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Dehydrogenase-Reduced Coenzyme Difference Spectra, Their Resolution and Relationship to the Stereospecificity of Hydrogen Transfer*

Harvey F. Fisher, Dismus L. Adija, and Dallas G. Cross

ABSTRACT: The binding of reduced diphosphopyridine nucleotide to any specific dehydrogenase causes changes in the ultraviolet absorption spectrum of the reduced coenzyme. The 340-mμ regions of the resulting difference spectra can all be resolved into two simple operations on a reduced diphosphopyridine nucleotide spectrum; a shift of the band itself to a higher or lower wavelength without any change in shape and a uniform hyper- or hypochromicity of that peak. We find that A stereospecific dehydrogenases produce blue shifts while B stereospecific dehydrogenases produce red shifts (with the possible exception of mitochondrial malate dehydrogenase). The size of most of the shifts observed here is sufficiently large that the concentration of reduced diphosphopyridine nucleotide involved can be calculated. We have previously shown that changes in conformation of reduced diphosphopyridine nucleotide produce difference spectra resolvable into various combinations of the same components. Most of the shifts of the coenzyme spectrum in dehydrogenase complexes, however, are far too large to be accounted for by a simple opening or closing of the reduced diphosphopyridine nucleotide molecule.

In previous studies we have shown that the shift in the ultraviolet absorption spectrum of DPNH produced by the binding of the reduced coenzyme to either liver alcohol dehydrogenase, liver L-lactate dehydrogenase, or mitochondrial malate dehydrogenase described by Theorell and Bonnichsen (1951), Chance and Neilands (1952), and Pfleiderer and Hohnholz (1959) is not a unique property of those three enzymes, but occurs in the binary complexes of other dehydrogenases (Fisher and Cross, 1966). In a more recent paper (Cross and Fisher, 1969), we have shown that changes in the conformation of DPNH (or TPNH) in solution generate rather similar difference spectra, and that all such spectra can be resolved into combinations of simple shifts and hypochromicities of the 340-mμ band of DPNH. While the difference spectra resulting from DPNH conformational changes differ from those due to dehydrogenase complexing, and while no two dehydrogenase binary complex difference spectra are themselves identical, all of these difference spectra can be resolved into different combinations and algebraic senses of the same two simple operations that sufficed for the resolution of conformation difference spectra.

We present here a general survey of the difference spectra of dehydrogenase-reduced coenzyme binary complexes, the resolutions of the reduced nicotinamide absorption regions of those difference spectra into two simple and physically relevant operations, and consider the possible implications of these resolutions. The scope of this particular paper is limited to those things which are characteristic of dehydrogenase-reduced coenzyme difference spectra as a group—more detailed studies of

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TABLE I: Resolution Components of the 340-mμ Region of Dehydrogenase-Coenzyme Difference Spectra.[a]

Dehydrogenase (mg/ml)[f]	pH	Coenzyme (μM)	Enzyme Stereo-specificity	Δλ (mμ) Required for Resolution	% Hypo- or Hyperchromicity Required for Resolution	Amount of Coenzyme Required for Resolution (μM)
Glucose 6-phosphate (≈1)[e]	8.0[b]	TPNH (9.9)	B	Red 37 ± 4	−44 ± 5	1.8 ± 0.6
Glutamate (1.0)	7.6[c]	TPNH (100)	B	Red 34 ± 2	−30 ± 7	2.4 ± 0.1
Glutamate (0.88)	7.6	DPNH (69)	B	Red 31 ± 2	−20 ± 5	2.0 ± 0.4
α-Glycerol phosphate (0.844)	7.6[c]	DPNH (9.9)	B	Red 31 ± 2	−39 ± 8	4.1 ± 0.2
20-β-Hydroxysteroid (≈1)[e]	7.7	DPNH (12.4)	B	Red 29 ± 2	−3 ± 4	2.8 ± 0.5
Glyceraldehyde-3-PO_4 (1.13)	8.7[c]	DPNH (134)	B	Red 11 ± 2	−25 ± 5	2.6 ± 0.5
Malate (mitochondrial) (1.27)	7.1	DPNH (25.6)	?	Red 22 ± 1	+12 ± 3	8.6 ± 1.0
Alcohol (liver) (1.0)	7.2	DPNH (10)	A	Blue 14 ± 1	−35 ± 2	10 ± 1
Alcohol (yeast) (0.98)	7.2	DPNH (73)	A	Blue 5 ± 1	−22 ± 2	13 ± 2
Lactate (0.93)	7.6[c]	DPNH (16)	A	Blue 2 ± 1	−2 ± 4	17 ± 2
Isocitrate (0.86)	7.4[d]	TPNH (10)	A	Blue 2 ± 1	−4 ± 8	13 ± 8
Malate (supernatant) (0.85)	7.6	DPNH (42)	A	Blue 4 ± 2	0 ± 2	15 ± 8

[a] The buffer used was 0.1 M potassium phosphate except: [b] 0.05 M Tris, [c] 0.2 M potassium phosphate, and [d] 0.25 M Tris. [e] Estimated from the dilution of stock solutions purchased from Sigma Chemical Corp. [f] Concentration of dehydrogenase was calculated from the mg/ml extinction coefficient at 280 mμ as follows: glutamate, 0.97 (Olson and Anfinsen, 1952); α-glycerophosphate, 0.75 (Beisenherz *et al.*, 1955); glyceraldehyde 3-phosphate, 2.1 (Krebs *et al.*, 1953); mitochondrial malate, 0.305 (Harada and Wolfe, 1968); liver alcohol, 0.42 (Shore and Theorell, 1966); yeast alcohol, 1.25 (Hayes and Velick, 1954); lactate, 1.42 (Velick, 1958); isocitrate, 0.90 (Moyle, 1965); and supernatant malate, 0.90 (P-L Biochemicals, Inc.).

specific features of individual enzyme–coenzyme and enzyme–coenzyme–substrate complexes and the relation of such features to the kinetics and mechanism of action of the enzyme will be the subject of later papers in this series.

