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Diaphorases from *Aerobacter aerogenes*[1]

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ABSTRACT

BERNOFSKY, CARL (The University of Kansas, Kansas City), AND RUSSELL C. MILLS. Diaphorases from *Aerobacter aerogenes*. J. Bacteriol. 92:1404–1414. 1966.—Five enzymes which catalyze the reduction of 2,6-dichlorophenol-indophenol by reduced nicotinamide adenine dinucleotide ($NADH_2$) have been separated from sonic extracts of *Aerobacter aerogenes* B199 by diethylaminoethyl (DEAE) cellulose chromatography. Three major chromatographic fractions (enzymes I, II, and III) account for most of the activity in the extract. Of the two minor fractions, one is associated with cytochrome b_1. The other is extremely labile, and was not studied further. The chromatographed diaphorases appear to have a specific requirement for flavin mononucleotide. They are also readily inactivated by dilution; however, this can be prevented by a combination of phosphate buffer, bovine serum albumin, and flavin mononucleotide. The different enzymes are clearly distinguishable by their activities with $NADH_2$ and reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$) in the presence of various electron acceptors (2,6-dichlorophenol-indophenol, ferricyanide, menadione, and cytochrome *c*), and by their responses to inhibitors (amobarbital, antimycin A, Atabrine, *p*-chloromercuribenzenesulfonate, dicumarol, and 2,4-dinitrophenol). With 2,6-dichlorophenol-indophenol as acceptor, enzymes I, II, and III have comparable activities with either $NADH_2$ or $NADPH_2$. With menadione and ferricyanide as acceptors, enzymes II and III exhibit very high, $NADH_2$-specific activities. When cytochrome *c* is the acceptor, however, enzyme III shows greater activity with $NADPH_2$ as the electron donor. Ferricyanide is the most active acceptor for the cytochrome b_1-containing fraction. Coenzyme Q_6 does not appear to serve as an acceptor. All the diaphorases, with the exception of that in the cytochrome b_1-containing fraction, are inhibited by *p*-chloromercuribenzenesulfonate. Amobarbital is relatively ineffective and inhibits only the indophenol reductase activity of enzyme I. The menadione reductase activity of enzymes I, and II, and the diaphorases in the cytochrome b_1-containing fraction are strongly inhibited by antimycin A, 2,4-dinitrophenol, dicumarol, and Atabrine. However, the menadione reductase activity of enzyme III is affected only by the last three of these inhibitors. The diaphorases in sonic-treated extracts do not appear to be associated with a particulate fraction.

A diaphorase may be defined as any flavoprotein which can catalyze the oxidation-reduction reaction between a reduced pyridine nucleotide and a suitable acceptor. Such enzymes have a wide distribution in nature, and characteristically exhibit a broad specificity for electron acceptors.

Well-known examples of flavoproteins which were first recognized as diaphorases before their physiological functions were understood are lipoyl dehydrogenase and the respiratory chain-linked reduced nicotinamide adenine dinucleotide ($NADH_2$) dehydrogenase. The latter enzyme is responsible for coupling $NADH_2$ oxidation to the electron transport system. When isolated from its mitochondrial association, this dehydrogenase is active with ferricyanide, but not with cytochrome *c*, as an electron acceptor (14). It is readily converted by mild treatment into a diaphorase that can function with cytochrome *c* as an acceptor (26).

Lipoyl dehydrogenase is a component of the pyruvate and α-ketoglutarate dehydrogenase complexes in which it serves to catalyze the oxidation-reduction reaction between $NADH_2$ and lipoic acid. This enzyme, first isolated and characterized as a diaphorase by Straub (21), was established in its present role through the work of Massey (13) and Searls and Sanadi (19).

[1] This paper was taken from a thesis submitted by Carl Bernofsky to the University of Kansas Graduate School in partial fulfillment of the requirement for the Ph.D. degree.

It is generally assumed that diaphorases are involved only in pathways concerned with the regeneration of pyridine nucleotides from their reduced forms. In the oxidation of α-keto acids, however, lipoyl dehydrogenase normally functions in the direction of nicotinamide adenine dinucleotide (NAD) *reduction* by dihydrolipoic acid (17), thus showing that a diaphorase can be involved in a reversible, pyridine nucleotide-linked reaction in which the isolated enzyme shows little specificity for the non-nucleotide reactant.

The lack of specificity for the non-nucleotide component is a property which enables diaphorases to be readily detected. However, it is also a property which obscures their physiological roles. Although such nonspecific enzymes may seem to be of questionable value to the cell, it is apparent from a consideration of lipoyl dehydrogenase that a flavoprotein which has little intrinsic specificity of its own can have specificity conferred upon it by virtue of its association with a larger complex in which its contact with substrates is governed by the specificities of other enzymes.

In the present study, extracts of *Aerobacter aerogenes* were examined for their content of diaphorases as a first step toward gaining an insight into the processes by which reduced pyridine nucleotides are oxidized by this bacterium. The assumption that these enzymes necessarily participate in oxidative pathways is, of course, subject to the above reservation.

It was found that sonic extracts of *A. aerogenes* contain five diaphorases which could be isolated by chromatography on diethylaminoethyl (DEAE) cellulose. One of these, because of its instability, was not studied further. The specificities of the other diaphorases for $NADH_2$ and reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$) have been determined with a number of electron acceptors, and an attempt has been made to ascertain whether these enzymes are associated with a particulate fraction.

