpreted by VELICK *et al.*[8] to mean that "the binding of 1 equivalent of DPN thus appears to increase

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the affinity of the protein for a second equivalent and the binding of a second equivalent appears to increase the affinity for a third". This interpretation can be modified to include the assumption that the only enzyme species present in appreciable concentrations were the free enzyme, TPD, and the fully saturatede nzyme TPD–$(DPN)_3$. This assumption allows the calculation of a combined dissociation constant for the overall reaction, TPD–$(DPN)_3 \rightarrow$ TPD + 3 DPN, by use of the following equation*:

$$K''' = \frac{[\text{TPD}]\ [\text{DPN}]^3}{[\text{TPD} - (\text{DPN})_3]} \tag{2}$$

The data of VELICK *et al.* yields K''' values equal to 1.4, 2.3, 1.9 and $2.2 \cdot 10^{-17}$ moles^3/liters^3, respectively. The constancy of the K''' values lends support to both the above quoted interpretation by VELICK *et al.* and to our modification of it.

Unfortunately, K''' cannot be used for calculating the degree of dissociation of the "native" complex of TPD and DPN, since this complex has only two binding sites for DPN. However, by analogy, it appears reasonable that the appropriate combined dissociation constant for the overall reaction in this case should be given by the equation:

$$K'' = \frac{[\text{TPD}]\ [\text{DPN}]^2}{[\text{TPD} - (\text{DPN})_2]} \tag{3}$$

The value of K'' may be determined from data obtained by VELICK *et al.*[8], whose measurements of dissociated DPN in the supernatant of an ultracentrifuged solution of TPD–$(DPN)_2$ gave K' values between 2 and $4 \cdot 10^{-7}$ mols/liter. The data being unrecorded, we assume that the TPD concentration in these experiments was $3. \cdot 10^{-5}$ M, in agreement with recorded concentrations in similar experiments. From the value of K' and the assumed TPD concentration, one may calculate the equilibrium concentrations of bound and dissociated DPN that must have been observed, and then obtain values for K'' between 0.7 and $1.9 \cdot 10^{-12}$ moles^2/liters^2.

In the experiments reported in the present paper, the concentration of bound nucleotide added lies in the range 0.5 to $1.5 \cdot 10^{-4}$ M. From equation (3), using an average value of $1.3 \cdot 10^{-12}$ for K'' one can calculate that the degree of dissociation of TPD–$(DPN)_2$ should range between 7.8 and 3.8% respectively. From equation (1), using an average value of $3 \cdot 10^{-7}$ for K', the calculated degree of dissociation ranges between 7.5 and 4.4% respectively. It is worth noting, however, that the two equations do not yield such similar results under other conditions. This point is illustrated by one experiment in which the substrate was TPD–$(DPN)_2$ from which approximately 60% of the DPN had been removed by charcoal treatment. If one assumes that this material consisted of a mixture of TPD$(DPN)_2$ and free TPD, then equation (3) can be applied, and it can be calculated that when the concentration of bound nucleotide is close to $1 \cdot 10^{-4}$ M, then the degree of dissociation of bound DPN is about 0.9%. If one assumes that the partial charcoal treatment had unmasked a third binding side, then equation (2) and (3) may be applied, with $K''' = 2 \cdot 10^{-17}$, to give a value of about 1.5% dissociation. On the other hand, if one uses equation (1) in this case, with $K' = 3 \cdot 10^{-7}$, only 0.2% dissociation is calculated. As will be shown in the experimental section, some data obtained with *Neurospora* DPNase are more compatible with the former values than with the latter and lend some support to the use of equations (2) and (3).

* We are indebted to Dr. ARDA A. GREEN for suggesting the use of this equation.

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TABLE I

DISSOCIATION CONSTANTS OF DPN AND DPNH OF VARIOUS ENZYMES

Enzyme	K_{DPN} *moles/liter*	K_{DPNH} *moles/liter*	*Reference**
Neurospora DPNase	$5 \cdot 10^{-4}$	—	KAPLAN *et al.*[12]
Snake venom pyrophosphatase	$3 \cdot 10^{-4}$	—	Determined in this study
DPN kinase	$6 \cdot 10^{-4}$	—	WANG AND KAPLAN[16]
Takadiastase deaminase	$18 \cdot 10^{-4}$	—	KAPLAN *et al.*[13]
Takadiastase deaminase	—	$7 \cdot 10^{-4}$	Determined in this study
Skeletal muscle lactic dehydrogenase	$1 \cdot 10^{-5}$	$5 \cdot 10^{-6}$	Determined in this study
DPN – *iso*citric dehydrogenase	$4 \cdot 10^{-4}$	—	Determined in this study
Horse liver alcohol dehydrogenase	$2.4 \cdot 10^{-5}$	$1.5 \cdot 10^{-5}$	THEORELL AND BONNICHSEN[1]
Yeast alcohol dehydrogenase	$9 \cdot 10^{-5}$	$3 \cdot 10^{-5}$	PULLMAN *et al.*[27]
Pseudomonas transhydrogenase	$7 \cdot 10^{-5}$	—	COLOWICK *et al.*[17]
Glutamic dehydrogenase**	$9 \cdot 10^{-3}$	$1 \cdot 10^{-4}$	OLSON AND ANFINSEN[32]

* The K_m values reported in the present work were obtained by conventional methods.
** The glutamic dehydrogenase used in this study was a gift of Dr. J. A. OLSON.