Materials and Methods

The following enzyme preparations were purchased from Sigma Chemical Co.; the descriptions of the properties and methods of preparation were supplied by Mr. Louis Berger of that company (personal communication).

Bovine liver L-glutamate dehydrogenase, type I, a highly purified, crystalline preparation is prepared substantially as described by Olson and Anfinsen (1952). The lactate dehydrogenase and malate dehydrogenase contamination amounts to less than 0.2% of the glutamate dehydrogenase activity.

Glucose 6-phosphate dehydrogenase, type VI, is a partially purified preparation from baker's yeast. Although its specific activity was somewhat low, it did have relatively low contaminating enzyme activities being substantially free of hexokinase and 6-phosphogluconic dehydrogenase. Purification was achieved primarily through the use of ammonium sulfate fractionation and column chromatography.

Liver alcohol dehydrogenase is a crystallized preparation, from horse liver prepared substantially as described by Bonnichsen and Brink (1955).

Isocitrate dehydrogenase, type IV, is a highly purified preparation from pig heart prepared substantially as described by Siebert *et al.* (1957). Contaminating enzymes such as aconitase and DPN-specific isocitrate dehydrogenase are of a low level (less than 0.2% of the TPN-specific isocitrate dehydrogenase activity).

Lactate dehydrogenase, type III, is a highly purified, crystalline preparation from beef heart prepared substantially as described by Meister (1952). Its pyruvate kinase content is less than 0.03% of its lactate dehydrogenase activity.

α-Glycerophosphate dehydrogenase is a highly purified, crystalline preparation from rabbit muscle. It has been purified substantially as described by Beisenherz *et al.* (1955). It is substantially free of aldolase, lactate dehydrogenase, pyruvate kinase, and glyceraldehyde phosphate dehydrogenase.

20β-Hydroxysteroid dehydrogenase, type II, is a highly purified preparation from *Streptomyces hydrogenans*. It is substantially free of DPNH oxidase.

Na_2DPNH (grade III, 98%) and TPNH (type II) were also products of the Sigma Chemical Corp. Yeast alcohol dehydrogenase was purchased from the Mann Chemical Corp. Mitochondrial L-malate dehydrogenase (batch 71), prepared from pig heart by the method of Thorne and Cooper (1963), was a product of Seravac Laboratories. Supernatant L-malate dehydrogenase (lot SM-3), prepared by the method of Thorne and Cooper, was a product of P-L Biochemicals, Inc. Yeast glyceraldehyde 3-phosphate dehydrogenase was a gift from Dr. Archie L. Murdock, University of Kansas School of Medicine.

All dehydrogenases were prepared by dialyzing them against their respective buffers shown in Table I. In addition, both yeast and liver alcohol dehydrogenases were pretreated with a

