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Fluorescence of Pyridine Nucleotides in Mitochondria*

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In mitochondria, the role of the pyridine nucleotides in electron transport and oxidative phosphorylation depends undoubtedly on the over-all structure of the particles and on the state of the pyridine nucleotides within the structure. Circumstantial evidence suggested a strong binding of diphosphopyridine nucleotide (DPN) (1, 2) and even firmer binding of DPNH in the mitochondria (3). Chance and Baltscheffsky (4) observed that the peak of the fluorescence emission of the reduced pyridine nucleotides in liver mitochondria is at 440 mμ, compared with 460 mμ for reduced pyridine nucleotides in solution. By analogy to similar shifts that are known to occur upon binding of DPNH to soluble enzymes (5), they interpreted their observation as an indication that DPNH is in the bound state in rat liver mitochondria. The state of reduced triphosphopyridine nucleotide (TPNH), which is the predominant type of reduced pyridine nucleotide in rat liver mitochondria (6), still remained obscure.

The present work is a further investigation of the state of DPNH and TPNH within the mitochondria. Fluorescence emission and excitation spectra of mitochondrial reduced pyridine nucleotide have been studied. The relative fluorescence yield of the total reduced pyridine nucleotide compared with that of DPNH or TPNH in solution has been determined, and the difference between the yields of DPNH and TPNH within the mitochondria has been estimated.

EXPERIMENTAL PROCEDURE

Materials—Sucrose (analytical grade); sodium succinate (purified); DL-β-hydroxybutyrate (Fisher Scientific Company); DPNH (98 to 100% purity); TPNH (type II, approximately 90% purity); phenazine methosulfate (Sigma Chemical Company); and yeast alcohol dehydrogenase (California Corporation for Biochemical Research) were the principal reagents. Other reagents used were chemically pure grade. TPN-specific glutamic dehydrogenase was prepared from bakers' yeast.[1]

Preparation of Mitochondria—Female rats weighing 200 to 250 g were decapitated. The liver or the demedullated kidney was forced through a metal tissue press and homogenized in 10 volumes of 0.25 M sucrose buffered with 0.05 M Tris, pH 7.4, containing 0.01 M β-hydroxybutyrate. The mitochondrial fraction was separated as described by Schneider and Hogeboom (8). Sucrose (0.25 M) buffered with 0.05 M Tris, pH 7.4, was used for washing and resuspension of the mitochondria. The final suspension contained approximately 40 mg of protein per ml.

Measurement of Fluorescence—1. Apparatus: Fluorescence emission spectra were determined by means of a fluorometer based on the design of Duysens and Amesz (9) for use with turbid suspensions. For excitation, the 334 mμ line from a mercury lamp (Hanovia AH-8) was isolated by a Schott interference filter. Partial transmission of the powerful 366 mμ Hg line by the filter contributed approximately 5% to the excitation of DPNH fluorescence. Fluorescent emission was collected from the same side of the sample that received the excitation beam and passed through a blue filter (Corning 9782) and a Bausch and Lomb grating monochromator (focal length, 500 mm). The fluorescence intensity in the spectral interval set by the monochromator was registered by a photomultiplier (Dumont 6292). Photomultiplier anode current was measured by a General Radio direct current amplifier and electrometer and recorded on a recording milliammeter (Esterline Angus or Texas Instruments). The over-all spectral sensitivity of the filter-monochromator-photomultiplier combination was determined and applied as a correction to express the emission spectra in units of relative energy per mμ (9). For reduced pyridine nucleotide, the fluorescence signal was proportional to the concentration from 10^{-6} M to 10^{-4} M. The sensitivity at 460 mμ in terms of anode current/(reduced pyridine nucleotide) × (band width)^2 is approximately 2×10^{-4} amperes M^{-1} $m\mu^{-2}$ with 1200 volts applied to the photomultiplier.

Fluorescence excitation spectra were recorded by a spectrophotofluorometer[2] that utilized the same geometry for excitation and collection of fluorescence as described above for the fluorometer used for emission spectra. The arc of a high pressure Xenon lamp (Osram XBO 162) was imaged on the entrance slit of a Bausch and Lomb grating monochromator (focal length, 250 mm). The excitation beam emerging from the exit slit was passed through an ultraviolet transmitting filter (Corning 9863) to remove stray light and was then directed onto the sample. The fluorescent emission from each sample of mitochondria was passed through an interference filter (Baltzer, 440 mμ) and a second monochromator to remove scattered excitation light. An RCA 6217 photomultiplier served as a detector. All excitation

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[1] The TPN-specific glutamic dehydrogenase used in these studies was separated from a DPN-linked glutamic dehydrogenase that was also present in yeast extracts. The details of this method and the properties of these two enzymes will be described elsewhere (7).

