

# Flavin Mononucleotide: The Coenzyme of Reduced Diphosphopyridine Nucleotide Dehydrogenase\*

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From the particulate reduced nicotinamide adenine dinucleotide (DPNH) oxidase of beef heart, Mackler (3) has obtained a soluble DPNH dehydrogenase which was estimated to be approximately 90 to 95% homogenous when examined electrophoretically.<sup>1</sup> Similarly, when the enzyme was chromatographed on diethylaminoethyl cellulose with phosphate buffer, pH 7.5, in a gradient elution method (0.001 M to 0.01 M), only a single, symmetrical peak with respect to protein and flavin was observed in the elution profile (4). The enzyme contains bound flavin and nonheme iron in the ratio of 1:2. The minimal molecular weight, calculated from the flavin content, is 100,000 which is in general agreement with a value of 80,000 to 90,000 obtained by sedimentation in the ultracentrifuge.<sup>1</sup>

The activity of the enzyme with 2,6-dichlorophenolindophenol as an electron acceptor is 40  $\mu$ moles of DPNH oxidized per minute per mg of protein. With ferricyanide and cytochrome *c* as acceptors, the DPNH oxidizing activities are 40 and 3, respectively, in the same units.

Although this enzyme is similar to other DPNH dehydrogenases described previously (5-11), it is difficult to compare the properties (molecular weight, pH optimum, turnover number, specificity for electron acceptors, etc.) of these various enzymes since, even when prepared from the same tissue, different methods of solubilization have been used. One of the principal discrepancies between the various DPNH dehydrogenases is the nature of the flavin component. It has been reported that only FAD is found in a soluble DPNH dehydrogenase obtained by treatment of beef heart electron transport particle with *Naja naja* venom (7, 9), and FAD has also been identified in a lipoflavoprotein DPNH dehydrogenase from beef heart (12). Earlier, Mahler *et al.* (5) had reported that the prosthetic group of the soluble DPNH-cytochrome *c* reductase from pig heart is a flavin dinucleotide not identical with FAD. On the other hand, we have reported in preliminary communications (1, 2) that FMN<sup>2</sup> is the flavin component of the soluble DPNH dehydrogenase prepared from DPNH oxidase, and King<sup>3</sup> has reached

a similar conclusion with a DPNH dehydrogenase prepared by a different procedure from beef heart (8).

The present paper extends our observations in support of the contention that FMN is the prosthetic group of the beef heart DPNH dehydrogenase. Several analytical techniques have been used to identify and quantitatively determine the flavin released from the enzyme by treatment with heat and acid. No evidence could be obtained to support the possibility that the enzyme contains bound FAD, which is broken down to FMN upon detachment from the protein. The enzyme has been resolved into an apoenzyme and FMN, and activity is restored to the apoenzyme by the addition of FMN, but not of FAD. Finally, various particulate precursors of the soluble DPNH dehydrogenase have been assayed for FAD and FMN and it has been shown that, during purification of the dehydrogenase, there is a progressive enrichment of FMN in the enzyme preparation.

## EXPERIMENTAL PROCEDURE

**Materials**—Chemicals and enzymes were obtained from the following commercial sources: DPN, TPN, DPNH, TPNH, and nucleotide pyrophosphatase (*Aspergillus oryzae*) from California Corporation for Biochemical Research; riboflavin, FMN, FAD, lactic dehydrogenase, alkaline phosphatase, and Trypsin-300 from Sigma Chemical Company; DEAE-cellulose from Schleicher and Schuell Company; AMP from Nutritional Biochemicals Corporation; 1,2-naphthoquinone-4-sulfonate from Eastman Organic Chemicals; Florisil from Floridin Company; catalase from Worthington Biochemical Corporation; pig kidney acetone powder from Biobin Laboratories. Brewers' yeast was kindly supplied by the Rainier Brewing Company. Nagarse mitochondria (13), particulate DPNH-coenzyme Q reductase, and particulate DPNH-cytochrome *c* reductase were kindly supplied by Drs. Y. Hatefi and D. E. Green.

**Methods**—D-Amino acid oxidase and TPNH-cytochrome *c* reductase were prepared from pig kidney and brewers' yeast, respectively, by the methods of Huennekens and Felton (14). For the assay of FAD by the D-amino acid oxidase, our previously described procedure (14) was adapted for spectrophotometry by coupling the reaction product, pyruvate, with excess lactic dehydrogenase according to the method of DeLuca, Weber, and Kaplan (15). The complete system contained in a cuvette of 1 cm light path: 0.4  $\mu$ mole of DPNH, 100  $\mu$ moles of sodium phosphate buffer, pH 7.5, 0.1 mg of crystalline lactic dehydrogenase, 150 units of crystalline catalase, sample containing 0.1 to 1.0  $\mu$ g of FAD, 0.1 ml of D-amino acid apo-oxidase, and water to make 3.0 ml. The optical blank was identical except for the omission of DPNH. One hundred micromoles of DL-

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<sup>1</sup> We are indebted to Mr. Roger Wade, Department of Biochemistry, for carrying out these analyses.