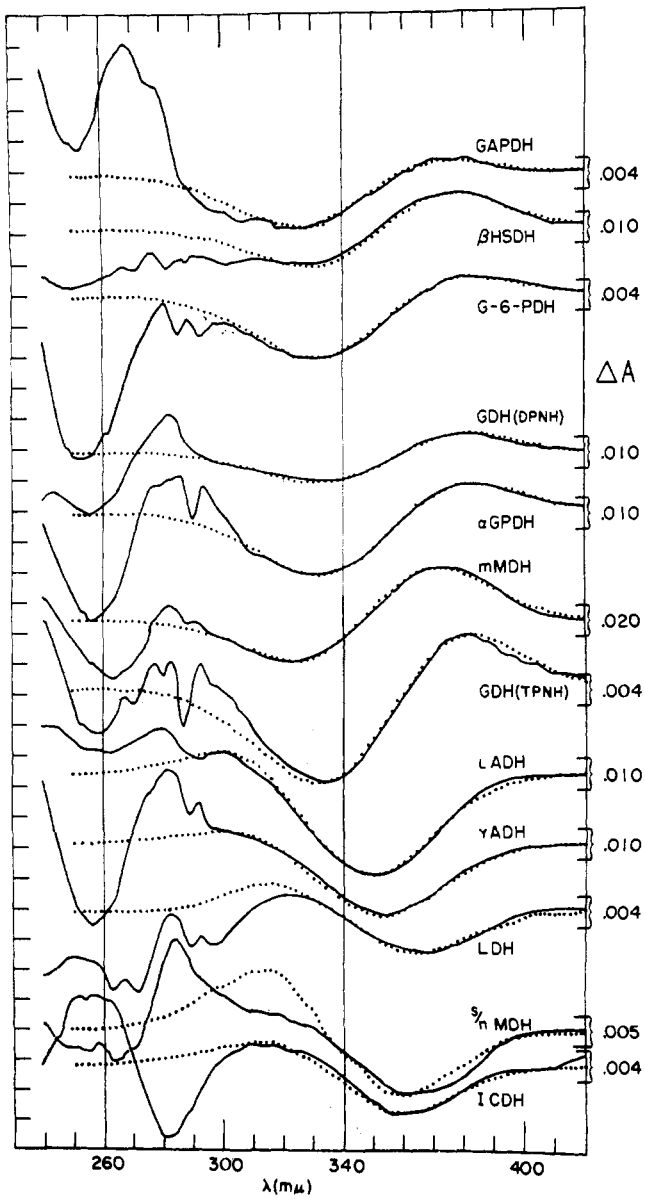

FIGURE 1: Dehydrogenase-reduced coenzyme difference spectra (solid lines) and the sum of the components required for resolution of the 340-mμ region (dotted lines). Conditions and concentrations of enzymes and coenzymes are described in the Materials and Methods section and in Table I. Enzyme abbreviations are as follows: GAPDH, yeast glyceraldehyde 3-phosphate; βHSDH, 20β-hydroxysteroid; G-6-PDH, glucose 6-phosphate; GDH, glutamate; αGPDH, α-glycerol phosphate; mMDH, mitochondrial malate; LADH, liver alcohol; YADH, yeast alcohol; LDH, lactate; s/n MDH, supernatant malate; and ICDH, isocitrate dehydrogenases.

5×10^{-5} M solution of DPNH before dialysis and with Norit A followed by filtration. After dialysis all enzymes were filtered through a 0.45 μ Millipore filter.

Difference spectra were recorded on a Cary Model 14 spectrophotometer using the zero- to one-tenth-scale expansion. Cuvets of 1.000-cm path length were used in a tandem cell arrangement as described by Herskovits and Laskowski (1961). The sample and reference compartments were thermostated to 20 ± 0.2°. Base lines were recorded with enzyme in both sample and reference compartments. Additions of coenzyme were made to the enzyme solution in the sample compartment and to a cuvet containing only buffer in the reference compartment. All other procedures have been previously described (Cross and Fisher, 1969).

Results

The difference spectra of eleven dehydrogenase-reduced coenzyme binary complexes (as referred to separated components) are shown as solid lines in Figure 1. The dotted lines represent summations of resolved components to be described later. Such difference spectra for supernatant malate, glucose 6-phosphate, 20-β-hydroxysteroid, and α-glycerolphosphate dehydrogenases have not been previously reported.

Resolution of ER Difference Spectra. PROCEDURE. In a previous paper (Cross and Fisher, 1969), we pointed out that any resolution of a complex curve is not in itself unique and proposed several criteria to ensure that the components and operations chosen would correspond to physically meaningful entities. We also showed that the 340-mμ region of all difference spectra generated by conformational changes of DPNH could be resolved into a shift in wavelength and a hypo- or hyperchromicity[1] of the original total spectrum. The use of these two operations was justified on the basis that a shift in wavelength is to be expected from *any* change in the intermediate environment of a chromophore; that the requirements for hypochromism as stated by Tinoco are present in the DPNH molecule and the phenomenon itself is common in polynucleotide systems; and that *all* difference spectra obtained could be successfully resolved without assuming that the 340-mμ band contained more than a single electronic transition.

While we will use only these same two operations to resolve the enzyme complex difference spectra, there is one important quantitative difference in the resolutions of the difference spectra reported previously and those described here. The difference spectra generated by conformational changes in the DPNH molecule could all be adequately resolved using only very small spectral shifts (<5 mμ). As has been noted by Chervenka (1959) and proved formally by Scheraga (1961), a difference spectrum produced by an infinitesimally small shift of a spectral peak measured against the original peak has the form of the first derivative of the absorption band itself, so that for very small finite shifts

$$\Delta A_\lambda = -\Delta\lambda\left(\frac{d\epsilon}{d\lambda}\right)_\lambda C \qquad (1)$$

where C is the molar concentration of the chromophore actually undergoing a spectral shift, $\Delta\lambda$; and ϵ_λ is the molar extinction coefficient. The difference spectrum will have positive and negative limbs with a null absorbance close to the peak of the spectral band of the chromophore (or, more exactly, at $\lambda = \lambda_{max} + \Delta\lambda/2$). An important consequence of eq 1 is that the product, $\Delta\lambda C$, cannot be resolved from information obtainable from the difference spectrum itself; two solutions, one involving half as much of the chromophore in an operation producing twice as large a shift as the other, would pro-

[1] We restrict our use of the terms "hypochromicity" and "hyperchromicity" to the phenomenon described by Tinoco (1960) as a proportional change in absorbance at all wavelengths without a shift in wavelength.

duce identical difference spectra. For this reason, we were able to use a solvent perturbation difference spectrum multiplied by a factor representing $\Delta\lambda C$ as a component in all the resolutions of the DPNH "denaturation" difference spectra described in the previous paper. However, Scheraga's equation does not describe difference spectra resulting from larger shifts. Scheraga used a Taylor series expansion disregarding all terms but the first to obtain eq 1. An equation valid for the larger shifts involved in enzyme–reduced coenzyme difference spectra can be obtained from this same expansion, retaining the first three terms. It can

$$\Delta\epsilon = \sum_{n=1}^{\infty} \frac{(\Delta\lambda)^n}{n!}\left(\frac{d^n\epsilon}{d\lambda^n}\right)_{\lambda_0} \quad (2)$$