The data provided by VELICK *et al.*[8] indicate that DPN–TPD$_i$ and DPN–TPD$_a$ are equally dissociable. The same authors measured by ultracentrifugation, the dissociation constant of DPNH–TPD and found K' to lie between 10^{-5} and 10^{-6} moles/liter. Although these experiments with the ultracentrifuge were termed somewhat unsatisfactory, data based on the inhibition by DPNH of glyceraldehyde oxidation also indicate that the value of K' for DPNH–TPD$_a$ lies close to 10^{-5} moles/liter. However, studies on the apparent equilibrium constant for the oxidation of glyceraldehyde-3-phosphate by TPD–(DPN)$_2$ in the presence or absence of phosphate[30,31] suggest that DPNH–TPD$_a$ and DPN–TPD$_a$ have the same dissociation constant. Other evidence in support of this conclusion, that the K' value for DPNH–TPD$_a$ is close to $3 \cdot 10^{-7}$ moles/liter, is based on studies of displacement of bound DPNH by DPN[11]. Because of the uncertainty concerning the K' value for DPNH–TPD$_a$, two calculations of DPNH–TPD$_a$ dissociation will be made based on the high and low K' value extremes, $1 \cdot 10^{-5}$ moles/liter and $3 \cdot 10^{-7}$ moles/liter, respectively.

Calculation of theoretical reaction rates for dissociated pyridine nucleotides

In order to estimate the extent to which apparent effects of various enzymes on bound pyridine nucleotides were actually due to dissociation, it was necessary to consider not only the degree of dissociation of the TPD–nucleotide complex but also the affinity of the dissociated pyridine nucleotide for the particular enzyme used. If v represents the initial velocity of the particular reaction at a concentration S_1 (equal to the amount of free pyridine nucleotide calculated to be present as a result of dissociation) while v_2 represents the velocity at a concentrations S_2 (equal to the total bound nucleotide added) then it follows from the Michaelis-Menton equation that

$$\frac{v_1}{v_2} = \frac{S_1(K_m + S_2)}{S_2(K_m + S_1)}$$

where K_m is the Michaelis constant for the enzyme used, and the ratio $v_1/v_2 \times 100$, referred to in this paper as "theory", is the theoretical value for the relative reaction rate of "bound DPN" that would obtain if only its dissociated DPN had been avail-

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able for reaction. Whenever the observed rate is appreciably higher than this theoretical rate, it may be concluded that DPN–TPD has reacted without undergoing prior dissociation.

RESULTS

Action of Neurospora DPNase on the bound DPN of TPD

Fig. 1 summarizes the data compiled in a series of experiments in which the initial rates of reaction of *Neurospora* DPNase on free and bound DPN were measured at various pH values. The graph records the ratio

$$\frac{\text{rate of splitting bound DPN}}{\text{rate of splitting free DPN}} \times 100$$

as a function of pH. Since the rate of splitting of free DPN is fairly independent of pH in this range[12], the data also reflects fairly accurately the actual changes in rate of splitting of bound DPN. The striking feature of the data is the change in the availability of bound DPN to DPNase as the pH is altered. It is also apparent that DPN–TPD_a is less reactive to the *Neurospora* enzyme than DPN–TPD_i, the difference becoming very pronounced between pH 7–8.

It seemed possible that the lowered rate of splitting DPN–TPD_a could be due to an inhibition of the DPNase by TPD_a. To test this, a mixture containing one-half free DPN and one-half DPN–TPD_a was compared with a solution of free DPN with respect to rate of splitting by DPNase at pH 8.0. The mixture containing half free and half bound DPN reacted at 65% the rate obtained with free DPN. This indicated the absence of DPNase inhibition by DPN–TPD_a.