[2] Constructed by H. Linschitz and J. M. Olson. Details of this instrument will be published elsewhere.

spectra were corrected to show relative fluorescence per unit of quantum intensity of excitation. The spectral distribution of quantum intensities for the excitation beam was determined by scanning the fluorescence signal (520 mμ) of 8-amino-1,3,6-naphthalenetrisulfonic acid (2×10^{-2} M in 0.02 N NaOH) over the excitation range of 240 to 400 mμ (10).

2. Conditions of measurement: The experiments were conducted aerobically. (At the highest mitochondrial concentration used in the assay, the oxygen pressure did not decrease by more than 10% of the original level during the time of measurement.) Samples of 0.5 ml were pipetted into a 2-mm thick quartz cuvette, which was placed in a cell holder cooled by circulating ice water that held the sample at approximately 2°. This low temperature was used throughout our work to avoid changes in fluorescence during the measurement of the fluorescence emission spectrum caused by a decay of the mitochondrial reduced pyridine nucleotide. Low temperature also slows down the equilibration between TPNH and DPNH presumed to occur through the mitochondrial pyridine nucleotide transhydrogenase. Thus, it becomes possible to oxidize enzymatically most of the DPNH without removing much of the TPNH. The marked effect of the temperature on the transhydrogenase can be explained plausibly by the finding made in this laboratory[3] that below 20° the activation energy of the TPNH-DPN transhydrogenase increases considerably. The difference in temperature coefficient between the system oxidizing DPNH and the transhydrogenase might account for the higher level of TPNH in isolated mitochondria.

Analytical Methods—For the assay of reduced pyridine nucleotide, the mitochondrial suspension (7 to 10 mg of protein per ml) was heated in a boiling water bath for 3 minutes with an equal volume of preheated 0.2 M Na_2CO_3. The alkali extract was then divided into three parts. In the first aliquot, DPNH and TPNH were oxidized with phenazine methosulphate (11); in the second, only DPNH was oxidized by yeast alcohol dehydrogenase with acetaldehyde as the hydrogen acceptor (12); and in the third, TPNH was selectively oxidized with a purified TPNH-specific glutamic dehydrogenase from yeast in the presence of α-ketoglutarate and NH_4Cl. After destruction of the nonoxidized nucleotides by acid treatment, the pyridine nucleotide in each aliquot was determined by fluorometric assay of the methyl ethyl ketone addition compound (12). The error in the assay method was less than ±0.1 mμmole of reduced pyridine nucleotide per mg of protein.

Protein was determined by the biuret method (13) after solubilization of the mitochondria with 3% sodium cholate.

Determination of Comparative Fluorescence Yield of Mitochondrial Reduced Pyridine Nucleotide—The fluorescence yield of the mitochondrial reduced pyridine nucleotide compared with that of reduced pyridine nucleotide in aqueous solution was determined in two ways.

Method 1: The fluorescence of a mitochondrial suspension of known protein content was measured at the peak of fluorescence emission spectrum and at 400 mμ, and the difference (I) was calculated. The sample was then analyzed for reduced pyridine nucleotide content, the fluorescence of a DPNH or TPNH solution of equivalent concentration was measured at 460 mμ and 400 mμ, and again the difference (II) was calculated. All of the conditions for the measurement of the mitochondrial fluorescence and of the fluorescence of the DPNH or TPNH solution were identical. Comparative fluorescence yield was calculated by dividing I by II.

Method 2: The fluorescence emission spectrum of the mitochondrial suspension was measured as in the first procedure. The reduced pyridine nucleotide was then removed from the mitochondria by Na_2CO_3 treatment, as described in "Analytical Methods." The separated reduced pyridine nucleotide-free mitochondria could be easily resuspended in the original volume of the suspending medium, and the fluorescence emission spectrum was measured again. The difference between the first and second measurements represents the fluorescence emission spectrum of the mitochondrial reduced pyridine nucleotide (III). The fluorescence emission spectrum of the alkali extract is not characteristic of the reduced pyridine nucleotides alone because the Na_2CO_3 liberates other material, perhaps flavin, that contributes very little to fluorescence in the mitochondrial state but becomes much more fluorescent after liberation. Therefore, after the fluorescence emission spectrum of the Na_2CO_3 extract was determined, the total reduced pyridine nucleotide was oxidized with beef liver glutamic dehydrogenase, which reacts with both TPNH and DPNH, plus α-ketoglutarate and NH_4Cl. After completion of the oxidation, the fluorescence emission spectrum was again determined. The difference between the last two spectra corresponded to the fluorescence emission spectrum of the reduced pyridine nucleotide liberated from the mitochondria (IV), and III divided by IV defines the comparative fluorescence yield.

In these measurements the assumption was made that reduced pyridine nucleotide has the same molar absorbancy index at 334 mμ in the mitochondria as in solution.