$$\Delta\epsilon \approx \left(\frac{d\epsilon}{d\lambda}\right)_{\lambda_0}\Delta\lambda + {}^1/_2\left(\frac{d^2\epsilon}{d\lambda^2}\right)_{\lambda_0}(\Delta\lambda)^2 + {}^1/_6\left(\frac{d^3\epsilon}{d\lambda^3}\right)_{\lambda_0}(\Delta\lambda)^3 \quad (3)$$

$$\Delta A \approx \left[\left(\frac{d\epsilon}{d\lambda}\right)_{\lambda_0}\Delta\lambda + {}^1/_2\left(\frac{d^2\epsilon}{d\lambda^2}\right)(\Delta\lambda)^2 + {}^1/_6\left(\frac{d^3\epsilon}{d\lambda^3}\right)(\Delta\lambda)^3\right]C \quad (4)^2$$

be seen from this equation that an increase in C does not produce the same effect as a corresponding increase in $\Delta\lambda$. Thus, any given finite value of $\Delta\lambda$ will produce a uniquely shaped difference spectrum, as shown in Figure 2.[3] Since $\Delta\lambda$ can be uniquely determined from a given difference spectrum, and since ϵ_λ is known from the absolute spectrum of the chromophore itself, it follows that C, the concentration of the chromophore involved in the shift-producing interaction, can also be uniquely determined and the larger the value of $\Delta\lambda$, the smaller will be the error in its determination. This property will be of some use in interpreting our resolutions.

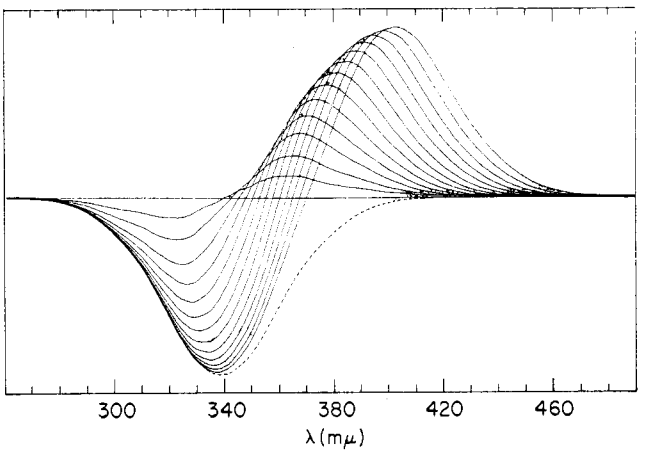

FIGURE 2: Difference spectra generated on a curve resolver by successive 5-mμ red shifts of a gaussian curve fitted to the 340-mμ portion of a DPNH spectrum, measured against an unchanged reference spectrum of DPNH (shown by the dotted line). Several general features may be noted. (1) The envelope of negative limbs, generated by the reference spectrum, corresponds to one edge of that reference spectrum; no such feature occurs anywhere in the positive limb, generated by the shifting "sample" spectrum. (2) The null point of the difference spectrum moves progressively in the direction of the shift and does so in a linear fashion; it occurs at $\lambda_0 + \Delta\lambda/2$ *only* for symmetrical peaks. The slope of the difference spectrum at the null point increases as $\Delta\lambda$ increases. (3) The absolute heights of the limbs of the difference spectra also increase with $\Delta\lambda$, and are almost proportional to $\Delta\lambda$ for small shifts; however, as $\Delta\lambda$ increases the ΔA increments decrease and the total ΔA for each limb approaches a limit, the value of A for the spectrum itself.

Thus we resolve the 340-mμ region of the enzyme DPNH–coenzyme complex difference spectra using only: (1) a change in the height of a spectrum of DPNH without any change of band width or shape, and (2) the shift to a new wavelength of that whole band—the same two operations we used in our previous paper. However, because of the properties conferred on the difference spectrum by large shifts, our resolution procedure becomes simpler than that used for infinitesimal shifts. The procedure is illustrated in Figure 3. Consider the absorption spectrum of a definite concentration of DPNH, labeled R, as shown by the dotted curve. Let this entire curve be shifted to a different wavelength by an amount $\Delta\lambda$ without any change in shape. Next, multiply the extinction coefficient of this shifted curve at each wavelength by the same constant. These two operations produce the spectrum ER, indicated by the solid line. Subtracting curve R from ER produces the difference spectrum ER–R indicated by the dashed line.

As indicated in the discussion of eq 4 for shifts greater than 5 mμ the resolution of the difference spectrum ER–R into spectra R and ER is unique; for such a difference spectrum plotted on a stated scale of ΔA, the concentration of reduced coenzyme *actually involved* in any interaction which affects its absorption properties is in fact the absolute value of A of the reference spectrum R divided by the value of ϵ at that wavelength of DPNH. (The absorbance of that portion of the DPNH in the cuvet containing ER which is *not* changed, of

[2] We are indebted to Dr. Ronald E. Gates for helpful discussions of this point.