MATERIALS AND METHODS

Culture. A. aerogenes B199, obtained from the Department of Bacteriology, University of Kansas, was cultured in a nutrient broth consisting of 0.5% NaCl, 1% glucose, 1% Peptone (Difco), and 0.7% Beef Extract (Difco), *p*H 7.4. The same medium, solidified with 2% agar (Difco), was used for the maintenance of stock cultures.

Fernbach flasks (2.8 liter) containing 500 ml of medium were incubated at 37 C for 24 hr on a reciprocating shaker. Inoculation was with 7 ml of a subculture previously incubated in test tubes on the shaker for 24 hr.

After centrifugation, the cells from each 2 liters of culture medium were washed in 150 ml of 0.01 to 0.02 M potassium phosphate (*p*H 7.4–8.0) and were centrifuged at 15,000 $\times$ *g* for 15 min. The concentration and *p*H were dependent on the intended subsequent treatment. All operations after the harvesting procedure were conducted at 0 to 5 C. The cells were resuspended in 75 ml of buffer and were centrifuged at 30,000 $\times$ *g* for 15 min. The usual yield of packed wet cells from 2 liters of culture medium was 30 g, 5% of which represented protein as measured by the method of Lowry et al. (10).

Extracts. The packed cells were suspended in an equal volume of buffer and subjected to sonic treatment for 10 min in a tap-water cooled Raytheon 9-kc magnetostriction oscillator, at a plate voltage of 100. After initial centrifugation at 30,000 $\times$ *g* for 30 min, the sonic-treated material was centrifuged at 105,000 $\times$ *g* for 2 hr to obtain a clear yellow supernatant fraction containing essentially all of the diaphorase activity. In preparation for chromatography, the supernatant fractions were dialyzed overnight with 0.01 to 0.02 M potassium phosphate (*p*H 7.4–8.0), depending upon the initial conditions of the chromatography.

Assays. The standard assay for measuring indophenol reductase activity in chromatographic fractions contained: 300 μmoles of potassium phosphate (*p*H 7.4), 6 mg of crystalline bovine serum albumin (BSA, Pentex Inc., Kankakee, Ill.), 0.4 μmole of flavin mononucleotide (FMN, Sigma Chemical Co., St. Louis, Mo.) 0.15 μmole of 2,6-dichlorophenol-indophenol (2,6-DCPIP, Fisher Scientific Co., Pittsburgh, Pa.), 0.48 μmole of $NADH_2$ (Sigma Chemical Co.), and enzyme, in a total volume of 3.0 ml. In practice, a stock solution containing the buffer, BSA, FMN, and 2,6-DCPIP was dispensed into a cuvette, followed by addition of the reduced pyridine nucleotide and enzyme. This stock solution was stable for at least 2 weeks at 0 to 5 C in total darkness; the 2,6-DCPIP underwent an FMN-catalyzed bleaching in light.

Other acceptors used in specificity studies were: menadione (Mann Fine Chemicals, Inc., New York, N.Y.), equine cytochrome *c* (Sigma Chemical Co.), and potassium ferricyanide. When ferricyanide was used, the amount of reduced pyridine nucleotide was doubled. $NADPH_2$ was the product of Calbiochem., Los Angeles, Calif.

The inhibitors used in this study were: antimycin A (Sigma Chemical Co.), in ethyl alcohol solution; Atabrine hydrochloride (Sterling-Winthrop, Rensselaer, N. Y.); amobarbital (Gane and Ingram, New York, N. Y.); *p*-chloromercuribenzene-sulfonic acid (PCMS, Sigma Chemical Co.); dicumarol (Nutritional Biochemicals Corp., Cleveland, Ohio), and 2,4-dinitrophenol. The last four inhibitors were used as their sodium salts, and all solutions were prepared with deionized distilled water.

Spectrophotometry. The amounts of the acceptors employed and the conditions used to measure the course of the various reactions are listed in Table 1. The concentrations of acceptors were chosen so that the maximal change in absorption which could occur was 1.00.

Initial rates of all enzymatic reactions were measured with a Bausch & Lomb Spectronic 505 recording

TABLE 1. *Summary of reactions investigated*

Acceptor	Reaction measured	Wavelength	Molar extinction coefficient	Amt of reactant per assay	Reference
		mμ		μmoles	
Menadione	Disappearance of $NADH_2$ or $NADPH_2$	340	6.22×10^3	0.482[a]	(9)
2,6-DCPIP	Disappearance of oxidized 2,6-DCPIP	600	20.0×10^3	0.150	(18)
Ferricyanide	Disappearance of ferricyanide	420	1.04×10^3	2.88	(8)
Cytochrome *c*	Formation of reduced cytochrome *c*	550	21.0×10^3[b]	0.143	(12)

[a] This figure is for $NADH_2$ and $NADPH_2$. The amount of menadione present per assay was 0.40 μmole, added in 0.04 ml of 95% ethyl alcohol solution. Oxygen was the final acceptor in this system, since the product of menadione reduction is rapidly autoxidized.