RESULTS

Effect of Variation in Reduced Pyridine Nucleotide Level and DPNH to TPNH Ratio on Fluorescence Emission Spectrum

According to Klingenberg, Slenczka, and Ritt (14), liver has the highest mitochondrial (TPN + TPNH)/(DPN + DPNH) ratio (mean value, 1.7) of all tissues. Kidney mitochondria, on the other hand, have a low (TPN + TPNH)/(DPN + DPNH) ratio (0.19). Both types of mitochondria have been used in the present work. The extent of reduction of the mitochondrial pyridine nucleotide could be increased by the addition of succinate or β-hydroxybutyrate, which can serve as hydrogen donors. Stepwise oxidation of the reduced pyridine nucleotide was effected through the mitochondrial enzymes. A specific hydrogen acceptor such as oxaloacetate was added for oxidation of DPNH by malic dehydrogenase, and α-ketoglutarate plus NH_4Cl were used for the oxidation of TPNH and DPNH by glutamic dehydrogenase.

Effect of Succinate and β-Hydroxybutyrate—It is apparent from Figs. 1 and 2 that in both liver and kidney mitochondria isolated by our procedure, the reduced pyridine nucleotide present is predominantly TPNH. After the addition of succinate, a new steady state level of pyridine nucleotide reduction was reached in a few minutes. In the experiment shown in the figures, the succinate-induced reduction resulted in an increase of 70% of the total reduced pyridine nucleotide in liver and an increase of 190% in kidney mitochondria. Correspondingly, the intensity

[3] Unpublished studies carried out in collaboration with Mr. Charles Allen of this laboratory.

of the fluorescence as measured at the peak increased by 80% in liver mitochondria and by 140% in kidney mitochondria. The changes in the DPNH content were from 8% of the total reduced pyridine nucleotide to 32% in liver and from 15% to 53% in kidney. Fig. 1 reveals no change in the shape of the fluorescence emission spectrum or the location of the maximum after the increase in the intensity of the fluorescence. In contrast, the peak of the fluorescence emission spectrum of kidney mitochondria shifts 5 to 10 mμ to a longer wave length after treatment with succinate. Some variation occurred in the initial reduced pyridine nucleotide content, in the DPNH to TPNH ratio, and in the extent of the reduction caused by succinate with various batches of liver or kidney mitochondria.

In accordance with the observation made by other workers (14, 15), β-hydroxybutyrate caused an appreciable reduction of pyridine nucleotide under aerobic conditions in liver mitochondria but not in kidney mitochondria. When experiments similar to that shown in Fig. 1 were conducted with the same preparation of liver mitochondria with β-hydroxybutyrate instead of succinate, the shape of the fluorescence emission spectrum after reduction and the location of the peak were the same as in Fig. 1, although the extent of increase in reduced pyridine nucleotide and in the intensity of the fluorescence was only 30 to 70% as great as that obtained with succinate.

Stepwise Oxidation of Mitochondrial DPNH and TPNH—After addition of oxaloacetate to rat liver mitochondria in which the DPNH level was increased by previous addition of β-hydroxybutyrate, selective oxidation of DPNH took place. When subsequently α-ketoglutarate and NH_4Cl were added simultaneously, the TPNH concentration dropped to approximately one-third of the initial value (see *bars* in Fig. 3). The absolute fluorescence emission spectra corresponding to the steady states of Fig. 3 are shown in Fig. 4, and the difference spectra reflecting the specific removal of DPNH or TPNH are plotted in Fig. 5. The difference spectra in Fig. 5 are very similar both in shape and in location of the peak.