[3] Chervenka (1959) in explaining difference spectra generated by structural changes in protein molecules, constructed theoretical models from 5-Å shift curves of the absolute spectra of tyrosine and tryptophan and compared these with a curve made by carrying out the same operation on a spectrum of chymotrypsinogen. In so doing, he assumed that difference spectra are represented by a function $\Delta\lambda \times d\epsilon/d\lambda$, where $\Delta\lambda$ is a small wavelength shift, and further stated that "essentially the same shape curves are obtained with shifts up to 40 Å with proportional changes in molar extinction." Chervenka noted that the protein difference spectra contained the same features present in the model difference spectra, except that all of the peaks in the protein difference spectrum were shifted some distance toward the longer wavelength end of the spectrum. He explained this discrepancy by observing that amino acid spectral peaks are known to "undergo shifts to longer wavelengths when incorporated into the protein structure" and on this basis shifted the entire model difference spectrum, *which had themselves been generated by just such a shift*, to correct for the discrepancy. There are numerous other cases in the literature of the matching of model difference spectra to protein operational difference spectra by such shifting of the entire model difference spectrum which have been justified on the same basis. Such a procedure would be valid only in the case of a highly perturbed (buried) chromophore undergoing a slight change in its state of perturbation. In the more usual case, the difference spectrum is produced by a large shift and, if properly generated, neither possesses nor requires the arbitrary freedom of movement along the wavelength axis. This illustrates the importance of the demonstration in Figure 2 of this paper that contrary to Chervenka's models, only very small shifts produce difference spectra of the same shape; and that as the shifts increase in magnitude, the peaks of the difference spectra move in the same direction, but with a change in shape and a progressive *decrease* in $d\epsilon/d\lambda$. The pertinence (and usefulness) of this characteristic will be seen in the resolutions presented below, since the difference spectra obtained can be resolved simply only by use of large shifts and not by a multiplication of the intensity of small shifts.

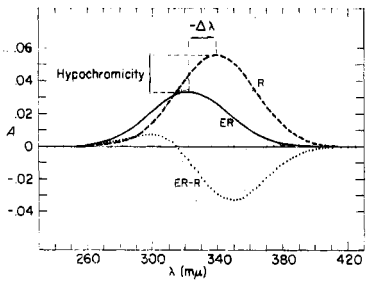

FIGURE 3: Illustration of the procedure for resolution of an enzyme–reduced coenzyme difference spectrum. (R) Spectrum of reduced coenzyme in solution, (ER) spectrum produced by the operations $-\Delta\lambda$ and hypochromicity, and (ER–R) difference spectrum resulting from the subtraction of R from ER.

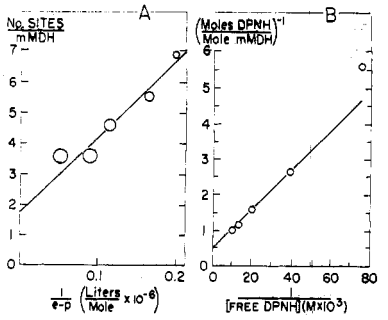

FIGURE 4: The spectrophotometric titration of mitochondrial malate dehydrogenase with DPNH. Part A is a Klotz plot as modified by Stockell (1959), where d/p is plotted *vs.* $1/(e-p)$ from the equation $d/p = (K'_{DPNH})/(e-p) + n$, where d = molar concentration of DPNH, $e = (\Delta A_{374-323\ m\mu})/(\Delta A_{374-322\ m\mu}) \times e$, e = molar concentration of the enzyme (mol wt 65,000), K' = apparent dissociation constant of bound DPNH, and n (ordinate intercept) = moles of DPNH bound per mole of enzyme. Part B is a double-reciprocal plot of the moles of DPNH required for resolution of the reduced nicotinamide portion of the enzyme–coenzyme difference spectrum per mole of enzyme *vs.* the concentration of free DPNH. The intercept on the ordinate is the reciprocal of the total moles of coenzyme bound per mole of enzyme.

course, balances an equivalent amount in the reference cuvet. This amount of DPNH, being unchanged by definition, makes no contribution to either the difference spectrum itself, or either of the component spectra.)

RESULTS OF RESOLUTIONS. The resolutions of the nicotinamide region of the difference spectra of enzyme–reduced coenzyme complexes for 11 different dehydrogenases using the procedures just described are listed in Table I. Summation curves, calculated by subtracting a spectrum of DPNH of the concentration listed from an identical spectrum of DPNH shifted in the amount and direction shown and increased or decreased in absorbance by the percentage hyper- or hypochromicity, are shown by the dotted curves in Figure 1 along with the experimental difference spectrum for each enzyme. The deviation between the summations and experimental curves in the 310–330-mμ region for several of the difference spectra is due to spectral contributions from enzyme and adenine chromophores. Such contributions from adenine chromophores have been discussed in a previous paper (Cross and Fisher, 1969). In all cases the concentration of coenzyme involved which appeared to be required for resolution of the experimental difference spectrum was either equal to or less than the total concentration actually present in the experiment. These resolutions are unique within the limits of error in Table I except for liver alcohol dehydrogenase–DPNH which has two possible resolutions, one of which gives a $\Delta\lambda = -2$ mμ and an A_{340} involved which is three times greater than that of the coenzyme present, and therefore was not used. The use of the resolution data is shown in Figure 4. Figure 4A shows the stoichiometry of binding of DPNH by mitochondrial malate dehydrogenase (mol wt 65,000) using the dependence of the total reduced nicotinamide difference spectra and Figure 4B shows the same stoichiometry using the amount of coenzyme involved as determined by resolution of the same difference spectra. While the 260- and 280-mμ regions of these difference spectra are, in theory, capable of resolution in the same manner, multiple transitions and mutual interference by different chromophores do not permit resolutions of the same rigor as those we have presented for the 340-mμ band.