[b] Based on the difference of molar absorption coefficients between the oxidized and reduced forms of cytochrome *c* at 550 mμ.

spectrophotometer which was modified by removal of tubes V-211 (6CM6) and V-210 (12AT7) from circuit board no. 2, and by connection of the instrument to a constant voltage transformer. This procedure disconnected the drum-braking mechanism and increased the uniformity of the drum speed.

Cuvettes of 1.00-cm light path were used, and the assays were conducted at room temperature. A cuvette containing the assay mixture, but no acceptor or enzyme, was placed in the reference beam of the spectrophotometer. After addition of enzyme to the sample cuvette, the change in absorption was recorded for 1 min. These rates were nearly always linear. The nonenzymatic reduction of 2,6-DCPIP by $NADH_2$, which was $\Delta A_{600} = 0.02$ per min, was subtracted from the enzymatic assays.

Units of activity. One unit of diaphorase (indophenol reductase) activity is defined as causing a ΔA_{600} per min of 1.00 in 3.00 ml of solution of 1.00-cm light path, when 2,6-DCPIP is the electron acceptor. Specific activity is the micromoles of acceptor reduced per min per milligram of enzyme protein, regardless of whether the electron change is two per mole (menadione and 2,6-DCPIP), or one per mole (cytochrome *c* and ferricyanide). It should be noted that, since substrate and acceptor concentrations were arbitrarily chosen, the latter on the basis of photometric convenience, the activities reported are not necessarily the maximal rates obtainable with the diaphorases.

DEAE-cellulose. Type 20 Selectacel-DEAE (Brown Co., Berlin, N. H.) was repeatedly washed with 1.0 M neutral phosphate buffer and filtered on a Büchner funnel until tan pigment was no longer extracted. After being washed with deionized water, the fines were removed and the product was suspended in water and stored in the cold. Fast-flowing columns were prepared by pouring the DEAE-cellulose at room temperature all at once and allowing it to settle by gravity. They were then equilibrated with the buffer used to dialyze the extract to be chromatographed.

Chromatography. All chromatography was performed in a cold room at 4 C by use of a Rinco fraction collector with a volumetric siphon which delivered an average of 5.8 ml per fraction. The apparatus for gradient elution consisted of an upper reservoir containing 0.25 M potassium phosphate (*p*H 8.0) which fed into the first two of mixing chambers in series, each containing 250 ml of the initial buffer used. The second mixing chamber discharged into the column. The flow rate was maintained at 15 to 20 ml/hr by adjustment at the reservoir.

RESULTS

Chromatographic separation. Chromatography of the supernatant fraction with DEAE-cellulose by a stepwise procedure resulted in the elution of the diaphorase activity between 0.05 and 0.15 M potassium phosphate (*p*H 8.0) with a recovery of 51%.

For separation of the various enzymes by gradient elution chromatography, 175 ml of a supernatant fraction containing 1.32 g of protein and 1,313 units of indophenol reductase activity was dialyzed with 0.02 M potassium phosphate (*p*H 8.0) and was adsorbed onto an 18 by 170-mm column of DEAE-cellulose. The upper surface was occasionally stirred to improve the flow rate. After the extract, 35 ml of 0.02 M buffer was passed through the column, and the gradient elution was begun. The mixing chambers contained 0.04 M potassium phosphate (*p*H 8.0) and the gradient was measured by phosphate analysis of about every 10th tube, by use of the method described by Taussky and Shorr (22).

The activity of the fractions, measured as indophenol reduction, is shown in Fig. 1 as open circles. Fifty-five per cent of the activity was recovered. The small peak labeled cytochrome b_1 was brown in color and had spectral characteristics identical with the cytochrome b_1 from *A. aerogenes* described by Smith (20). The difference spectrum (Fig. 2) was taken at room temperature with a Zeiss PMQ II spectrophotometer by placement of the peak fraction into two microcuvettes and addition of a small pinch of dithionite to the one in the sample position.

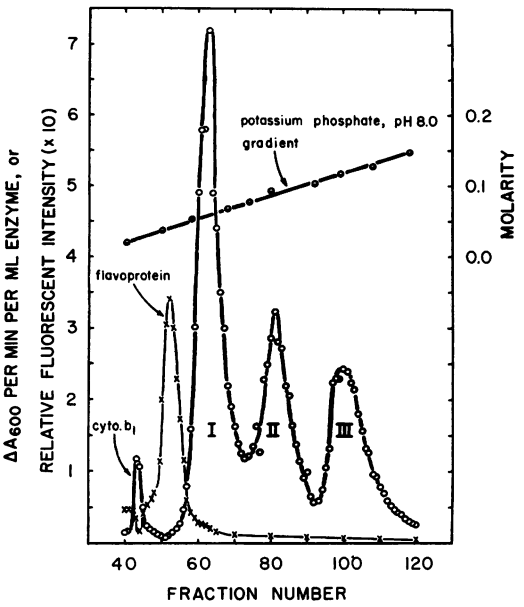

FIG. 1. *Gradient-elution chromatography of a sonic extract of Aerobacter aerogenes. Activity measured with 2,6-DCPIP. Symbol: × = fluorescent emission at 517 mμ upon activation at 444 mμ.*

The three major fractions of diaphorase activity shown in Fig. 1 are labeled I, II, and III, in order of their emergence from the chromatographic column. In addition, a fluorescent yellow fraction (labeled flavoprotein) which possessed no diaphorase activity was eluted between the cytochrome b_1 and enzyme I peaks. When activated with light at 444 mμ, this fraction fluoresced with an emission peak at 517 mμ, as measured with an Aminco-Bowman Spectrophotofluorometer.