In order to obtain further evidence that the low reactivity of bound DPN was not attributable to inhibition of DPNase by the TPD protein, the effects of *p*-chloromercuribenzoate (PCMB) and iodoacetate on the availability of bound DPN to DPNase were studied. VELICK[7] has reported that PCMB quantitatively displaced DPN from TPD. Either $1.2 \cdot 10^{-4}$ *M* PCMB or $5 \cdot 10^{-3}$ *M* iodoacetate was incubated with DPN–TPD_a ($1 \cdot 10^{-4}$ *M* bound DPN) in 0.1 *M* Tris buffer at pH 7.4 for 38 minutes at 0° C and 2 minutes at 37° C prior to the addition of DPNase. Free DPN, serving as control was treated in the same manner. The DPN which was or had been bound to TPD was split at 60% the rate of free DPN. Comparison of this figure with those obtained in the absence of PCMB or iodoacetate indicates that the sulfhydryl inhibitors cause a considerable portion of the bound DPN to react like free DPN. It is clear, therefore, that the low rate of cleavage of DPN–TPD_a by DPNase in the neutral pH range is a result of DPN-binding rather than being due to inhibition of DPNase by the TPD.

An important question to answer is how much of the observed reaction rate of "bound DPN" was due to DPN which had dissociated from the TPD. We have listed in Table II under the column "theory" a theoretical value for the relative reaction rate of "bound DPN" that would obtain if only its dissociated DPN had been available for reaction. Comparison of the calculated relative reaction rate of dissociated DPN with the observed reaction rates of bound DPN presented in Table II and Fig. 1, indicates that DPN bound to TPD_i can be split by DPNase without prior dissociation at any pH tested, DPN bound to TPD_a can be split outside the

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pH range 7.4–8.0, but within this pH range DPN bound to TPD_a cannot be split by DPNase. Similar comparisons of the data obtained from charcoal-treated TPD (retaining 40% of its original DPN content) led to the same conclusions. It can be seen that in all cases charcoal treatment led to a marked decrease in the rate of cleavage of the remaining bound DPN, although the concentration of bound DPN was the same in all tests. In the case of DPN–TPD_a at pH 7.4, the observed rates of cleavage by DPNase before and after charcoal treatment are both in good agreement with the values calculated from equations (2) and (3) for reaction due to dissociated DPN.

TABLE II

ACTION OF DPNASE ON BOUND DPN

Temp. 37° C, total volume 1 ml: DPN–TPD_a was twice treated with Norit A (13.5 mg/ml) a pH 6.0. DPN–TPD_i was treated once with charcoal. Approximately 60% of the bound DPN was removed from both forms of TPD. Reaction mixtures contained DPN, free or bound, $(1.3 \cdot 10^{-4}\ M$ for DPN–TPD_a, DPN–TPD_i and DPN–TPD_i (charcoal-treated); $0.9 \cdot 10^{-4}\ M$ for DPN–TPD_a (charcoal-treated)), buffer (0.1 M), and the reaction was started by the addition of DPNase. Buffers: KH_2PO_4 and pH 7.4 Tris.

pH	DPN–TPD_a*		DPN–TPD_i	
	Native (*Theory* = 7**)	*Charcoal-treated* (*Theory* = 1–2)	*Native* (*Theory* = 7)	*Charcoal-treated* (*Theory* = 1–2)
5.0	30	11	—	—
7.4	8.5	2.5	29	11

* Values represent $\frac{\text{rate bound DPN} \times 100}{\text{rate free DPN}}$.

** Theory represents rate which would be expected from the dissociation of DPN from the TPD.

For the following reasons we have rejected the possibility that the increased availability of both DPN–TPD_a and DPN–TPD_i to DPNase at pH values outside the neutral pH range is simply due to dissociation of the complex. (a) The dissociation constant of the DPN–TPD_i complex is independent of pH in the pH range 7.0 to 8.3 according to the direct measurements of VELICK *et al.*[8]; (b) As will be demonstrated in a later section, DPN–TPD_a is unavailable to the action of deaminase throughout the pH range 5.9 to 8.5.

The increased availability of DPN–TPD to DPNase outside the neutral pH range must therefore be attributed to some subtle change in the linkage between DPN and TPD rather than to actual dissociation. That this change, whatever its nature, is a reversible one, is indicated by the following experiment. DPN–TPD_a (DPN conc. $1.7 \cdot 10^{-4}\ M$) was incubated at pH 5.8 in 0.05 M phosphate for thirty minutes at 0° C and then three minutes at 37° C prior to neutralization to pH 7.8 with pH 8.0 buffer (final Tris conc. 0.15 M). The reneutralized DPN–TPD_a was then treated with the DPNase. Free DPN, serving as a control was handled in the same way. The bound DPN was split by DPNase at 10% the rate of free DPN. This indicated that the effects of acid pH on the availability of bound DPN are reversible.