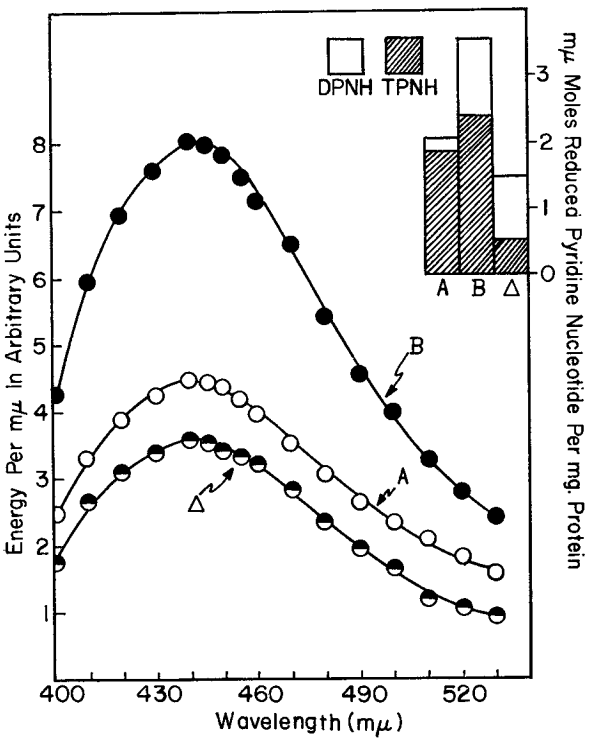

FIG. 1. Effect of succinate on the fluorescence emission spectrum of rat liver mitochondria. The reaction mixture contained mitochondria (9.7 mg of protein per ml) suspended in 0.25 M sucrose buffered with 0.05 M Tris, pH 7.4. *Curve A*, absolute fluorescence emission spectra before addition of succinate; *Curve B*, the same after addition of 8 μmoles per ml of succinate. △, Difference between spectra *A* and *B*. Temperature was 2°. The *bars* in the upper right-hand corner show the reduced pyridine nucleotide composition of the mitochondria in the states corresponding to the three curves

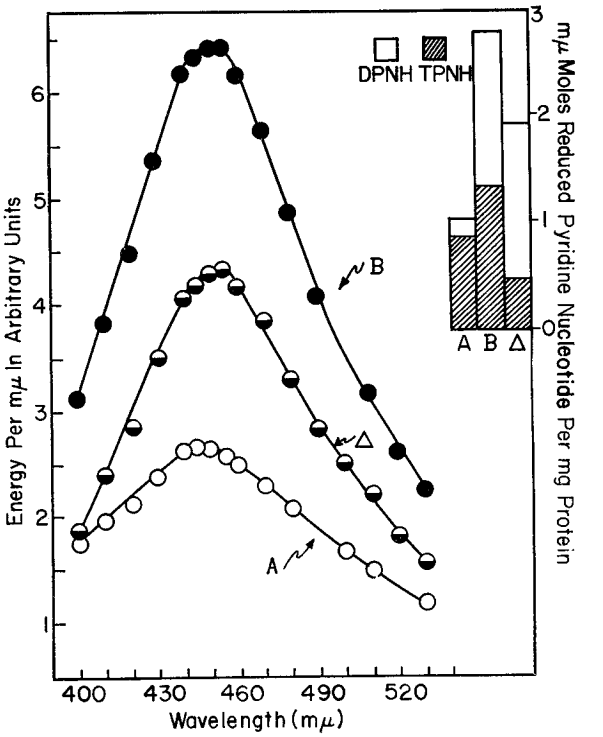

FIG. 2. Effect of succinate on the fluorescence emission spectrum of rat kidney mitochondria. Rat kidney mitochondria were suspended in the same medium as in Fig. 1 (8.8 mg of protein per ml). All of the symbols used and the conditions were as described in Fig. 1.

Comparative Fluorescence Yield of Mitochondrial Reduced Pyridine Nucleotide

From experiments similar to those shown in Figs. 1 and 2, the comparative fluorescence yield was calculated for various states of oxidation of pyridine nucleotides by Method 1 (see "Experimental Procedure"). The over-all comparative fluorescence yield of mitochondrial reduced pyridine nucleotide varied in a range between 6.0 and 8.5 for liver mitochondria (eight experiments) and between 7.0 and 8.0 for rat kidney mitochondria (four experiments). Somewhat lower results (values between 5 and 6) for the comparative fluorescence yield were obtained for rat liver mitochondria by Method 2.

In principle, the comparative fluorescence yield for DPNH alone and TPNH alone can be calculated from the analytical determinations and the fluorescence data for two states of the same batch of mitochondria. In practice, the precision of our analytical methods for assaying mixtures of reduced nucleotides was sufficient to show that the yield for TPNH was less than the yield for DPNH in each of five batches of mitochondria.

We estimate that at 2° the ratio of TPNH yield to DPNH yield probably lies between 0.4 and 0.8.

Effect of Serum Albumin and Lecithin on Fluorescence of Reduced Pyridine Nucleotide

In view of the finding of Fisher and McGregor (16) that nonspecific binding of DPNH may cause a shift in the fluorescence emission spectrum of DPNH toward the blue region and may increase the comparative fluorescence yield, the effect of serum albumin on the fluorescence of TPNH and DPNH has been examined. As seen from Table I, serum albumin causes a blue shift in the fluorescence spectrum of TPNH and of DPNH, which is similar in magnitude to the shift observed for the mitochondrial reduced pyridine nucleotide. Serum albumin also causes the appearance of an excitation peak at 280 mμ that is approximately as high as the 340 mμ peak in the excitation spectrum of TPNH. It is of interest that the increase in comparative fluorescence yield caused by serum albumin is twice as great for TPNH as for DPNH. The effect of serum albumin on the fluorescence of reduced pyridine nucleotide could be observed only at low ionic strength and is not obtained in 0.1 M phosphate buffer. The effect of lecithin on nucleotide fluorescence was also examined in view of the high lipid content of mitochondria. In contrast to serum albumin, lecithin increased the comparative fluorescence yield without causing a shift in the location of the peak of