Flavin requirement. Although the dialyzed supernatant fraction did not show a dependence for added flavin coenzymes, the diaphorases obtained by DEAE-cellulose chromatography appeared to have an absolute requirement for FMN. The flavin requirement was determined with a preparation from a preliminary chromatography which failed to separate the activities. Figure 3 shows the dependence on FMN of this enzyme mixture. The apparent K_m for FMN, as calculated from a double reciprocal plot, was 1.9×10^{-5} M. FAD was inactive.

In this study, a coupled menadione-2,6-DCPIP assay was used in which 0.4 μmole of menadione in 0.04 ml of ethyl alcohol was added to the standard indophenol reductase assay. This system, which essentially measured menadione reduction, was followed at 600 mμ and depended upon the rapid nonenzymatic reduction of 2,6-DCPIP by reduced menadione. In later studies, menadione reductase activity was measured directly by following $NADH_2$ oxidation at 340 mμ.

It should be pointed out that, although the mixed diaphorase fraction had no significant

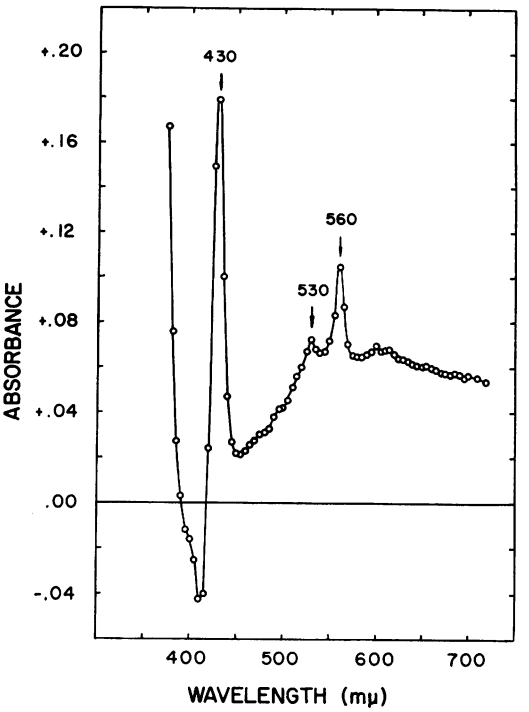

FIG. 2. *Difference spectrum of cytochrome b_1 obtained from chromatography illustrated in Fig. 1.*

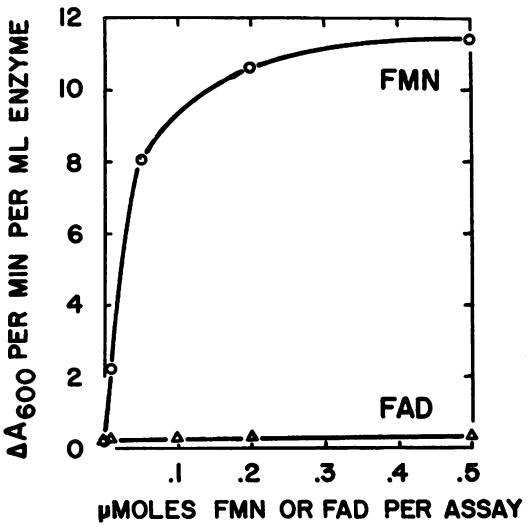

FIG. 3. *Flavin dependence of an unfractionated peak of diaphorase activity eluted from DEAE-cellulose. Activity measured with menadione and 2,6-DCPIP.*

menadione reductase activity in the absence of added FMN, the apparent K_m obtained probably reflected the activity of the component with the smallest catalytic requirement for FMN. The flavin dependencies of the individual diaphorases were not determined.

Effect of dilution. Another characteristic of the chromatographed diaphorases was their instability to dilution. This property was studied with the same diaphorase preparation described above. In the experiment shown in Fig. 4A, the mixed diaphorase fraction in 0.05 M potassium phosphate (*p*H 7.5) was diluted fivefold at 0 C with the same buffer, and was assayed at varying time intervals with the coupled menadione-2,6-DCPIP assay. There was initially a rapid inactivation followed by a period during which the activity did not change. The undiluted enzyme preparation showed no loss of activity after 4 hr. Although the inactivation described here probably reflected the behavior of the most unstable component with menadione reductase activity, more than one diaphorase was found to be unstable to dilution as shown below.

With use of the activity remaining 120 min after fivefold dilution as a quantitative measure of inactivation, it was found (Fig. 4B) that increasing concentrations of phosphate buffer could stabilize the preparation. Additional effects of BSA and FMN are given in Table 2. The final combination shown in Table 2 which resulted in satisfactory stabilization for fivefold dilution was not as effective where dilutions of 20-fold were required, and, in these cases, stabilization was achieved by substituting 0.5 M potassium phosphate for the 0.1 M buffer.