The pH dependence of the rate of splitting of bound DPN suggested that at pH values in the neutral range, a new group on TPD becomes available for binding DPN in a manner which makes it inaccessible to the action of DPNase. A model for

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this kind of binding is provided by the known reaction between DPN and bisulfite[26]. The absorption of light at 340 mμ by the product is maximal at pH 6.8 and falls with change of pH. Fig. 2 shows the inhibitory effect of bisulfite on the rate of DPNase action as a function of pH. The curve for inhibition of DPNase by bisulfite corresponds closely to the curve for formation of the DPN-bisulfite complex, as measured by 340 mμ absorption. This indicates that the DPN-bisulfite complex cannot be split by DPNase, the reaction rate being due to free DPN. Two conditions might combine to give a pH optimum for DPN-bisulfite complex formation. The first is that, as in other addition reactions with DPN, H ions should be released. Decreasing the pH would favor dissociation of the addition compound. On the other hand, increasing

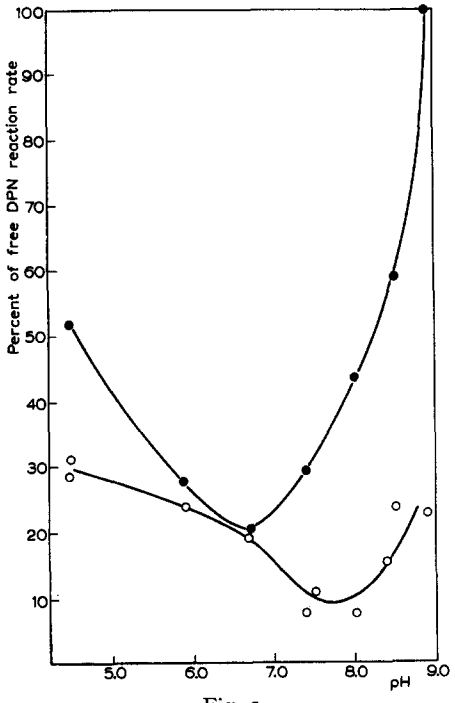

Fig. 1.

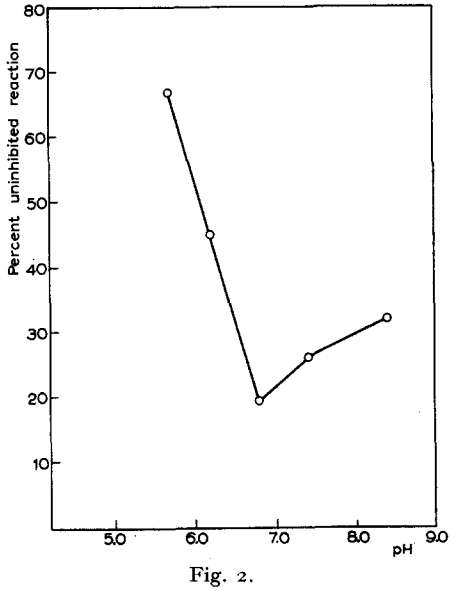

Fig. 2.

Fig. 1. Rate of reaction of DPNase on bound DPN as a function of pH. With the rate of reaction of DPNase on free DPN arbitrarily set at 100 for each pH, the curves give the relative rates of reaction of DPNase on bound DPN. Systems contained (final concentrations) DPN (1.1 to $1.5 \cdot 10^{-4}$ *M*), buffer and sufficient DPNase to split 1/2 the free DPN in 2 to 5 minutes. Total volume 1 ml. Temp. 37° C. Buffers: pH's 4.5, 5.9 and 6.7, phosphate (0.09 *M*); pH 7.5, phosphate (0.04 *M*); pH's 7.4 and 8.0, Tris (0.1 *M*); pH 8.4, pyrophosphate (0.07 *M*); pH's 8.5 and 8.9, glycine (0.09 *M*). Aliquots taken ato. 5, 1, 2, 4.5, 10 and 30 minutes. ● Inactive TPD-bound DPN, ○ Active TPD-bound DPN.

Fig. 2. Inhibition of DPNase by bisulfite as a function of pH. The pH is plotted versus the ratio:

$$\frac{\text{DPNase activity in presence of bisulfite}}{\text{DPNase activity in absence of bisulfite}} \times 100$$

Systems contained DPN ($1.2 \cdot 10^{-4}$ *M*), bisulfite when present (1%) buffer and DPNase. Temp. 37° C, total volume 1 ml. Buffers: pH's 6.2, 6.8, 7.4 and 8.4, Tris (0.1 *M*); pH 5.7, phosphate (0.1 *M*). Aliquots taken at 0.5, 1.0, 1.5, 2.5 and 5 minutes after the addition of DPNase. Prior to DPNase addition, mixture with and without bisulfite were incubated 15 to 30 minutes at 0° C and 2 minutes at 37° C.

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the pH would favor the formation of sulfite from bisulfite with a consequent decrease of the addition compound. The similarity of the pH activity curves for DPNase action on the DPN bisulfite mixture and on DPN–TPDi suggests the analogy that an addition compound between bound DPN and an ionizable side group of TPD could account for the results obtained.