<sup>2</sup> The abbreviation used is: FMN, flavin mononucleotide (riboflavin 5'-phosphate).

<sup>3</sup> T. E. King, private communication.

alanine were added to each cuvette in order to start the reaction and the change in absorbancy at 340 m $\mu$  was recorded over a 5-minute period. FMN was measured by the reactivation of the TPNH-cytochrome *c* aporeductase as described previously (14). These enzymatic assays are very specific for FAD and FMN, and no inhibition is observed when the inactive nucleotide (FMN in the FAD assay, or FAD in the FMN assay) is present in a 10-fold excess. Microbiological assays for riboflavin with *Lactobacillus casei* were carried out by the Wisconsin Alumni Research Foundation. Total flavin in a sample was determined by the reduction in absorbancy at 450 m $\mu$  upon the addition of sodium hydrosulfite; a value of  $\Delta\epsilon = 11.3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  per mole was used for the difference in extinction coefficient between the oxidized and reduced flavin (16). AMP was measured enzymatically with adenylyc deaminase according to the method of Nikiforuk and Colowick (17). Adenine-containing compounds were detected by a chemical method in which the sample was hydrolyzed for 6 hours with 6 N HCl in a sealed tube, and the degradation product, 4-amino-5-imidazole carboxamide, was chromatographed on paper ( $R_F = 0.69$  in *n*-butanol-acetic acid-water, 4:1:5) and made visible by spraying with Folin's reagent (18).

The reaction mixture for the hydrolysis of flavin coenzymes by nucleotide pyrophosphatase contained 100  $\mu$ moles of flavin, 100  $\mu$ moles of sodium phosphate buffer, pH 8.3, and 6  $\mu$ g of enzyme in a total volume of 1.0 ml. After incubation of the mixture at 30° for 10 minutes, the fluorescence of a suitable diluted aliquot was measured in a Farrand spectrofluorometer with the appropriate riboflavin filters.

The hydrolysis of flavins by alkaline phosphatase was carried out in the following reaction mixture: 100  $\mu$ moles of Tris buffer, pH 8.5, 3.0  $\mu$ g of alkaline phosphatase, and 1.0  $\mu$ mole of flavin in a total volume of 1.0 ml. The reaction mixtures were incubated at 30° and aliquots were withdrawn at various time intervals and assayed for residual FMN with the TPNH-cytochrome *c* reductase system.

*Isolation and Assay of DPNH Dehydrogenase*—The enzyme was solubilized by incubating the DPNH oxidase at 44° for 15 minutes in the presence of 9% ethanol, as described earlier (3). The enzyme was lyophilized to remove ethanol, taken up in water, and fractionated with saturated ammonium sulfate, pH 7.0; the 0 to 60% fraction was used in all experiments reported in this paper. DPNH oxidizing activity with cytochrome *c* and dichlorophenolindophenol were measured spectrophotometrically, as described previously (3). For assay with ferricyanide as the acceptor, the cuvettes contained: 400  $\mu$ moles of potassium buffer, pH 7.5, 1.3  $\mu$ moles of sodium ferricyanide, 10  $\mu$ moles of sodium azide, and 0.8  $\mu$ mole of DPNH. The blank was identical except for the omission of ferricyanide. The nonenzymatic reduction of ferricyanide was observed by the change in absorbancy at 410 m $\mu$  for 1 minute after the addition of DPNH, whereupon the enzyme was added and the reaction rate followed at 15-second intervals.

*Release of Enzyme-bound Flavin*—The flavin of the DPNH dehydrogenase was completely released by heating the enzyme, approximately 6 mg per ml, in a boiling water bath for 6 minutes, followed by cooling to 5° and acidification with perchloric acid to a final concentration of 10%. After centrifugation, the clear yellow supernatant fluid was adjusted to pH 7.5 to 8.0 with 6 N KOH and potassium perchlorate was removed by centrifugation. Alternatively, particulate preparations, 50 mg

of protein, were incubated with 10 mg of trypsin at 37° for 90 minutes (19) before the above heat and acid treatment.