Effect of stabilization. To assess the extent of inactivation which occurred between elution and assay of the diaphorases, 12-ml samples of the supernatant fraction containing 200 mg of protein and 242 units of indophenol reductase activity were dialyzed with 0.01 M potassium phosphate (*p*H 8.0) and were adsorbed onto each of two identical 11 by 120-mm columns of DEAE-cellulose. These were then eluted in the usual manner by use of 0.01 M buffer in the mixing chambers. The eluate from one column was collected into empty receiving tubes, whereas the eluate from the other was collected into tubes containing 1.2 ml of 1.0 M potassium phosphate (*p*H 7.4) and 5.84 $\times 10^{-4}$ M FMN. As far as possible, all other conditions were kept the same.

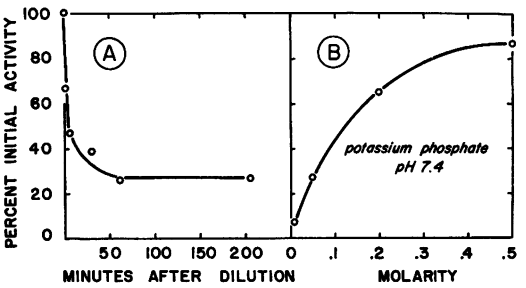

FIG. 4. *Effect of dilution on an unfractionated peak of diaphorase activity in 0.05 M phosphate (pH 7.4) eluted from DEAE-cellulose. Activity measured with menadione and 2,6-DCPIP. (A) Effect of five-fold dilution with 0.05 M potassium phosphate (pH 7.4) on activity. (B) Activity remaining 120 min after fivefold dilution with various concentrations of potassium phosphate (pH 7.4). Abscissa indicates final molarity.*

TABLE 2. *Stabilization of a mixed diaphorase fraction toward dilution*[a]

Final concn in reaction mixture	Activity remaining 120 min after five-fold dilution
	%
0.01 M KPO_4, *p*H 7.4	7
0.05 M KPO_4, *p*H 7.4	27
0.05 M KPO_4, *p*H 7.4 + 1.0% BSA	46
0.05 M KPO_4, *p*H 7.4 + 0.13 mM FMN	70
0.10 M KPO_4, *p*H 7.4 + 1.0% BSA + 0.13 mM FMN	100

[a] See text for experimental procedure.

The recovery from the stabilized fractions was 83%, compared, with 40% for the unstabilized fractions; this increase was not uniform among all the fractions (Fig. 5). From these data, it appears that enzyme I was the most stable, that enzyme III and the enzyme associated with cytochrome b_1 were the least stable, and that enzyme II was of intermediate stability. Figure 5 also shows the presence of a previously unobserved fraction of diaphorase activity which passed directly through the cellulose exchanger. This activity was extremely unstable, and no further characterization was attempted. This unstable diaphorase was not detected when chromatography was carried out in the absence of stabilizing solutions.

Activities with various acceptors. The enzymes used for this study were the peak fractions of diaphorase activity obtained from the DEAE-cellulose chromatography illustrated in Fig. 1. Table 3 lists the specific activities of the diaphorase fractions with $NADH_2$ and $NADPH_2$, and with the various acceptors. The activity of the cytochrome b_1 fraction was too small in some cases to be measured accurately.

It can be seen that for the general purpose of assaying the chromatographic fractions, 2,6-

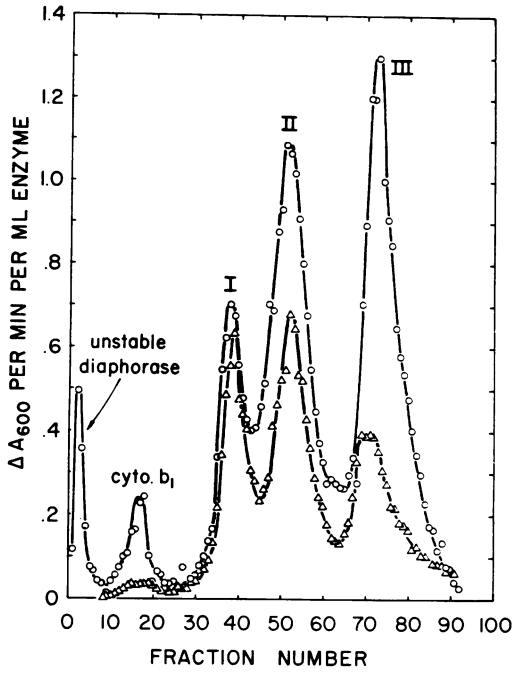

FIG. 5. *Gradient-elution chromatography of a sonic extract of Aerobacter aerogenes. Activity was measured with 2,6-DCPIP, and 242 units were applied to each column. Symbols:* ○ = *fractions (5.8 ml) collected into 1.2 ml of 0.58 mM FMN; 1.0 M potassium phosphate (pH 7.4). Corrections were made for dilution.* △ = *fractions collected into empty receiving tubes. See text for conditions of chromatography.*

DCPIP was the acceptor of choice, since enzymes I, II, and III had similar specific activities, in addition to having comparable activities with either pyridine nucleotide. It is interesting to note that the rates of menadione and ferricyanide reduction closely paralleled one another, and that enzyme II was highly specific for $NADH_2$ with these acceptors.