Action of snake venom pyrophosphatase on the bound DPN of TPD

In Table III are presented the results of a series of experiments in which the initial rates of splitting of bound and free DPN by snake venom pyrophosphatase are compared. Over the pH range used, both forms of bound DPN could be split readily without prior dissociation from TPD. It is not certain whether there is a real effect of pH on the reaction rate of bound DPN with pyrophosphatase, but it should be noticed that variation of pH differently affected the actions on bound DPN of DPNase and pyrophosphatase. As the pH was increased from neutrality, DPN–TPD_i, in comparison to free DPN, became more available to DPNase and less available to pyrophosphatase. Around pH 8.5, pyrophosphatase did not distinguish, in terms of relative reaction rate, DPN–TPD_a from DPN–TPD_i, whereas DPNase at this pH split DPN–TPD_i much more rapidly than DPN–TPD_a. DPN–TPD_a was more readily available to pyrophosphatase than to DPNase, the availability to pyrophosphatase being close to that measured for DPN–TPD_i.

TABLE III

ACTION OF SNAKE VENOM PYROPHOSPHATASE ON BOUND DPN

The reaction mixtures contained $MgCl_2$ (0.05 *M*), buffer (0.1 *M*), bound or free DPN ($1.1 \cdot 10^{-4}$ *M* in experiments with DPN–TPD_a, and $1.3 \cdot 10^{-4}$ *M* in experiments with DPN–TPD_i), and sufficient pyrophosphatase to split half the free DPN in 3–5 minutes. Total volume 1 ml. Temp. 37° C. Buffers: pH's 7.4 and 8.0, Tris; pH 6.7, phosphate; pH 8.5, glycine. Rates are expressed as in Table II. Theory = 8.

Substrate	*pH*			
	6.7	*7.4*	*8.0*	*8.5*
DPN–TPD_a	37	33	34	45
DPN–TPD_i	—	67	57	48

Study of the relative rates of splitting of bound DPN by crude snake venom and Armour's intestinal phosphatase yielded the same numerical results obtained with snake venom pyrophosphatase. This indicates that the relative rates of splitting of bound DPN are determined by the DPN bond under attack and not by the nature of the pyrophosphatase used.

Studies with DPN kinase

In experiments with DPN kinase it was found the DPN–TPD_i was converted to TPN faster than was DPN–TPD_a. Both forms of bound DPN apparently could be converted to TPN without prior dissociation of DPN from TPD. This would indicate that although TPN has been found by others[8] not to bind to TPD, conditions have been found which allow the deduction that a TPN–TPD complex was formed, at least transiently. The experimental data are given in Table IV.

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TABLE IV

ACTION OF DPN KINASE ON BOUND DPN

In a total volume of 1 ml at 37° C were DPN, free or bound ($0.7 \cdot 10^{-4}$ *M*), $MnCl_2$ ($2 \cdot 10^{-3}$ *M*), nicotinamide ($1 \cdot 10^{-2}$ *M*), ATP ($8 \cdot 10^{-3}$ *M*), Tris buffer pH 7.4 (0.1 *M*), and the reaction was started by the addition of DPN kinase.

Substrate	$\frac{\text{Rate bound DPN}}{\text{Rate free DPN}} \times 100$
$TPD\text{–}DPN_a$	20
$TPD\text{–}DPN_i$	47
Theory	8

Effect of takadiastase deaminase on bound DPN

When the relative rates of deamination of bound DPN were measured (Table V), it was found, in agreement with the results obtained with other enzymes, that $DPN\text{–}TPD_i$ was attacked faster than $DPN\text{–}TPD_a$. In contrast to studies with *Neurospora* DPNase, it was found that the rate of deamination of $DPN\text{–}TPD_a$ was independent of pH. Comparison of the observed rates with the rate calculated for dissociated DPN led to the conclusion that $DPN\text{–}TPD_a$, unlike $DPN\text{–}TPD_i$, cannot be attacked by deaminase without prior dissociation. The similarity of the calculated and observed relative reaction rates and the pH independence of the latter, indicate that the dissociation constant for $DPN\text{–}TPD_a$, which can be calculated from our rate data, is in agreement with that derived from equilibrium data of VELICK *et al.*[8] for $DPN\text{–}TPD_i$, and is independent of pH.