*Reversible Resolution of Enzyme*—A rapid resolution of the enzyme into apoenzyme and flavin was achieved by passing 2.0 ml of the preparation, 10 mg of protein per ml, through a 1- $\times$  7-cm Florisil column in the cold. The flavin was retained at the top of the column, and after the column had been washed with 3.0 ml of water, the pale yellow, protein-containing eluate was recycled through the same column to yield approximately 7.0 ml of a colorless eluate representing the DPNH apodehydrogenase, 2.5 mg of protein per ml.

*Column Chromatography of Flavins*—Commercial DEAE-cellulose was washed repeatedly with 5.0 M K<sub>2</sub>HPO<sub>4</sub>, pH 8.5, until a clear supernatant fluid was obtained after the suspension had settled. The material was then washed with water until the filtrate had a pH of approximately 7.0 and showed essentially no absorbancy at 260 m $\mu$ . A thin slurry of purified DEAE-cellulose was packed under gravity into a 2- $\times$  15-cm glass column to a height of 14 cm and washed with 500 ml of water. The flavin, approximately 0.1 to 10.0 mg in 1 to 2 ml, was added carefully to the top of the column, and water was used initially to elute riboflavin. The solvent was then changed to 0.05 M K<sub>2</sub>HPO<sub>4</sub>, pH 5.5, whereupon FMN and FAD were eluted consecutively.

*Paper Chromatography of Flavins*—Descending paper chromatography of flavins was performed on 28- $\times$  42-cm sheets of Whatman No. 1 or No. 3 paper, with 5% Na<sub>2</sub>HPO<sub>4</sub>, *n*-butanol-acetic acid-water (4:1:5, volume for volume), or isobutyric acid-ammonia-water (66:1:33) as the solvent systems (20). In addition, circular paper chromatography was also used (21). The latter method involves application of the flavins to the circumference of a small circle with a radius of approximately 2 cm, the center of which coincides with the center of a circular filter paper 24 cm in diameter.

## RESULTS AND DISCUSSION

### Identification of Dehydrogenase Flavin as FMN

*Total Flavin*—The flavin of the DPNH dehydrogenase was completely released by heating the enzyme at 100° for 6 minutes followed by acidification with perchloric acid to a final concentration of 10%. No additional flavin was released when the heat- and acid-denatured protein was subjected to tryptic digestion. The flavin content of both the intact dehydrogenase and the supernatant fluid obtained from the denatured enzyme was measured by means of a microbiological assay with *L. casei* and also by the change in absorbancy at 450 m $\mu$  after treatment with hydrosulfite. Data presented earlier (1, 2) demonstrated an average content of 9.8  $\mu$ moles of flavin per mg of protein. Comparably, 10  $\mu$ moles of flavin per mg of protein were found in the DPNH-cytochrome *c* reductase from pig heart (16), whereas when the DPNH dehydrogenase is obtained by treatment of beef heart electron transport particle with snake venom (10), only 1  $\mu$ mole of flavin per mg of protein is present (22). Thus, the latter preparation may actually be an apodehydrogenase with respect to flavin. Further evidence on this point will be discussed in a subsequent section of this paper.

*Enzymatic Assay*—Assay systems which respond specifically to FMN and FAD revealed that essentially all of the flavin released from the DPNH dehydrogenase by the above treatment could be accounted for as FMN (see Huennekens *et al.* (1),

TABLE I  
Paper chromatography of dehydrogenase flavin

Solvent system	$R_F \times 100$			
	Dehydrogenase flavin	FMN	FAD	Riboflavin
A. Descending chromatography				
<i>n</i> -Butanol-acetic acid-water	10	10	5	30
Na <sub>2</sub> HPO <sub>4</sub>	52	50	40	30
Isobutyric acid-ammonia-water	32	33	36	58
B. Circular chromatography				
<i>n</i> -Butanol-acetic acid-water	42	40	25	55
Na <sub>2</sub> HPO <sub>4</sub>	68	70	55	45

Table I). A small amount of FAD, approximately 5% of the total, was encountered occasionally in dehydrogenase preparations, but never when the enzyme had been fractionated with ammonium sulfate after solubilization. It should be noted that when FAD was admixed with the intact enzyme before the heat and acid denaturation, it was recovered quantitatively as judged by enzymatic assay. This experiment demonstrated that *external* FAD, at least, is not broken down under conditions which liberate the enzyme-bound flavin.

*Paper Chromatography*—When examined by paper chromatography with three different solvent systems, the dehydrogenase flavin migrated with the same  $R_F$  value as FMN (Table I). No separation was observed when the dehydrogenase flavin was admixed with FMN before chromatography.

