The Purification and Properties of Formate Dehydrogenase and Nitrate Reductase from Escherichia coli*

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HARRY G. ENOCH† AND ROBERT L. LESTER
From the Department of Biochemistry, College of Medicine, University of Kentucky, Lexington, Kentucky 40506

SUMMARY

The membrane-bound formate dehydrogenase of Escherichia coli grown anaerobically in the presence of nitrate was solubilized with deoxycholate and purified to near homogeneity. The purification procedure included ammonium sulfate fractionation and chromatography on Bio-Gel A in the presence of the nonionic detergent, Triton X-100. This detergent caused a significant decrease in the molecular weight of the soluble formate dehydrogenase complex and allowed the enzyme then to be resolved from other membrane components. Anaerobic conditions were required throughout due to the sensitivity of the enzyme to oxygen inactivation. Formate dehydrogenase was judged to be at least 93 to 99% pure by the following procedures: polyacrylamide gel electrophoresis in the presence of Triton X-100 and sodium dodecyl sulfate, gel filtration, and sedimentation velocity procedures. The purified enzyme exists as a detergent-protein complex (0.20 g of Triton X-100/g of protein) which has an $s_{20,w}$ of 18.1 S and a Stokes radius of 76 A. This corresponds to a molecular weight of 590,000 ± 59,000. The enzyme had an absorbance spectrum of a b-type cytochrome which could be completely reduced by formate. The heme content corresponds to an equivalent weight of 154,000 which suggests a tetrameric structure for the enzyme. Formate dehydrogenase was found to contain (in relative molar amounts): 1.0 heme, 0.95 molybdenum, 0.96 selenium, 14 non-heme iron, and 13 acid-labile sulfide. Neither FAD nor FMN could be detected. The enzyme contains three polypeptides, designated α, β, and γ, whose molecular weights were estimated by gel electrophoresis in the presence of sodium dodecyl sulfate to be 110,000, 32,000, and 20,000, respectively. After separation of the polypeptides by gel filtration in the presence of sodium dodecyl sulfate $α$, $β$, and $γ$ were found in 1:1.2:0.85 molar ratios. A study of the enzyme obtained from cells grown with $[^{75}S]$selenite showed that only the $α$ polypeptide contained significant amounts of selenium. The enzyme will catalyze the formate-dependent reduction of phenazine methosulfate, dichlorophenolindophenol, methylene blue, nitroblue tetrazolium, benzyl viologen, methyl viologen, ferricyanide, and coenzyme Q. Cyanide, azide, $p$-hydroxymercuribenzoate, iodoacetamide, and oxygen inhibit the enzyme.

The procedure which was designed for the purification of formate dehydrogenase also yields a highly purified preparation of nitrate reductase. This nitrate reductase has been shown to contain significant amounts of heme (ENOCH, H. G., AND LESTER, R. L. (1974) Biochem. Biophys. Res. Commun. 61, 1234-1241). The enzyme contains three polypeptides with molecular weights of 155,000, 63,000, and 19,000. When measured in the presence of Triton X-100 the Stokes radius of nitrate reductase is 75 A and the $s_{20,w}$ is 16 S which corresponds to a molecular weight of 498,000.

Formate dehydrogenase plays a versatile role in the oxidative metabolism of Escherichia coli. Depending upon the growth conditions formate can provide electrons for the reduction of oxygen to water (formate oxidase), nitrate to nitrite (formate-nitrate reductase), and $H^+$ to hydrogen (formate hydrogenlyase). The activity of the formate dehydrogenase(s) of each of these membrane-associated electron transport systems requires the presence of trace amounts of selenium, molybdenum, and iron in the culture medium (1-3). Previous studies (4) have shown that the absence of selenium and molybdenum in the aerobic culture medium has no effect on the activity of a number of oxidative enzyme systems or on the growth rate and cell yield obtained with a number of different carbon sources. This suggests that formate dehydrogenase may be a unique metalloenzyme in E. coli. Definition of the molecular characteristics of formate dehydrogenase requires highly purified preparations of the enzyme not heretofore obtained. Partially purified preparations have been shown to contain cytochrome $b$ (5, 6), nitrate reductase (5), flavin (5, 6), and selenium (7). Difficulties in purifying the enzyme have been due in part to its oxygen sensitivity and its firm association with other membrane components. We undertook to purify formate dehydrogenase from cells grown anaerobically in the presence of nitrate. These conditions lead to the formation of high levels of formate dehydrogenase, cytochrome $b$, and nitrate reductase (3, 8, 9) which are thought to be the essential components of the formate-nitrate reductase system (10). We now report a procedure for the preparation of highly purified cytochrome-containing forms of formate dehydrogenase and nitrate reductase. In addition to being a hemo-
protein formate dehydrogenase contains significant amounts of selenium, molybdenum, and non-heme iron, but no flavin. Preliminary accounts of part of this work have been reported (11, 12).