With cytochrome *c* as an acceptor, the existence of $NADPH_2$-specific activity in the original extract was indicated by the $NADH_2$-$NADPH_2$ ratio of 0.25. However, only enzyme III, with a $NADH_2$-$NADPH_2$ ratio of 0.37, appeared to be significantly $NADPH_2$-specific. It is possible that a pathway for $NADPH_2$ oxidation (i.e., transhydrogenation) which was present in the extract was lost upon chromatography.

Inhibition studies. The enzymes for these experiments were obtained from a number of DEAE-cellulose fractionations. The analogous enzyme peaks were pooled, precipitated by 90% saturation with ammonium sulfate, and dissolved in a small volume of stabilizing diluent (last line, Table 2). Before use, the enzymes were diluted so that 0.1 ml would produce a Δ A per min of 0.250 to 0.400 in the absence of inhibitor.

In the typical assay, the enzyme was put into a small tube together with the assay medium, but without $NADH_2$. After addition of the inhibitor and incubation for 3 min at 37 C, $NADH_2$ was added. The reaction mixture was then transferred to a cuvette, and the optical density was recorded.

The inhibitors were tested with $NADH_2$ only,

TABLE 3. *Specific activities of chromatographed diaphorases with various acceptors*[a]

Conditions		Diaphorase fraction				
Electron acceptors	Coenzyme	Before chromatography	Cytochrome b_1	Enzyme I	Enzyme II	Enzyme III
2,6-DCPIP.....	$NADH_2$	0.16	0.084	0.71	0.56	1.00
	$NADPH_2$	0.12	0.041	0.54	0.60	0.82
	$NADH_2$-$NADPH_2$	1.29	2.05	1.32	0.94	1.22
Cytochrome *c*....	$NADH_2$	0.015	—[b]	0.11	0.18	0.015
	$NADPH_2$	0.061	0.007	0.10	0.18	0.040
	$NADH_2$-$NADPH_2$	0.25	—	1.05	0.95	0.37
Menadione.....	$NADH_2$	1.05	0.09	1.75	17.7	5.49
	$NADPH_2$	0.44	—[b]	0.54	1.32	4.44
	$NADH_2$-$NADPH_2$	2.40	—	3.27	13.4	1.24
Ferricyanide.....	$NADH_2$	1.76	0.37	4.44	59.0	12.8
	$NADPH_2$	0.76	0.32	1.97	4.50	7.50
	$NADH_2$-$NADPH_2$	2.32	1.17	2.25	13.1	1.71

[a] See text for experimental details.
[b] Activity too small to measure.

with menadione and 2,6-DCPIP as acceptors. An attempt was made to determine those concentrations which caused an inhibition of 50%. However, where this was not achieved, the per cent of inhibition at the highest concentration of inhibitor used is reported (Table 4).

Dicumarol generally caused initial precipitation which, in the more dilute cases, dissolved during incubation. The photometric interference of persistent precipitates and the ultraviolet absorption of dicumarol were compensated for by positioning a cuvette in the reference chamber so that the ground-glass sides blocked part of the light beam. Erratic tracings were repeated over each other by use of the displacement control to reposition the pen over the previous tracing. Reproducible data were obtained. Amobarbital also generally caused precipitation; however, little or no inhibition by this barbiturate was observed.

The data (Table 4) clearly indicate that the enzymes in the various fractions are quite different from each other. In general, indophenol reductase activities are less affected by the inhibitors than are menadione reductase activities, PCMS inhibits all of the enzymes except the cytochrome b_1 fraction, and amobarbital does not appreciably inhibit any of the diaphorases, with the exception of the indophenol reductase activity of enzyme I. Of particular interest is the relatively strong inhibition of the menadione reductase activities of enzymes I, II, and the enzyme in the cytochrome b_1 fraction by antimycin A, 2,4-dinitrophenol, dicumarol, and Atabrine. The menadione reductase activity of enzyme III is not inhibited by antimycin A.

Distribution study. An attempt was made to demonstrate an active particulate fraction. Bacterial cells were subjected to sonic disintegration in 0.02 M potassium phosphate (*p*H 8.0), and the extract was centrifuged at 20,000 × *g* for 20 min, yielding a highly turbid supernatant fraction. Two-thirds of this low-speed supernatant fraction was centrifuged at 105,000 × *g* for 4 hr. After dialysis with the above buffer, the supernatant fraction from the low-speed centrifugation (10.0 ml) and that from the high-speed centrifugation (11.5 ml), each containing 279 units of indophenol reductase activity, were applied to 11 by 120-mm columns of DEAE-cellulose. A 50-ml amount of 0.02 M potassium phosphate (*p*H 8.0) was put through the column, followed by 75 ml of 0.2 M buffer; 5.8-ml fractions were collected into 1.2 ml of the stabilizing medium described earlier. Turbidity was measured with a 10-mm round cuvette by use of a Klett colorimeter with a 660-mμ filter.

Most of the material responsible for the turbidity of the low-speed supernatant fraction (cross-hatched areas) passed directly through the column without being adsorbed (Fig. 6A). Immediately after this fraction, there appeared a small peak of diaphorase activity which was only slightly retarded by the column. Addition of the 0.2 M buffer immediately eluted the remaining diaphorases, together with a small amount of particulate matter. The total activity recovered was 147% of that placed on the column.