TABLE V

RATE OF REACTION OF BOUND DPN AND DPNH WITH TAKADIASTASE DEAMINASE*

The reaction mixtures contained: DPN or DPNH (bound or free) ($1 \cdot 10^{-4}$ *M* in experiments with $DPN\text{–}TPD_a$, $1.5 \cdot 10^{-4}$ *M* in experiments with $DPN\text{–}TPD_i$ and $0.5 \cdot 10^{-4}$ *M* in experiments with $DPNH\text{–}TPD_a$), buffer and sufficient takadiastase deaminase to split half the free nucleotide in 2 to 5 minutes. Total volume 1 ml. Temp. 37° C. Buffers: pH's 7.4 and 8.0, Tris (0.1 *M*); pH's 5.9, 6.6 and 7.5, phosphate (resp. 0.1 *M*, 0.08 *M* and 0.04 *M*); pH 6.7, glyglyglycine (0.06 *M*); pH 8.5, pyrophosphate (0.07 *M*).

Substrate	*pH*				
	5.9	*6.6*	*7.4*	*8.0*	*8.5*
$DPN\text{–}TPD_a$	7.8**	8.0	5.0	5.0	5.0
$DPNH\text{–}TPD_a$	—	—	70	—	—
$DPN\text{–}TPD_i$	—	26	40	—	—

* Values represent $\frac{\text{rate bound nucleotide}}{\text{rate free nucleotide}} \times 100$.

** The theory for $DPN\text{–}TPD_a$ or DPN_i is 5–8; that for DPNH–TPD 8 ($K' = 3 \cdot 10^{-7}$) or 38 ($K = 1 \cdot 10^{-5}$).

Since $DPN\text{–}TPD_i$ can, without prior dissociation, be deaminated and $DPN\text{–}TPD_a$ cannot, it is suggested that activation of TPD results in the formation of a new enzyme-DPN bond which serves to protect the adenine amino group from reaction with deaminase. Reduction of $DPN\text{–}TPD_a$ to yield $DPNH\text{–}TPD_a$ resulted in a greatly increased availability to deaminase. Regardless of the K' for DPNH–TPD used,

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calculations of theoretical reaction rates due to dissociated DPNH indicate the bound DPNH must, without prior dissociation, be readily available to deaminase. This indicates that the bond which protects the adenine amino group of DPN–TPD_a is not present in DPNH–TPD_a.

Action of dehydrogenases on bound DPN

When the reduction of bound DPN was catalyzed by various dehydrogenases, it was found that DPN–TPD_i was more rapidly reduced than DPN–TPD_a (Table VI). This information agrees with the generalization that has developed in this paper that DPN–TPD_i is more readily available to other enzymes than DPN–TPD_a. Comparison of the observed reaction rates with the theoretical rates calculated for dissociated

TABLE VI

ACTION OF DEHYDROGENASES ON BOUND NUCLEOTIDES

Total volume 1 ml. Room temp. *Lactic dehydrogenase*: DPN reduction: the reaction mixture contained DPN, free or bound ($0.72 \cdot 10^{-4}$ *M*), sodium lactate (0.1 *M*), hydroxylamine (0.08 *M*), buffer (0.1 *M*), and lactic dehydrogenase. The reaction was started with the addition of enzyme. Buffers were pH 6.7, phosphate; pH's 7.2, 8.0, Tris; pH's 8.8, 9.9, glycine. DPNH oxidation: the reaction mixture contained DPNH, bound or free ($4.5 \cdot 10^{-5}$ *M*), Tris buffer pH 7.4 (0.1 *M*), sodium pyruvate (0.02 *M*), and lactic dehydrogenase. The reaction was started by the addition of enzyme. *Yeast DPN isocitric acid dehydrogenase*: the reaction mixture contained DPN ($0.72 \cdot 10^{-4}$ *M*), $MgCl_2$ (0.006 *M*), 5′-adenylic acid ($5 \cdot 10^{-5}$ *M*), KCN (0.01 *M*), sodium *iso*citrate (0.0025 *M*), potassium phosphate buffer pH 6.7 (0.1 *M*), and the reaction was started by the addition of *iso*citric dehydrogenase. *Pseudomonas transhydrogenase*: the reaction mixture contained DPN ($0.72 \cdot 10^{-4}$ *M*), TPN ($0.3 \cdot 10^{-4}$ *M*), $MgCl_2$ (0.003 *M*), 2′-adenylic acid (0.0032 *M* (when present)), sodium *iso*citrate (0.0015 *M*), KCN (0.01 *M*), Tris buffer pH 7.4 (0.1 *M*) and sufficient pig heart extract to reduce all the TPN in 15 seconds. After TPN reduction, the transhydrogenase reaction was started by the addition of enzyme. *Horse liver and yeast alcohol dehydrogenase*: DPN reduction: the reaction mixtures contained DPN ($1.1 \cdot 10^{-4}$ *M*), ethanol (0.34 *M*) and Tris pH 10.1 (0.35 *M*), in addition to enzyme used to start the reaction. DPNH oxidation: the reaction mixtures contained DPNH ($4.5 \cdot 10^{-5}$ *M*), acetaldehyde (0.05 *M*), Tris buffer pH 7.4 (0.1 *M*), in addition to enzyme used to start the reaction. *Glutamic acid dehydrogenase*: DPN reduction: the reaction mixture contained DPN ($0.69 \cdot 10^{-4}$ *M*), histidine buffer pH 8.1 (0.1 *M*), potassium glutamate (0.01 *M*), and enzyme. The reaction was started by the addition of glutamate. DPNH oxidation: the reaction mixture contained DPNH ($4.5 \cdot 10^{-5}$ *M*), histidine buffer pH 8.1 (0.1 *M*), α-ketoglutaric acid (0.01 *M*), ammonium sulphate (0.02 *M*), and the reaction was started by the addition of enzyme.