In order to investigate whether the migration of the flavin on paper chromatograms could be affected by the prior denaturation treatment, aliquots of the enzyme were exposed to various periods of heating, with and without subsequent acid treatment. A solution of the enzyme was placed in a boiling water bath and two aliquots were withdrawn at each of the following time intervals: 30 seconds, and 1, 2, 4, and 6 minutes. One of the two samples was treated with perchloric acid and the precipitated protein was removed by centrifugation. The second heat-deproteinized sample was centrifuged to remove denatured protein but was not treated with acid. Both samples were subjected to circular paper chromatography with the *n*-butanol-acetic acid-water solvent system. From the data in Fig. 1, it is evident that shorter periods, 30 seconds to 2.5 minutes, of heat denaturation unaccompanied by acid treatment resulted in the flavin migrating with an  $R_F$  value which more nearly resembled that of FAD than FMN. Even when the acid treatment was included with samples heated for less than 2 minutes, an  $R_F$  value intermediate between those of FAD and FMN was obtained. With increasing periods of heat denaturation, however, the  $R_F$  value increased progressively and reached that of FMN. This anomalous chromatographic behavior could be misleading in elucidating the identity of the dehydrogenase flavin, but when the above samples were analyzed before chromatography, or when the flavin spots were eluted from the paper and analyzed similarly, only FMN was detected in all instances by the specific enzymatic assays. Although the reason for the anomalous migration is not

yet clear, it is possible that incomplete denaturation of the protein may hinder the movement of the flavin.

*Chromatography on DEAE-cellulose*—The heat- and acid-liberated flavin from 90 mg of enzyme was adsorbed on a 1- $\times$  10-cm Florisil column (20), and 100 ml of water were passed through in order to remove salts. The adsorbed flavin was then eluted with pyridine, and after the removal of the pyridine and reduction of the volume by partial lyophilization, the flavin solution was chromatographed on DEAE-cellulose as described in "Experimental Procedure." In the elution profile the dehydrogenase flavin emerged as a single, symmetrical peak indistinguishable in its position from FMN (cf. Fig. 2). Admixing FMN with the dehydrogenase flavin before chromatography did not result in separation of the two substances.

*Fluorescence Studies*—Since the molal fluorescence of FAD at pH 7.0 is only approximately 10 to 15% as large as that of FMN (23), fluorescence measurements can be used to obtain additional evidence on the nature of the dehydrogenase flavin. Samples of FMN, FAD, and the dehydrogenase flavin were incubated with a purified nucleotide pyrophosphatase from *Aspergillus oryzae*. The fluorescence of each solution was measured before the addition of enzyme and again after incubation for 10 minutes at 30°. The fluorescence of the FAD sample increased approximately

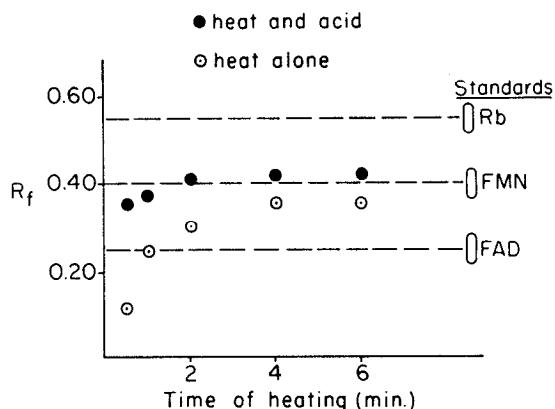


FIG. 1. Effect of denaturation process on  $R_F$  of released flavin

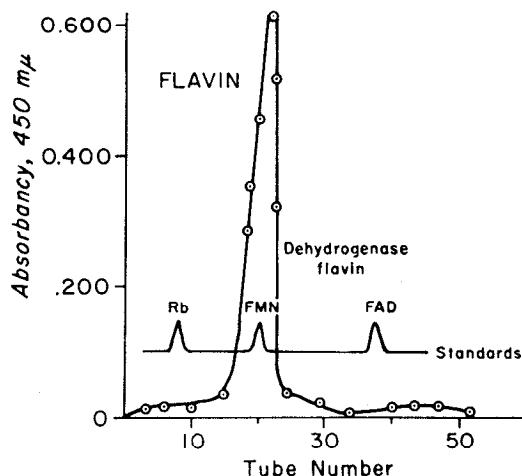


FIG. 2. Chromatography of dehydrogenase flavin on DEAE-cellulose. Flavin from 90 mg of DPNH dehydrogenase was chromatographed as described in "Experimental Procedure." Aliquots, 2.5 ml, were collected in each tube. The positions of riboflavin, FMN, and FAD are shown in the elution profile.