Chromatography of the high-speed supernatant fraction into the stabilizing medium is shown in Fig. 6B. It is apparent that, whereas the high-speed centrifugation removed the material responsible for the turbidity, it did not affect the activity of the diaphorases. Upon addition of the 0.2 M buffer, a small amount of particulate material was eluted. The total recovery of activity was 161% of that added.

Another portion of the high-speed supernatant fraction, dialyzed with 0.005 M potassium phosphate (*p*H 8.0) was chromatographed as above, except that the fractions were not collected into the FMN-phosphate stabilizing medium. Stepwise elution was carried out with 50 ml of 0.005 M, 50 ml of 0.02 M, and 75 ml of 0.2 M potassium phosphate (*p*H 8.0). The initial peak of activity previously seen was essentially absent (Fig. 6C). Full activity, however, was retained in the peak after the addition of the 0.2 M buffer. It is assumed that the unadsorbed diaphorase decomposed rapidly in the absence of the stabilizing medium.

These experiments failed to demonstrate the association of diaphorase activity with a particulate fraction not sedimented at 20,000 × *g* under the conditions stated. The unexpected increases in activity after chromatography may have been due to the removal of some inhibitor, or to the dissociation of the natural flavin followed by recombination with FMN, with which the activity was greater, or to some other cause. In contrast to these high activities obtained when the enzymes were on the column only a short time, recovery was only 81% from the gradient elution collected into stabilizing medium. It is apparent that considerable losses of activity resulted when the diaphorases were on the column for extended periods of time.

DISCUSSION

The present work furnishes another example of the observation that microorganisms contain a number of enzymes which can catalyze the oxidation of reduced pyridine nucleotides. Robinson and Mills (16) isolated three diaphorases from the soluble fraction of *Pasteurella tularensis*, and a fourth one by deoxycholate extraction of the particulate fraction. These diaphorases were characterized by their coenzyme specificities and transhydrogenase activities. Dolin (4) reported

TABLE 4. *Summary of diaphorase inhibition*

Inhibitor	Cytochrome b_1				Enzyme I				Enzyme II				Enzyme III			
	2,6-DCPIP		Menadione		2,6-DCPIP		Menadione		2,6-DCPIP		Menadione		2,6-DCPIP		Menadione	
	Per cent[a]	Amt	Per cent	Amt	Per cent	Amt	Per cent	Amt	Per cent	Amt	Per cent	Amt	Per cent	Amt	Per cent	Amt
Amobarbital	32	1.0×10^{-2}	28	1.0×10^{-2}	50	2.2×10^{-3}	31	1.0×10^{-2}	NI[b]	1.0×10^{-2}	NI	1.0×10^{-2}	NI	1.0×10^{-2}	NI	1.0×10^{-2}
Antimycin A	NI	1.1×10^{-4}	50	8.5×10^{-5}	23	1.1×10^{-4}	50	1.0×10^{-4}	NI	1.1×10^{-4}	50	9.4×10^{-5}	NI	1.1×10^{-4}	16	1.1×10^{-4}
Atabrine	33	2.0×10^{-3}	50	4.3×10^{-4}	36	2.0×10^{-3}	50	3.6×10^{-4}	50	1.5×10^{-3}	50	3.0×10^{-4}	28	2.0×10^{-3}	50	5.2×10^{-4}
p-Chloromercuribenzenesulfonate	11	1.6×10^{-3}	20	1.6×10^{-3}	50	3.0×10^{-4}	50	3.0×10^{-4}	50	1.6×10^{-4}	50	1.3×10^{-4}	50	1.6×10^{-4}	50	8.2×10^{-5}
Dicumarol	NI	1.0×10^{-3}	50	2.9×10^{-4}	50	3.2×10^{-5}	50	8.0×10^{-5}	48	1.0×10^{-3}	50	2.9×10^{-4}	NI	1.0×10^{-3}	50	2.5×10^{-4}
2,4-Dinitrophenol	NI	1.0×10^{-3}	50	1.0×10^{-4}	50	9.0×10^{-5}	50	4.0×10^{-5}	50	2.5×10^{-4}	50	9.0×10^{-5}	24	1.0×10^{-3}	50	1.0×10^{-4}

[a] Activity inhibited at the molar concentration of inhibitor listed.
[b] Not inhibited.

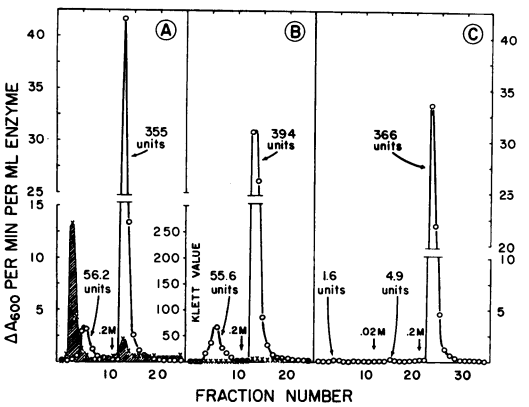

FIG. 6. *Stepwise-elution chromatography of low- and high-speed supernatant fractions from a sonic-treated extract. Symbols:* O = *units of diaphorase activity per milliliter in each fraction;* × = *turbidity with 660-mμ filter. (A) Low-speed supernatant fraction, collected into FMN-phosphate medium. (B) High-speed supernatant fraction, collected into FMN-phosphate medium. (C) High-speed supernatant fraction, not collected into FMN-phosphate medium. Corrections made for volume. See text for conditions of chromatography.*

the presence of five $NADH_2$-oxidizing enzymes in *Streptococcus faecalis*. One of these, an FMN-containing diaphorase which was purified 600-fold, was active with 2,6-DCPIP, ferricyanide, and certain benzoquinones and naphthoquinones. The other four enzymes were characterized as being an oxidase, a peroxidase, a menadione reductase, and a cytochrome *c* reductase, respectively.