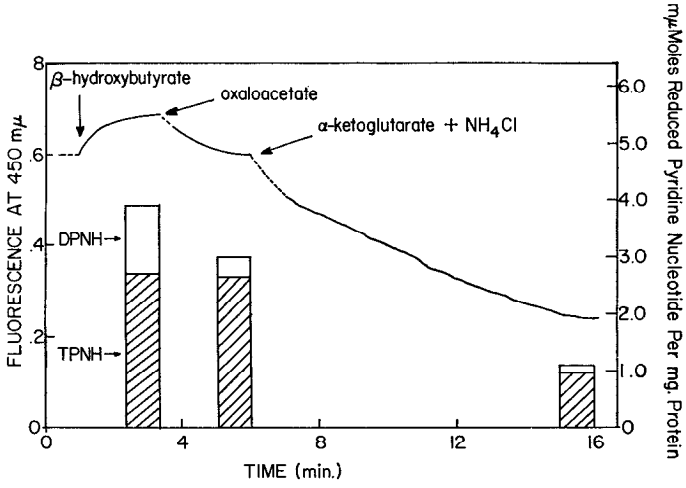

FIG. 3. Effect of stepwise oxidation of reduced pyridine nucleotides of rat liver mitochondria on the fluorescence emission spectrum and on the composition of reduced pyridine nucleotides. The kinetic trace shows the changes in the intensity of fluorescence at 450 mμ when the substrates indicated in the figure were added. The substrates were added, per ml, in the following amounts: DL-β-hydroxybutyrate, 16 μmoles; oxaloacetate, 2 μmoles; α-ketoglutarate, 2 μmoles; and NH_4Cl, 20 μmoles. The *bars* show the reduced pyridine nucleotide composition in the steady states attained after the addition of the substrates. The eaction mixture contained rat liver mitochondria (6.0 mg of protein per ml) suspended in 0.25 M sucrose buffered with 0.05 M Tris, pH 7.4.

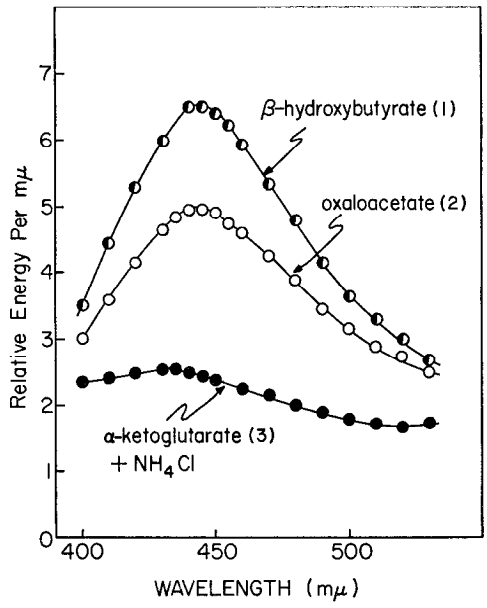

FIG. 4. Absolute fluorescence emission spectra corresponding to the steady states in Fig. 3.

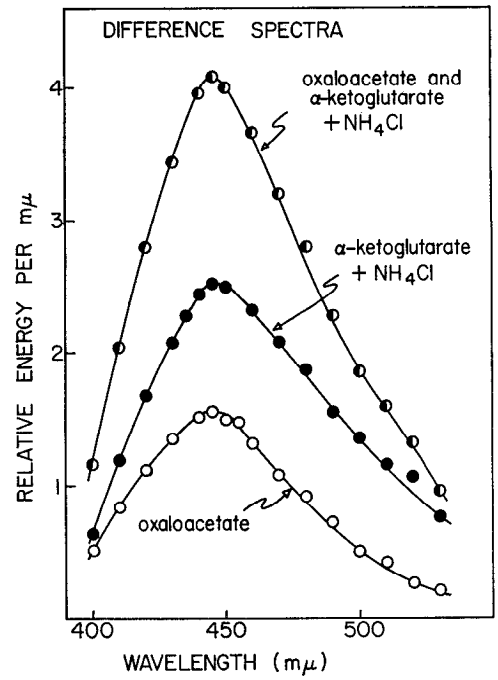

FIG. 5. Difference spectra obtained by plotting the difference between the absolute spectra of Fig. 4. *Curve* ○——○ corresponds to (1) − (2); *Curve* ◐——◐ corresponds to (1) − (3); and *Curve* ●——● corresponds to (2) − (3).

TABLE I

Effect of serum albumin and lecithin on fluorescence emission spectrum of reduced pyridine nucleotide

Serum albumin*	Lecithin†	TPNH	DPNH	Wave length of maximum	Comparative fluorescence yield‡
$\times 10^{-4}$ M	mg/ml	$\times 10^{-5}$ M	$\times 10^{-5}$ M	mμ	
2.5		5.0		445	3.1
2.5			5.0	445	1.5
	6.0	5.0		460	2.2
2.5	6.0	5.0		445	4.2

* Neutralized with KOH to pH 7.0.