Discussion

The shift of the reduced nicotinamide absorption peak of DPNH in combination with a specific dehydrogenase has long-been considered an exclusive property of only three such enzymes, liver alcohol dehydrogenase, beef liver lactate dehydrogenase, and malate dehydrogenase. The demonstration of such difference spectra from the complexes of the three dehydrogenases we reported recently, the five new ones described in detail here, as well as two additional complexes we have not listed (17-β-hydroxysteroid dehydrogenase and muscle glyceraldehyde 3-phosphate dehydrogenase) now make it clear that such shifts of the absorption spectrum of DPNH are a quite general consequence of the formation of dehydrogenase complexes.

Table I shows that the resolved difference spectra fall into two distinct groups. All of the difference spectra in one group are characterized by rather large red-shift components. Those in the other group contain blue shifts of varying size. It is clear that the group of red-shifting dehydrogenases corresponds to the group of dehydrogenases previously characterized as B stereospecific, while the group of blue-shifting dehydrogenases corresponds to the group of A stereospecific dehydrogenases with the possible exception of mitochondrial malate dehydrogenase.[4]

[4] The assignment of A stereospecificity to L-malate dehydrogenase rests solely on two investigations: one by Loewus *et al.* (1955) on a preparation from wheat germ and another on a Straub pig heart preparation by Graves *et al.* (1956). Since that time it has been shown that both pig heart malate dehydrogenase (Chan and Schellenberg, 1968) and the bovine heart enzyme (Siegel and Englard, 1962) each consist of a mixture of two enzymes, a mitochondrial and a supernatant malate dehydrogenase. This work has been reviewed by Kun (1963). The mitochondrial and supernatant enzymes resemble each other sufficiently to have caused controversy up to the present day and there are important differences between the two. Despite the vast amount of effort that has been expended to distinguish between these two enzymes, there has been no determination of the A, B stereospecificity of any purified preparation. The original Straub pig heart preparation probably contained both forms of the enzyme, and the results do not suggest the presence of significant amounts of a B stereospecific enzyme as suggested by the difference spectrum of a Straub preparation reported here. This would suggest a single exception to the relationship we have proposed, of blue shifts from A enzyme and red shifts from B enzyme. It has been

While the resolutions make the distinction between these two groups of dehydrogenases more obvious, it is apparent even from the unresolved difference spectra themselves (Figure 1) that the upper group of spectra are (in the longer wavelength region) rough inversions of each other. Thus it would appear possible to predict the stereospecificity of a dehydrogenase from the difference spectrum of its reduced coenzyme binary complex alone.[5]

Because of the extremely general nature of the causes of spectral shifts, it is difficult to determine a specific mechanism from a general survey of difference spectra such as we present in this paper. However, the magnitude of the shifts (particularly the red shifts) does permit us to at least exclude some possibilities. In a previous paper (Cross and Fisher, 1969) we have shown that changes in conformation of the DPNH molecule give rise to difference spectra resolvable (in the 340-mμ region) into simple red or blue shifts and hypo- or hyperchromicities. While the difference spectra generated by combinations of DPNH with dehydrogenases can be resolved into combinations of the same components required to resolve difference spectra generated by changes in coenzyme conformation, the enzyme-induced red shifts are, in general, so very large that any signal due to changes in coenzyme conformation alone would generally be masked. The specific causes of these large shifts, then, must be sought in phenomena other than the changes in conformation of the reduced coenzyme.

Thus, the large red shifts could be caused by a burying of the nicotinamide ring in a hydrophobic pocket in the enzyme, by the binding of one face of that ring to an extremely close fitting and very nonpolar patch on the enzyme surface, or even by the close proximity in the binary complex of an electron donating group. On the other hand, among the group of blue-shifting enzymes, the shifts caused by lactate, isocitrate, supernatant malate, and possibly yeast alcohol dehydrogenases are 5 mμ or less—the same magnitude as that found when the average conformation of DPNH is changed to the open form (Cross and Fisher, 1969). The small blue shift observed in the binding of DPNH by lactate dehydrogenase could be accounted for by the binding of the coenzyme in an open conformation as described by Velick (1961) using polarization fluorescence. Only one enzyme, liver alcohol dehydrogenase, has been found to produce a large blue shift. This shift (-14 mμ) cannot be accounted for by general environmental changes and indicates a change in electronic configuration, possibly through the participation of an electron-withdrawing group as discussed by Kosower (1962).

Whatever the specific nature of the causes of these red and blue shifts, since they are closely linked to the stereospecificity of the transfer of one of two hydrogen atoms on the C-4 position of the reduced nicotinamide ring, they must, by that very fact, reflect entities able to produce electron shifts in the nicotinamide chromophore; and these entities must have a very well-defined spatial position very close to the nicotinamide binding site itself.

All of the dehydrogenase–DPNH binary complex difference spectra showed hypochromisms of amounts varying from 2 to 44% with the single exception of mitochondrial L-malate dehydrogenase, whose DPNH complex showed a hyperchromism of 10%. Aside from the direction of shift of the 340-mμ peak; no other feature of the difference spectra (shifts of the 260-mμ peak, hypochromicity of either peak, or protein chromophore signals) shows any correlation with stereospecificity.