Two fractions which specifically catalyze the oxidation of $NADH_2$ have been isolated from *Lactobacillus casei* by Walker and Kilgour (24, 25). The first requires FMN for maximal activity and can utilize 2,6-DCPIP, ferricyanide, or *p*-benzoquinone as electron acceptors. The other fraction, which contains bound FAD, possesses both peroxidase and oxidase activities which could not be separated.

Of special interest are the diaphorases which can utilize as acceptors the simple benzoquinones and naphthoquinones that are models of compounds having a very broad distribution in nature. Many of the organisms surveyed by Wosilait and Nason (28) were found to contain such activities, and these investigators isolated from *Escherichia coli* both menadione reductase and a quinone reductase. Menadione reductase from this source has been further purified and studied by Bragg (2). In addition, Asano and Brodie (1) isolated menadione reductases from extracts of *Mycobacterium phlei*. An $NADH_2$-specific menadione reductase from *Hydrogenomonas eutropha* has also been described by Repaske and Lizotte (15).

Wosilait (27), in his studies of menadione reductase from dog liver, found that this enzyme was active with both $NADH_2$ and $NADPH_2$, and that it was able to reduce vitamin K_1. Märki and Martius (11) described a similar vitamin K_1 reductase which they were able to obtain in a very highly purified form from bovine liver. Ernster et al. (5) reported on the occurrence of an abundant "DT diaphorase" from rat liver which was active with both $NADH_2$ and $NADPH_2$. This enzyme could utilize menadione as well as 2,6-DCPIP and ferricyanide as electron acceptors, and was inhibited by Dicumarol.

In the present study, enzyme II is seen to be a very active menadione reductase, relatively specific for $NADH_2$. It is inhibited by Dicumarol, 2,4-dinitrophenol, PCMS, Atabrine, and antimycin A, but not by amobarbital. Enzyme III, also an active menadione reductase, functions almost equally with either $NADH_2$ or $NADPH_2$. It is not inhibited by antimycin A. These enzymes from *A. aerogenes* differ from the menadione reductase isolated from *E. coli* by Bragg (2), not only in their sensitivity to inhibitors, but also in the relative ease with which they are obtained in the soluble state. Menadione reductase from *E. coli* must be extracted from a particulate fraction.

This difference between the two bacteria with regard to intracellular associations is not limited to menadione reductase. Fujita et al. (6) found that, in *E. coli*, cytochome b_1 is also localized in the particulate fraction and requires vigorous conditions for its solubilization. Preparations have been obtained which contain this cytochrome in association with either formate dehydrogenase or formate dehydrogenase plus nitrate reductase. In contrast, cytochrome b_1 is readily obtained in a soluble state from *Micrococcus denitrificans* and *Pseudomonas denitrificans* (23), as well as from *A. aerogenes*.

From the data in the present study, one cannot determine whether cytochrome b_1 is itself catalytically active, or whether it co-chromatographs with another diaphorase. In either case, the cytochrome-containing fraction is relatively inactive as a diaphorase under the conditions used. It is of interest that cytochrome b_1 from *E. coli* is active as an electron acceptor with purified menadione reductase from the same organism (3).

The requirement for FMN and the relative ease with which the native flavin is dissociated upon chromatography appear to be general properties of the diaphorase fractions prepared from *A. aerogenes*. Flavin is not lost appreciably upon overnight dialysis, and it is likely that the cellulose exchanger is directly involved in separating

the flavins from the enzymes. This situation resembles that of the vitamin K_1 reductase from dog liver (27), in which the enzyme does not exhibit a requirement for flavin until after chromatography on DEAE-cellulose. In the latter case, the enzyme is more strongly activated by FAD than by FMN.

The yellow fluorescent band shown in Fig. 4 may be free flavin, since the conditions under which it appears are similar to those described by Huennekens et al. (7) for the chromatography of FMN on DEAE-cellulose. The sensitivity to FMN of the FMN-specific diaphorase system reported here is about 10-fold that of the $NADPH_2$-cytochrome *c* reductase of yeast (7); this may be useful as an analytical tool for flavin identification.

The instability to dilution is another characteristic of diaphorases from *A. aerogenes*. As seen in Fig. 5, the presence of FMN and concentrated phosphate buffer in the collecting tubes appears to minimize the dilution-inactivation which probably is responsible for the poor recoveries in the experiments illustrated in Fig. 1. The unstable diaphorase (Fig. 5) is apparently an extreme example of this lability.

The physiological roles of the isolated diaphorases are, of course, still unknown, since what was measured is the ability of these enzymes to catalyze the transfer of electrons from $NADH_2$ and $NADPH_2$ to arbitrarily chosen electron acceptors. Elucidation of the pathways of reduced pyridine nucleotide oxidation will require further studies both of the isolated components of the systems and of their interactions within the cell.

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