† Evaporated from a benzene solution to dryness and emulsified in water.

‡ Measurements carried out at 2°.

TPNH. It is also apparent from Table I that the combined effect of serum albumin and lecithin on the comparative fluorescence yield of TPNH is greater than that of serum albumin or lecithin alone, although the effect is not strictly additive.

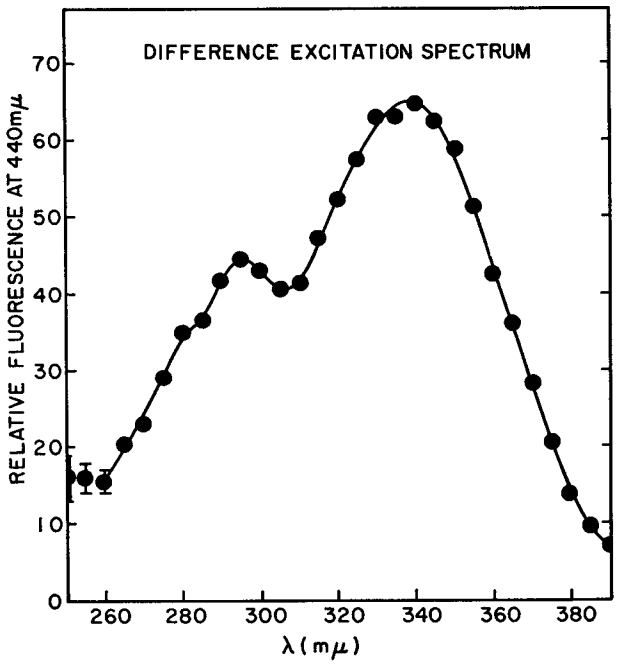

FIG. 6. Increase in excitation spectrum caused by addition of succinate. A mitochondrial suspension (9 mg of protein per ml) was treated with succinate as described in Fig. 1, and the curve represents the difference in excitation spectra before and after succinate treatment. The succinate treatment caused an 84% increase in fluorescence at 345 mμ.

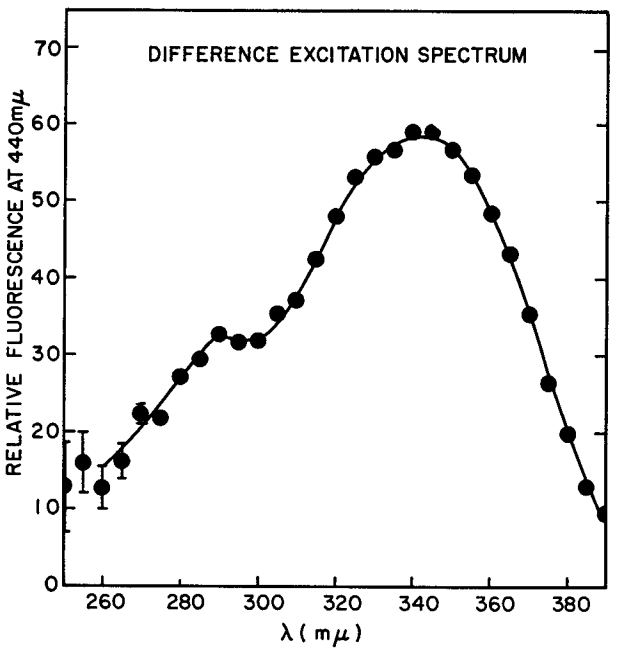

FIG. 7. Decrease in excitation spectrum caused by H_2O_2. A mitochondrial suspension (9 mg of protein per ml) was treated with 10^{-4} M H_2O_2 at 0° for 5 minutes, and excess catalase was added. The curve represents the difference in excitation spectra before and after the oxidation by H_2O_2.