In a previous section we have shown that sufficiently large shifts of an absorption band produce difference spectra unique for some single and determinate concentration of the chromophore-containing molecule. The demonstration that the resolution data of the mitochondrial malate dehydrogenase–DPNH difference spectra in Figure 4 shows two binding sites in agreement with Thorne and Cooper (1963) and Pfleiderer and Auricchio (1964), and the demonstration that the resolutions of the other dehydrogenase–coenzyme difference spectra in Table I show amounts of coenzyme involved in binding less than the one per 40,000 molecular weight, generalized for all dehydrogenases by Pfleiderer and Auricchio (1964), is of rather special significance. We believe that this is the only procedure devised up to now that permits such a calculation. All other procedures fall into one of two groups. All "binding" measurements, including direct methods, such as equilibrium dialysis, and methods which depend upon the mass law effect of free chromophore, such as Klotz plot measurements, can determine only the *total* amount of chromophore bound and must by their nature include all bound forms, whether they all contribute to a given signal or not. Other methods which rely on a specific signal, spectrophotometric or otherwise, require some assumption of a proportionality constant (such as a differential extinction coefficient); such values are not generally obtainable directly whereas this method uses the extinction coefficient of the unperturbed coenzyme. (It is precisely this problem which limits our interpretation of the DPNH difference spectra we reported in the previous paper; while the signals themselves were quite large, they were resolvable by very small shifts, which lack the uniqueness we stress here.) Thus where large shifts do occur, as in most of these enzyme–coenzyme interactions, the resolution procedure we have described permits the calculation of the concentration of a chromophore actually involved in producing that particular phenomenon, and thus may be very useful in distinguishing between different types of binding sites and the number of ligands bound to each.[6]

reported, however, by Wolfe and Neilands (1956), by Alberty (1956), and by Davies and Kun (1957) that at high concentrations of oxalacetate, malate dehydrogenase is strongly inhibited and most particularly the mitochondrial form. Thus, the experiment of Loewis *et al.* contained sufficient oxalacetate to inhibit the mitochondrial enzyme, and the results might then reflect the stereospecificity of the supernatant enzyme alone. We have shown here that the supernatant enzyme is indeed a "blue-shifting" enzyme as are the other A enzymes. Thus, the stereospecificity of L-malate dehydrogenases must be considered as being at least somewhat uncertain at present.

[5] The difference spectrum will reflect all coenzyme binding, while the stereospecificity will, of course, depend upon only coenzyme bound at the active site. Thus, secondary binding sites could cause confusion in making such assignments. Thus far, however, all secondary site binding which we have observed has involved only the adenine portion of the reduced coenzyme molecule; this has made the relationship we have stated quite obvious from inspection. However, the possibility of nicotinamide involvement at secondary sites must be kept in mind.

[6] There are two theoretical limitations to this calculation. If an equilibrium exists between two forms of a molecule whose spectra differ, and if the binding to an enzyme results in a bound form whose spectrum closely resembles that of one of the free forms, then the calculation we have described provides the concentration of the isomer actually converted and not the total amount bound. DPNH has in fact been shown to exist as just such an equilibrium system (Cross and Fisher, 1969; Jardetzky and Wade-Jardetzky, 1966; Czerlinski and Hommes, 1964; Sarma *et al.*, 1968). However, in all of the cases de-

The 260–290-mμ region of the spectra contains contributions from tyrosyl and tryptophanyl residues of the enzyme and from at least two electronic transitions of the adenylate moiety of the coenzyme. This multiplicity of absorption components precludes resolutions of the binary complex difference spectra in this region of the same rigor as that employed for the simpler 340-mμ band. Nevertheless, we can identify certain general features of the difference spectra.

For example, in an earlier paper (Fisher and Cross, 1966) we have already noted the much larger contribution of the adenylate moiety to the difference spectrum of the GDH[7]–DPNH complex as compared with that of the GDH–TPNH complex. This feature of the GDH–DPNH difference spectrum appears to contain at least a red shift and hypochromicity of the adenylate 260-mμ peak. Concentration dependence and inhibition studies (to be reported in detail elsewhere) show that most of the signal in the 260-mμ region of this difference spectrum is due to a secondary binding site not present in the corresponding GDH–TPNH difference spectrum. Pantaloni and Iwatsubo (1967) showed that ADP does bind to GDH and produces a signal in the 260-mμ region quite similar to that of the GDH–DPNH binary spectrum shown in our earlier paper (Fisher and Cross, 1966).

In the case of the alcohol dehydrogenases, both of which produce a blue shift of the 340-mμ band, the yeast enzyme appears to produce a red shift and hypochromicity of the 260-mμ peak. The 260-mμ region of the liver enzyme difference spectrum, on the other hand, consists mostly of a small unresolved signal. Thus it can be seen that there is no general relationship between the appearance or algebraic sense of 260- and 340-mμ signals in the dehydrogenase-reduced coenzyme binary difference spectra.

Many, but not all, of these difference spectra contain small sharp peaks in the 275–295-mμ region. These are sometimes identifiable as blue or red shifts of tyrosyl and tryptophanyl residues of the enzymes themselves. One such interaction (in a ternary enzyme–reduced coenzyme–reduced substrate complex) has been described previously (Fisher and Cross, 1965).

We have, then, reached the limit of our general description of features of these phenomena which are common to the dehydrogenases as a group. Some of these enzymes are known to possess secondary coenzyme binding sites; the difference spectra we have shown may be due to binding only at the active site (or sites), to binding only at a secondary site, or to binding at both kinds of sites. Having reached this point it will now be more profitable to consider the resolved components of various difference spectra generated by one specific dehydrogenase system in a more detailed way, further separating the components by their concentration dependence, by the kinetics of their formation, and their pH dependence, and relating such information to the kinetic properties of the system. A beginning to such a study for bovine L-glutamate dehydrogenase will be described in the near future.

scribed here with the exception of a few of those enzymes producing small shifts, the signals produced by enzyme binding are so much larger than those due to coenzyme conformer equilibrium that no problem exists. Secondly, if there were two separate binding sites, one of which caused *only* a hypochromicity without a shift, that too would produce a lower calculated value of the concentration of coenzyme involved.