		$\frac{\text{Rate bound nucleotide}}{\text{Rate free nucleotide}} \times 100$						
		DPN–TPD_a		DPNH–TPD_a			DPN–TPD_i	
Dehydrogenase	pH	found	theory	found	theory		found	theory
					$K' = 3 \cdot 10^{-7}$	$K' = 1 \cdot 10^{-5}$		
Lactic dehydrogenase	6.7	25	39					
	7.2	27	39				49	39
	8.0	31	39					
	8.8	34	39					
	9.9	23	39					
	7.4	—	—	80–100	46	84		
*Iso*citric dehydrogenase	6.7	15	8.5				28	8.5
Liver alcohol dehydrogenase	10.1	52	26				78	26
	7.4	—	—	100	25	69	—	—
Yeast alcohol dehydrogenase	10.1	18	12				67	12
	7.4	—	—	70–100	17	59		
Transhydrodenase	7.4	14	14				63	14
Glutamic acid dehydrogenase	8.1	15	8	80–100	11	46		

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DPN revealed that DPN bound to inactive TPD can without prior dissociation be reduced by other dehydrogenases. The reaction rates measured when DPN–TPD_a served as substrate indicated that DPN bound to active TPD could not be reduced by other dehydrogenases and that only dissociated DPN was available for reaction. While the observed and calculated values show some disagreement, it is clear that in those cases in which relatively high rates of reduction of DPN–TPD_a were observed (liver ADH and lactic dehydrogenase), the theoretical rates due to dissociation were also relatively high.

From the results obtained in the experiments with adenosine deaminase, it was concluded that the dissociation of DPN from TPD_a was independent of pH. Since the reaction rate of DPN–TPD_a with the various dehydrogenases was due only to dissociated DPN, the reaction rate should be independent of pH. When the reduction of DPN–TPD_a by lactic dehydrogenase was studied over the pH range 6.7–9.9, the rate of reduction relative to free DPN was indeed found to be independent of pH.

In marked contrast to the results obtained with bound DPN, the various dehydrogenases catalyzed the oxidation of DPNH–TPD_a 80–100% as fast as free DPNH. These data are in agreement with the work of CORI *et al.*[11] who studied the oxidation of DPNH–TPD_a by lactic dehydrogenase, as well as the more recent work of MAHLER[33], who studied the oxidation of DPNH–TPD_a by cytochrome *c* reductase. Estimation of the dissociation constant of DPNH–TPD_a—which was considered to be equal to the dissociation constant of DPN–TPD_a—led CORI *et al.*[11] to conclude that the concentration of dissociated DPNH could not account for the observed rate of oxidation of DPNH–TPD_a. If we consider that K' given by CORI *et al.*[11] for DPNH–TPD is equal to K' for DPN–TPD, then our data obtained with four enzymes supports the conclusion that bound DPNH may be oxidized without prior dissociation. Corroborative evidence is supplied by our data with glutamic acid dehydrogenase even when K' is assumed to be 10^{-5} moles/l*.

DISCUSSION

The most striking and consistent result that we have obtained is that substitution of active TPD for inactive TPD is attended by a decreased availability of the bound DPN to other enzymes. This was the case for all enzymes that we have used with the possible exception of snake venom pyrophosphatase. From this fact we might predict that agents such as glutathione which activate sulfhydryl enzymes should also indirectly conserve DPN *in vivo*. Since it is probable that in muscle a large part of the DPN is bound to TPD, the different reactivities of DPN–TPD_a and DPN–TPD_i could be the basis of a regulatory mechanism by which DPN would be protected from degradative enzymes when TPD is active and would become available for reduction by other enzymes when TPD is inactive.

The difference in reactivity of DPNH and DPN bound to TPD_a may also be of some physiological significance. If under anaerobic conditions, muscle TPD is in the active form, one might expect that a large part of the DPN would be linked to the TPD in such a manner that it would not be available to other enzymes. This might result in a preferential utilization by muscle of an oxidative pathway that involves

* Since this work has been completed, NYGAARD AND RUTTER[34] have reported experiments which are similar to ours with respect to reaction of the bound DPN of TPD with dehydrogenases.