TABLE II  
Rate of degradation of FMN and dehydrogenase flavin by alkaline phosphatase

Time of incubation	Hydrolysis	
	Dehydrogenase flavin	FMN
min	%	%
0	0	0
5	25	30
15	55	65
30	75	85
60	100	100

10-fold after this treatment owing to the production of FMN, whereas that of FMN and the dehydrogenase flavin each remained essentially constant.

**Degradation Studies**—Equimolar quantities of FMN and the dehydrogenase flavin were incubated with alkaline phosphatase at 30°. Aliquots were withdrawn at 0, 5, 15, 30, and 60 minutes and the amount of FMN was estimated by the TPNH-cytochrome *c* aporeductase assay system. As shown in Table II, both samples were dephosphorylated at essentially the same rate. When the aliquots of these reaction mixtures were subjected to paper chromatography in three solvent systems, only FMN and riboflavin were found.

**Possibility that Enzyme-bound Flavin is FAD**—The above results provide strong evidence that when the DPNH dehydrogenase is denatured by heat, by acid, or by a combination of these procedures, the original amount of enzyme-bound flavin is accounted for quantitatively as FMN in the supernatant fraction. Furthermore, when the flavin is removed by other techniques, including dialysis or treatment with Florisil (see next section), only FMN is found. Even when the flavin is bound to the protein, it can be shown to be FMN by assay with the yeast

TPNH-cytochrome *c* aporeductase. In the experiment described in Fig. 3, all of the components of the FMN assay system were present initially except for FMN. Addition of 0.2 µg of FMN, *Curve A*, or two levels of DPNH dehydrogenase containing 0.25 and 0.5 µg of flavin, respectively, *Curves B* and *C*, caused the reduction of cytochrome *c*. No activity was observed, however, with the DPNH dehydrogenase samples when TPNH or the TPNH-cytochrome *c* reductase was omitted. Separate experiments, in which flavin binding is measured fluorometrically (4), have established that the flavin *does not* dissociate from the protein under the conditions of this experiment. Thus, reactivation of the TPNH-cytochrome *c* aporeductase must be caused by the *bound* flavin, obviously FMN. This experiment is similar to the demonstration by Mahler (24) that DPN, bound to rabbit muscle 3-phosphoglyceraldehyde dehydrogenase, can be utilized as a substrate by the DPNH-cytochrome *c* reductase.

These observations, leading to the conclusion that FMN is the prosthetic group of the beef heart DPNH dehydrogenase, are further strengthened by finding, to be described below, that the apodehydrogenase is reactivated almost completely by FMN, but not by FAD. King<sup>3</sup> has also concluded that his DPNH dehydrogenase preparation from beef heart contains FMN as the prosthetic group. Finally, it would not be unexpected to find FMN involved with a mammalian DPNH dehydrogenase since it is well established that the classical TPNH-cytochrome *c* reductase (TPNH dehydrogenase) from yeast has FMN for its prosthetic group (25).

Despite the weight of this evidence, the possibility must be considered that the enzyme-bound flavin is actually FAD, or an FAD-like dinucleotide, and that removal of the flavin from the protein by any method results in its breakdown to FMN. In support of this hypothesis are the observations of Mahler *et al.* (5) and Singer *et al.* (7, 9), who have reported finding approximately equimolar quantities of flavin and adenine in other