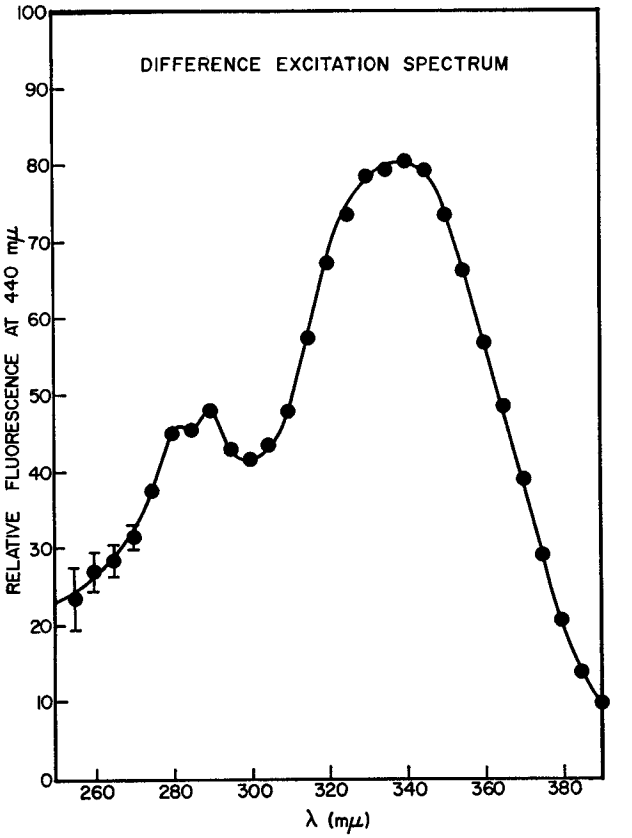

FIG. 8. Increase in excitation spectrum on treatment of H_2O_2-oxidized mitochondria with succinate and β-hydroxybutyrate. Mitochondria were treated as described in Fig. 7, and 8 mM succinate and 16 mM DL-β-hydroxybutyrate were added and incubated until there was no further increase in fluorescence at 340 mμ. The curve represents the difference in excitation spectra before and after reduction.

Excitation Spectra of Mitochondrial Reduced Pyridine Nucleotide

The absolute excitation spectrum of a fresh liver mitochondrial preparation showed approximately equal maxima at 290 and 335 mμ when the fluorescence emission was measured at 440 mμ. Upon oxidation of the mitochondrial reduced pyridine nucleotide by the addition of H_2O_2, the excitation spectrum peaks shifted to 288 and 325 mμ, with the 288 mμ peak approximately 80% higher than the 325 mμ peak. This latter spectrum indicated the probability that fluorescent material other than reduced pyridine nucleotide contributes to the fluorescence emission at 440 mμ in all absolute excitation spectra. The 288 mμ peak undoubtedly is caused by a very high protein fluorescence, of which a small fraction is emitted at 440 mμ.

In order to eliminate the fluorescence contribution of the interfering material, changes in excitation spectrum corresponding to changes in the level of mitochondrial reduced pyridine nucleotide were measured. These difference spectra of fluorescence excitation should, therefore, indicate the characteristics of reduced pyridine nucleotide without significant interference from other fluorescent material present in the mitochondria.

Difference spectra of excitation corresponding to the following changes in reduced pyridine nucleotide level were obtained: (*a*) reduction of pyridine nucleotide by addition of succinate to fresh mitochondria (Fig. 6), (*b*) oxidation of reduced pyridine nucleo-

tide by addition of H_2O_2 to fresh mitochondria (Fig. 7), and (*c*) reduction of pyridine nucleotide by addition of succinate and β-hydroxybutyrate to mitochondria after treatment with H_2O_2 and catalase (Fig. 8).

In each case, the major peak appeared at 340 mμ, and a minor one was found around 290 ± 5 mμ. The ratios of the 290 mμ maximum to the 340 mμ maximum for these three cases were 0.69, 0.55, and 0.60, respectively.

DISCUSSION

Chance (17) has shown that the absorption maximum of DPNH in liver mitochondria is very close to 340 mμ. Our difference spectra of fluorescence excitation indicate that both DPNH and TPNH have maximal absorption at this wave length. There is little reason to suppose that the absorbancy index differs significantly from the value of 6.2 mM^{-1} cm^{-1} for aqueous solution. In agreement with Chance and Baltscheffsky (4), we have found a blue shift in the fluorescence emission spectrum of mitochondrial DPNH, and in addition we have observed the same shift for TPNH. However, in kidney mitochondria, the DPNH formed by the addition of succinate shows only a slight blue shift in comparison with the DPNH of liver mitochondria.

The occurrence of a peak at approximately 290 mμ in the difference spectra of fluorescence excitation demonstrates that energy transfer to reduced pyridine nucleotide takes place in the mitochondria. Most probably, the energy is transferred from some protein component(s). Since the absorbancy of the mitochondria is undoubtedly much higher in the 290 mμ region than in the 340 mμ region, the relatively lower excitation maximum at 290 mμ compared with that at 340 mμ shows that the average efficiency of the energy transfer from all of the proteins containing aromatic amino acid residues is quite low. This low value for the average efficiency may be due to the fact that efficient energy transfer requires an appropriate spatial relationship between donor and acceptor. Probably only a small proportion of the total mitochondrial protein absorbing at 290 mμ has this appropriate spatial relationship to the mitochondrial reduced pyridine nucleotide.