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triose phosphate since DPN would be only reduced by TPD. The bound DPNH formed in such a reaction could then readily be oxidized by pyruvate and lactic dehydrogenase. Under aerobic conditions, it is possible that the TPD may exist largely in the inactive form, leading to a decrease in the rate of glycolysis. If this were the case, then the DPN would not be linked by the bond which is present in DPN–TPD_a, and would be available to other dehydrogenases. For example, the DPN could become available to enzymes in the citric acid cycle which would operate under aerobic conditions. Hence the additional DPN binding in TPD_a may be a factor in the operation of the Pasteur effect.

The data presented offer information on the mode of binding of DPN to TPD. It was found that DPN–TPD_a had to dissociate before it could be deaminated or reduced by various dehydrogenases, whereas DPNH–TPD_a could be deaminated or oxidized as rapidly as free DPNH. This indicated the existence of a DPN–TPD bond which disappears upon reduction of DPN to DPNH. Since DPN–TPD_i could be deaminated or reduced without prior dissociation, the bond must also disappear when TPD becomes inactive. RACKER AND KRIMSKY[35] and VELICK[7] have provided spectrophotometric evidence for the existence of a bond between DPN and active TPD. Observed spectral changes indicated that addition of sulfhydryl inhibitors or acyl phosphates abolished the bond[35]. It should be stressed that the bond being discussed is not the only link between pyridine nucleotides and TPD, since DPN or DPNH are bound to TPD in situations where the secondary bond under discussion is absent. Some information as to the nature of this primary binding in DPN–TPD_i will be described elsewhere.

The great importance of the secondary bond is the intimate association with enzyme activity. RACKER AND KRIMSKY[35] have suggested that the bond is formed between enzyme sulfhydryl group and position 4 of the nicotinamide ring which is eventually hydrogenated[36]. Our data may be interpreted to agree with their theory since we have shown that the bond was broken when the nicotinamide group was reduced or the enzyme sulfhydryl group was absent. Our data obtained with dehydrogenases and deaminase, confirm RACKER AND KRIMSKY's spectral data of a pH-independent bond present between DPN and active TPD. However, formation of a bond between nicotinamide and sulfhydryl should release hydrogen ion and consequently the bond formation should be pH-dependent. In fact, the data obtained with DPNase can be interpreted to indicate that a pH-dependent bond is formed between TPD and the nicotinamide group of DPN. It is difficult to extend RACKER AND KRIMSKY's theory to explain why in DPN–TPD_a, DPN–TPD_i, and DPNH–TPD_a, the adenine amino group reacts differently. The relationship of the adenine group of the DPN to the sulfhydryl group of the protein is a problem which appears to merit further clarification since it has recently been shown that the adenine group of DPN is involved in a link with an SH group of yeast alcohol dehydrogenase[37].

SUMMARY

1. It has been found that the *Neurospora* DPNase can cleave the bound DPN of inactive muscle triose phosphate dehydrogenase (TPD_i) without prior dissociation of the pyridine nucleotide. The ratio, rate of splitting DPN–TPD_i/rate of splitting free DPN, is pH-dependent, a minimum being observed at pH 6.7. DPN bound to active triose phosphate dehydrogenase (TPD_a) is also cleaved without prior dissociation if the reaction is run outside the pH range 7.4–8.0. Within that pH range, DPN bound to TPD_a is not split by DPNase, the limited reaction observed being due to dissociated DPN.

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2. Takadiastase deaminase will readily deaminate the bound DPN of TPD_i although at a rate considerably slower than free DPN. On the other hand, no deamination of DPN–TPD_a occurs. Since TPD_a has additional SH groups, some type of relationship is suggested between a protein SH group and the amino group of the adenine moiety of the oxidized DPN. DPNH bound to TPD_a is deaminated by the takadiastase enzyme at a rate comparable to free DPNH, suggesting that reduction of DPN alters the interaction between the adenine amino group and the protein.

3. Snake venom pyrophosphatase splits the DPN bound to TPD_a and TPD_i. The rate of splitting is somewhat slower with TPD_a.

4. The bound DPN can also be phosphorylated by ATP in the presence of DPN kinase to form TPN. This phosphorylation occurs without prior dissociation of the DPN and occurs at a faster rate with TPD_i than with TPD_a.

5. The bound DPN of TPD_i is available to a number of dehydrogenases, but the reaction rate with these dehydrogenases is slower than that of free DPN. DPN–TPD_a does *not* react with other dehydrogenases. However DPNH–TPD_a is readily available to these same dehydrogenases.

6. It is concluded from the above studies that the DPN found on TPD_a is bound to the protein through at least two linkages. One is through a binding found also in TPD_i and a second through a grouping which may involve the nicotinamide moiety and which has also been indicated by the studies of RACKER AND KRIMSKY[35].

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