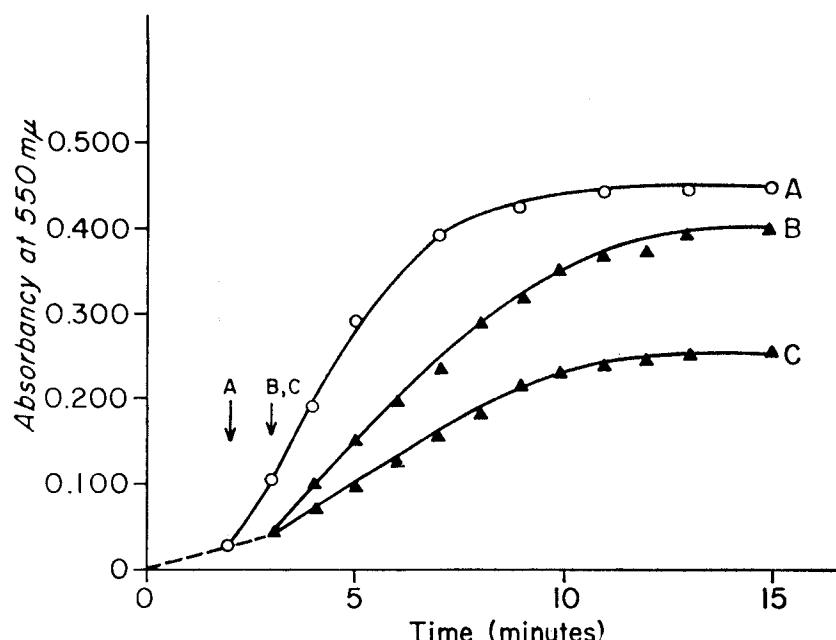


FIG. 3. Enzymatic estimation of FMN bound to DPNH dehydrogenase. Standard TPNH-cytochrome *c* reductase assay as described in "Experimental Procedure." In Experiment A, 0.2 µg of FMN was added at the time indicated by the arrow; in Experiments B and C, DPNH dehydrogenase samples containing 0.5 and 0.25 µg of FMN, respectively, were added at the arrow.

DPNH dehydrogenase preparations. An attempt was made, therefore, to detect AMP or other adenine-containing fragments in the present DPNH dehydrogenase, especially after release of FMN had occurred. With adenylic deaminase as the assay system (17), no AMP could be detected in the native or denatured enzyme, or in the flavin extracts. Alternatively, a chemical method (18) was used which responds to adenine, adenosine, or AMP. Samples of the intact enzyme, the dehydrogenase flavin, FMN, and FAD were hydrolyzed with 6 N HCl in a sealed tube at 100° and 4-amino-5-imidazole carboxamide was estimated with the Folin reagent. A positive reaction was obtained only with FAD. These results appear to exclude the possibility that FMN originates from cleavage of bound FAD during the denaturation process.

#### Reconstitution of Dissociated DPNH Apodehydrogenase with FMN

As a consequence of the above findings, which demonstrated that the dehydrogenase contains only FMN, attempts were made to dissociate the bound flavin from the enzyme by relatively mild procedures and thereafter to reactivate the resulting apoenzyme with added FMN. It was found that dialysis of the enzyme for 24 hours against water resulted in a progressive loss of flavin which paralleled the loss of DPNH dehydrogenase activity with dichlorophenolindophenol or cytochrome *c* as acceptors (Fig. 4). The activity was not restored, however, by the addition of either FMN or FAD to the apoenzyme. When the enzyme was dialyzed for shorter time intervals (less than 12 hours), resulting in only a partial loss of flavin and activity, there was considerable stimulation of cytochrome *c* reductase activity upon the addition of FMN, but not FAD. Activity with dichlorophenolindophenol, on the other hand, could not be restored by addition of either flavin.

A rapid and more satisfactory method for the removal of flavin consisted of passing the enzyme through a small Florisil column. The flavin was adsorbed at the top of the column while the protein emerged in the effluent. Repetition of this procedure gave rise to a suitably resolved apoenzyme, recovered in about an 80% yield as judged by protein analysis, and from which about 90% of the flavin had been removed.

When the apoenzyme, prepared by the above method, was tested in the presence of added FMN, approximately 75% of the original activity with cytochrome *c* as acceptor was restored

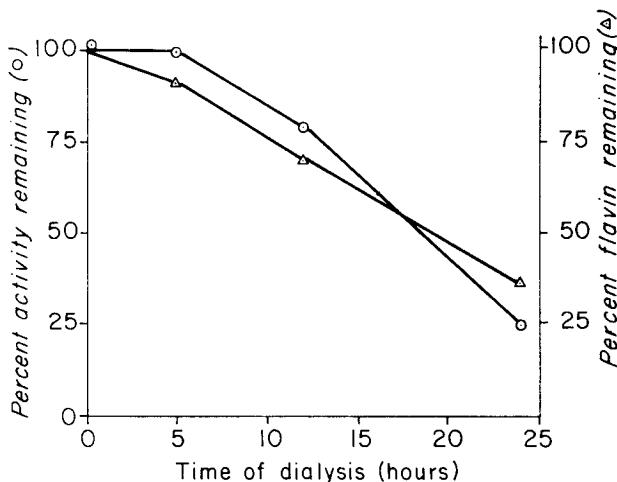


FIG. 4. Effect of dialysis on flavin content and enzyme activity

TABLE III

#### Effect of removal of flavin on activity with various acceptors

The holoenzyme and the apoenzyme contained 8.0 and 0.4  $\mu$ moles of flavin, respectively, per mg of protein. Preparation of the apoenzyme and enzymatic assays were performed as described in "Experimental Procedure."

Electron acceptor	Activity*		
	Holoenzyme	Apoenzyme	Apoenzyme plus FMN
Cytochrome <i>c</i> .....	3.9	0.3	3.2
Dichlorophenolindophenol.....	40	0.4	0.4
Ferricyanide.....	38	32	32

\* Activity is expressed as micromoles of DPNH oxidized per minute per mg of protein.