In the excitation spectrum for the fluorescence increase caused by the addition of succinate to fresh mitochondria (Fig. 6), the 290 mμ to 340 mμ ratio is considerably higher than in the excitation spectrum for the fluorescence decrease caused by addition of H_2O_2 (Fig. 7). In the former case, most of the pyridine nucleotide involved is DPNH, whereas in the latter case, mainly TPNH is involved. This suggests the possibility that the efficiency of energy transfer to DPNH may be higher than the efficiency of transfer to TPNH within the mitochondria.

Energy transfer from protein to reduced pyridine nucleotide is often associated with the formation of a specific protein-nucleotide complex in which the protein fluorescence is quenched (18) and the nucleotide fluorescence is enhanced. The appearance of a protein peak in the fluorescence excitation spectra of DPNH bound to yeast alcohol dehydrogenase has been shown (19). However, relatively nonspecific association of protein and pyridine nucleotides, as in the case of serum albumin and TPNH, can also give rise to a protein peak in the excitation spectrum.

Reduced pyridine nucleotide shows an unusually high fluorescence in mitochondria. The comparative fluorescence yield values of 6 to 8 obtained are more than twice the values of 2 to 3 for complexes of DPNH with a number of dehydrogenases (20–22). DPNH in ethylene glycol monomethyl ether (23) has a comparative yield of 4, and the TPNH yield in mixtures of serum albumin and lecithin may go up to 4.2. The substantially higher fluorescence yields observed in mitochondria are probably due predominantly to mitochondrial DPNH. The fluorescence yield of DPNH may be up to 2.5 times as high as the yield of TPNH in mitochondria at 2°, although the exact relationship is still under investigation. Recent data of Estabrook (24) indicate that at room temperature mitochondrial DPNH may have a yield 4 times as high as the yield for mitochondrial TPNH.

It should be emphasized that the shift of fluorescence emission toward the blue does not necessarily accompany an enhancement of fluorescence yield, since each phenomenon reflects a different aspect of interaction between the fluorescent molecule and its environment. This lack of correlation is clearly illustrated in the case of a model compound, 4-dihydro-*N*-methylnicotinamide, dissolved in various solvents (25), and in the case of mixtures of TPNH with serum albumin or lecithin. In this latter case, serum albumin alone causes a shift and enhancement; lecithin causes only an enhancement.

The mitochondrial reduced pyridine nucleotide shows a shift to the blue, an extraordinary yield, and an excitation spectrum indicative of energy transfer from material absorbing in the 290 mμ region. Although the crude model system consisting of reduced pyridine nucleotide, serum albumin, and lecithin also shows the blue shift, high yield, and energy transfer, it differs from the mitochondria at least in one respect. Whereas in the mitochondria, DPNH shows a higher yield than TPNH, the opposite is true for the serum albumin-reduced pyridine nucleotide system. Since TPNH has one more negative charge than DPNH, this difference between the two systems may indicate that in the artificial system mainly electrostatic binding forces are involved, whereas within the mitochondria more specific types of binding are also present in addition to these forces; *i.e.* binding to apoenzymes may occur. The unusually high fluorescence yield of the mitochondrial reduced pyridine nucleotide compared with that found with purified enzymes may be due in part to the influence of the mitochondrial lipids in the system. A number of DPN-linked dehydrogenases are known to be present in mitochondria; however, only isocitric dehydrogenase and the TPNH-DPN transhydrogenase (26) have TPN specificity. As yet, no studies have been carried out on the binding properties of these enzymes isolated from mitochondria.

SUMMARY

The fluorescence emission and excitation spectra of the reduced di- and triphosphopyridine nucleotides (DPNH and TPNH) of rat liver and rat kidney mitochondria have been studied. Variations in the level of the reduced pyridine nucleotides and in the DPNH to TPNH ratio were induced enzymatically, and the accompanying changes in the location of the peak of the fluorescence emission spectrum and of the intensity of the fluorescence were noted.

In both liver and kidney mitochondria, the peak of the fluorescence emission spectrum is shifted by approximately 20 mμ toward shorter wave lengths in comparison with DPNH or TPNH in aqueous solution. In liver mitochondria, the fluorescence emission spectrum is unaffected by a change in the reduced pyridine nucleotide level or by variation in the DPNH to TPNH ratio. In kidney mitochondria, reduction of the DPN by succinate causes a slight shift toward longer wave lengths

Excitation spectra of mitochondrial fluorescence indicate an energy transfer from protein to reduced pyridine nucleotide.

The average fluorescence yield of the reduced pyridine nucleotide in mitochondria is 6 to 8 times the yield of DPNH and TPNH in aqueous solution. The yield of mitochondrial DPNH is substantially higher than the yield of mitochondrial TPNH. Serum albumin increases the fluorescence yield of reduced pyridine nucleotide in solution and in addition gives a blue shift similar in magnitude to that obtained with mitochondria. Lecithin increases the comparative fluorescence yield but fails to cause a blue shift.

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