[7] Abbreviation used is: GDH, glutamate dehydrogenase.

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Two-Stage Photosensitive Label for Antibody Combining Sites*

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ABSTRACT: Dinitrophenylglycine diazoketone, dinitrophenyl alanine diazoketone, and their tritiated analogs were synthesized and used to label, in two steps, the combining sites of rabbit antidinitrophenyl antibodies. Diazoketones are unreactive with proteins, but when photolyzed, they yield carbenes and by rearrangement, ketenes. Such derivatives are reactive with most amino acid side chains. The dinitrophenylamino acid diazoketones enter the antidinitrophenyl site. Excess reagent may then be removed. The diazoketones are then photolyzed within the site, using light of 300–400 mμ. Up to 47% of the sites are covalently labeled. The label is chiefly on the γ-globulin heavy chains, and is limited to the Fab fragments.

The effectiveness of any covalent label for the reactive site of a protein is limited by (i) its ability to localize at the active site and (ii) its covalent reactivity with nearby amino acid residues. One label which has been used for antibodies is the diazonium affinity label (Wofsy *et al.*, 1962, 1967; Metzger *et al.*, 1963a), which has yielded information on those peptides in the combining site containing tyrosine or histidine (Singer *et al.*, 1967; Metzger and Potter, 1968; Koyama *et al.*, 1968).

We have developed another antibody labeling reagent which potentially can react with most amino acid residues (Vaughan and Westheimer, 1969). This reagent, a DNP-diazoketone, enters the anti-DNP combining site. Once in the site, it is converted into reactive carbene and ketene derivatives by ultraviolet irradiation.

Evidence for the synthesis of these labels and their ability to react specifically with the anti-DNP combining site is presented in this paper.

Materials and Methods

Anti-DNP-BGG.[1] New Zealand white male rabbits were immunized in all four footpads with 1 ml of complete Freund's adjuvant containing 5 mg of DNP-BGG (38 moles of DNP/mole of γ-globulin) and 6 mg of desiccated mycobacterium tuberculosis. They were boosted at 5 weeks after immunization with 0.5 mg of alum-precipitated DNP-BGG in PBS in the marginal ear veins. Anti-DNP-BGG was purified from sera of a single animal at 8 and 12 weeks by the method of Farah *et al.* (1960). Fluorescence quenching measurements (Aminco-Bowman or Farrand Mark I spectrofluorometers) gave Q_{max} = 65–75% (uncor) with ε-DNP-lysine (Eisen, 1964). Equilibrium dialysis with [^{3}H]ε-DNP-lysine (Richards *et al.*, 1969) gave an association constant, K_A, = 1.1×10^8 l./mole at 8 weeks. At 12 weeks, $K_A = 1.1 \times 10^8$ l./mole for both [3,5,-6-^{3}H]DNP-D-alanine and [3,5,6-^{3}H]DNP-L-alanine. In the experiments described below, the 8-week anti-DNP-BGG preparation was employed.

DNP-glycine Diazoketone. FDNB (20 ml) in 30 ml of absolute ethanol was added to 10 g of glycine and 42 g of Na_2CO_3 in 250 ml of water and stirred in the dark for 2 hr. The precipitate was washed with ether, redissolved in water, and acidified to pH 1.0 to reprecipitate the DNP-glycine. The infrared spectrum (KBr disk, Perkin-Elmer Model 257 grating infrared spectrophotometer) and melting point after recrystallization from 50% methanol agreed with the literature (Kimmel and Saifer, 1964; Fraenkel-Conrat *et al.*, 1955). A single component was detected on polyamide thin-layer chromatography in solvent systems I and II,[2] paper chromatography (*t*-amyl alcohol saturated with pH 6.0 sodium phthalate buffer), and electrophoresis at pH 1.8, 3.0, and 6.0 (Evered, 1960).

To make the acid chloride, 0.25 g of DNP-glycine was dissolved in 300 ml of dry benzene and 1.0 ml of $SOCl_2$ in 20 ml of benzene was added slowly. The mixture was refluxed for 2 hr and then stored at −20° overnight. The $SOCl_2$ was redistilled (Vogel, 1956) and then could be stored without decomposition in glass-stoppered bottles in a dry box at −20°.

DNP-glycine acid chloride is readily hydrolyzed to DNP-glycine (identified by polyamide thin-layer chromatography in solvents I and II; by electrophoresis at pH 6.0; and by the infared spectrum (Kimmel and Saifer, 1964)). Therefore to determine the yield, the acid choride was used to acylate excess rosaniline acetate, a red amine dye. It was shown that all of the DNP-glycine acid chloride reacts with rosaniline ace-

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[1] Abbreviations used are: BGG, bovine γ-globulin; FDNB, 1-fluoro-2,4-dinitrobenzene; PBS, 0.01 M phosphate buffer (pH 7.4)–0.15 M in respect to NaCl; Q_{max}, maximum quenching of fluorescence.

[2] Solvent I, benzene–glacial acetic acid (80:20, v/v); solvent II, 1-butanol–glacial acetic acid (90:10, v/v).