(*cf.* Huennekens *et al.* (1), Fig. 1); the apoenzyme was not reactivated by FAD or riboflavin. This demonstration of the restoration of activity of the apoenzyme with FMN further establishes the identity of the prosthetic group of DPNH dehydrogenase.

The Michaelis constant of  $2.3 \times 10^{-4}$  M for the reactivation of the apoenzyme by FMN is relatively large for flavoproteins, and provides an explanation for the facile dissociation of the coenzyme from the protein during dialysis. It should be noted that approximately 15 to 20 times as much FMN as was present originally in the holoenzyme must be added to fully reconstitute the activity of the apoenzyme.

The activity of the enzyme with dichlorophenolindophenol as acceptor drops from 40 to 3  $\mu$ moles of DPNH oxidized per minute per mg of protein upon removal of the flavin, and in contrast to the results with cytochrome *c*, this activity is not restored upon the addition of FMN or FAD. On the other hand, the activity with ferricyanide as acceptor is not appreciably diminished when the flavin is removed from the enzyme and, hence, no increase in activity is observed upon the addition of FMN or FAD. These results are summarized in Table III. The finding that DPNH oxidation coupled with ferricyanide is apparently not dependent upon the presence of bound flavin provides further evidence that the DPNH dehydrogenase of Ringler, Minakami, and Singer (7) could be an apodehydrogenase with respect to flavin since these investigators have routinely used a ferricyanide assay.<sup>4</sup>

<sup>4</sup> Since this manuscript was submitted for publication, a further communication from the laboratory of Drs. Singer and Kearney has appeared (H. Watari, E. B. Kearney, T. P. Singer, D. Basinski, J. Hauber, and C. J. Lusty, *J. Biol. Chem.*, **237**, PC1731 (1962)). The properties of a high molecular weight DPNH dehydrogenase, believed by these authors to be the "respiratory chain DPNH dehydrogenase," are compared with those of several low molecular weight DPNH dehydrogenases, including the present enzyme. The high molecular weight enzyme ( $s_{20} = 12.6$ ) contains FAD and nonheme iron in the ratio of 1:16, but the functional nature of the flavin has never been demonstrated since the enzyme apparently cannot be reversibly resolved, and, as shown above, assay with ferricyanide does not require the presence of flavin. Watari *et al.* have shown further that treatment of their enzyme with acid-ethanol leads to lower molecular weight enzyme ( $s_{20} = 5.6$ ) with the following properties: an FMN to iron ratio of 2 or 4:1, reversible resolution and reactivation with FMN, and ability to use cytochrome *c* as acceptor.

*Flavin Content of Various Particulate Precursors of DPNH Dehydrogenase*

The identification of FMN as the DPNH dehydrogenase flavin has made it of interest to determine the FMN and FAD content of various particulate preparations which are precursors of, or related to, the soluble dehydrogenase. Of the following preparations which were assayed, all but the last two were particulate: beef heart homogenate, beef heart mitochondria, Nagarse mitochondria, electron transport particle, DPNH oxidase, DPNH-cytochrome *c* reductase (Amytal- and antimycin-sensitive), DPNH-coenzyme Q reductase, DPNH-cytochrome *c* reductase from pig heart (5), and succinic dehydrogenase from electron transfer particle (26). Each sample was digested with trypsin, followed by denaturation with heat and acid to release the total flavin, which was determined by the change in absorbancy at 450 m $\mu$  upon addition of hydrosulfite. The same treatment, but omitting the trypsin step, gave a value for the heat- and acid-released flavin alone, whereas the difference between these two values presumably represented peptide-bound flavin. With each preparation, both the total flavin extract and the heat- and acid-released extract were also analyzed enzymatically for FMN and FAD. It should be noted that the bound flavin released by tryptic digestion does not estimate either as FMN or FAD. Results of these analyses are presented in Table IV.

Data from the heat- and acid-extract of beef heart homogenate revealed that the flavin was distributed as follows: 90% FAD, 8% FMN, and less than 1% riboflavin. These values are in agreement with the data of Bessey *et al.* (23, 27) and Cerletti and Ipata (28), all of whom used the partition coefficients of flavins between water and benzyl alcohol as the basis for analysis. Our values were also confirmed by chromatography of the extract on a DEAE-cellulose column.

The flavin analyses of mitochondria, prepared via the con-

TABLE IV  
*Flavin content\* of various particulate precursors of DPNH dehydrogenase and other related preparations*

Preparation	Treatment					
	Trypsin, heat, and acid			Heat and acid		
	Total flavin	FAD	FMN	Total flavin	FAD	FMN
Homogenate.....	—†	—	—	6.4	5.8	0.28
Mitochondria.....	0.30	0.10	0.03	0.12	0.08	0.03
Nagarse mitochondria.....	0.44	0.3	0.2	0.55	0.31	0.27
DPNH oxidase.....	0.57	0.08	0.11	0.23	0.12	0.11
Electron transport particle.....	0.46	0.17	0.08	0.19	0.10	0.09
DPNH-cytochrome <i>c</i> reductase.....	0.84	0.03	0.8	1.0	0.08	0.80
DPNH-Q reductase.....	1.4	0.03	1.3	1.3	0.05	1.3
DPNH dehydrogenase from DPNH oxidase.....	8.2	0.3	7.6	8.2	0.3	7.8
DPNH-cytochrome <i>c</i> reductase from pig heart.....	5.3	0.2	4.8	5.2	0.2	4.8
Succinic dehydrogenase.....	0.8	0.09	0.05	0.1	—	0.05

\* Flavin content is expressed as millimicromoles per mg of protein.

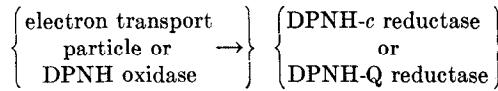
† —, Value not determined.

TABLE V  
*Recovery of flavin and enzyme activity during solubilization of DPNH dehydrogenase*

Preparation	DPNH oxidase			DPNH dehydrogenase			Recovery	
	Protein	Activity	FMN	Protein	Activity	FMN	Activity	FMN
I	5,600	1620	567	45	1570	365	96	65
II	7,000	2240	840	66	2100	441	93	53
III	12,000	3840	1350	73	2700	688	70	52
Average							86	63

ventional sucrose method, or of Nagarse mitochondria (13), indicated that approximately equal amounts of FMN and FAD were present; in addition, there is a large amount, 50 to 60%, of the peptide-bound flavin. The DPNH oxidase and electron transport particle have a similar distribution of flavins. On the other hand, the particulate DPNH-cytochrome *c* reductase, the particulate DPNH-coenzyme Q reductase, and the soluble DPNH-cytochrome *c* reductase from pig heart, each contain approximately 90% FMN and less than 10% of FAD. Finally, in confirmation of Kearney's results (19), soluble succinic dehydrogenase contains only peptide-bound flavin which cannot be estimated as either FMN or FAD. From the data in Table IV, it is seen that although the initial heart homogenate contains much greater amounts of FAD than FMN, the derived particles in the sequence

Homogenate → mitochondria →



contain progressively larger amounts of FMN relative to FAD, and this is paralleled by the increase in DPNH dehydrogenase activity. Finally, all of the bound flavin is FMN in the highly purified, soluble DPNH dehydrogenase which presumably has no other enzymatic activities.

It is of interest to calculate the recovery of flavin and enzymatic activity (DPNH oxidation linked to cytochrome *c* or dichlorophenolindophenol) during the solubilization of the dehydrogenase from the DPNH oxidase (Table V). In three experiments, an average of 86% of the activity was recovered with a retention of 63% of the flavin. Since the amount of FMN in the dehydrogenase did not exceed in any instance the amount originally present in the DPNH oxidase, further evidence is thereby provided that FAD is not broken down during solubilization of the enzyme. The lower recovery of flavin, relative to the recovery of activity, may be due to the fact that all of the FMN in the particulate DPNH oxidase is not associated with the DPNH dehydrogenase.

#### SUMMARY

1. The flavin component of the soluble reduced nicotinamide adenine dinucleotide dehydrogenase from beef heart was shown to be flavin mononucleotide (FMN), as judged by enzymatic, chromatographic, fluorometric, and degradative methods.
2. The enzyme was resolved into an aporeductase and FMN

by passage through a Florisil column; FMN, but not flavin adenine dinucleotide (FAD), reactivated the aporeductase ( $K_m = 2.3 \times 10^{-4}$  M) with cytochrome *c* as the terminal electron acceptor. The aporeductase retained almost all of its activity with ferricyanide as the acceptor and FMN or FAD did not stimulate this system.

3. The possibility that enzyme-bound FAD was degraded to FMN upon release from the enzyme was invalidated by the inability to find adenosine 5'-phosphate after dissociation of the flavin.

4. FMN was estimated quantitatively in various particulate precursors of the reduced nicotinamide adenine dinucleotide dehydrogenase, and was shown to be concentrated, relative to FAD, during the course of purification of the enzyme.

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