**EXPERIMENTAL PROCEDURE**

**Materials**

Triton X-100, seinitation grade (Research Products International), Brij detergents (Atlas Chemical Industries), sodium dodecyl sulfate (Fisher Scientific Co.), and deoxycholic acid (Armour Pharmaceuticals) were used without further purification. Tobacco mosaic virus was a generous gift of Dr. John Shaw. [Se]Selenite and [14C]bicarbonate were purchased from New England Nuclear. Bio-Gel A-1.5m, Bio-Gel A-15m, and DEAE Bio-Gel A were purchased from Bio-Rad Laboratories; coenzyme Q10, p-hydroxymercuribenzoate, isodesmoside, N-ethylmaleimide, n-heptyl hydroxyquinoline-N-oxide, FAD, FMN, phenazine methosulfate, dichloroindolindolophenol, nitroblue tetrazolium, and all proteins (unless otherwise noted) were from Sigma Chemical Co.; methyl viologen and benzyl viologen were from Schwarz/Mann; and methylene blue was from Fisher Scientific Co.

**Sedimentation Coefficients**

Density gradient centrifugation was performed by the method of Martin and Ames (13). A sample (0.1 ml) was layered on a 12-ml density gradient of sucrose (15 to 50%) in 50 mM Tris-HCl, pH 8. The tubes were centrifuged at 35,000 rpm for 16 hours at 4°C in a Beckman SW 41 rotor. Each sample included β-galactosidase (kindly provided by A. S. L. Hu, University of Kentucky) as a marker; β-galactosidase, s20,W = 10.1 S (14), was assayed with o-nitrophenyl-β-D-galactoside (15).

The sedimentation coefficient was also determined in a Beckman model E analytical ultracentrifuge using an An-E rotor. The enzyme solution (2 ml) containing 0.3 mg of purified formate dehydrogenase/ml in 0.1% Triton X-100/10 mM NaCl/10 mM Tris-HCl, pH 6.8, was placed in a 30-mm-single-sector cell. Centrifugation was conducted at 5°C and 42,040 rpm. A single, symmetrical peak was observed which remained unchanged throughout the experiment.

**Polyacrylamide Gel Electrophoresis**

Triton gel electrophoresis was carried out in 3% polyacrylamide gels (5 × 65 mm) in 0.1% Triton X-100/0.1 M NaCl/10 mM Tris-HCl, pH 8. A sample (0.1 ml) was layered on a 12-ml density gradient of sucrose (15 to 50%) in 50 mM Tris-HCl, pH 8. The tubes were centrifuged at 35,000 rpm for 16 hours at 4°C in a Beckman SW 41 rotor. Each sample included β-galactosidase (kindly provided by A. S. L. Hu, University of Kentucky) as a marker; β-galactosidase, s20,W = 10.1 S (14), was assayed with o-nitrophenyl-β-D-galactoside (15).

**Filtration in Presence of Sodium Dodecyl Sulfate**

A column (0.9 X 92 cm) of Bio-Gel A-1.5m was equilibrated with 0.1% sodium dodecyl sulfate/50 mM sodium phosphate, pH 7.2. The column was operated at room temperature with a flow rate of 2.9 ml/hour.

**Detergent Studies**

The amount of Triton X-100 bound to formate dehydrogenase was determined by a modification of the method described by Helenius and Simons (26) using gel filtration or density gradient centrifugation instead of gel filtration to ensure the separation of Triton X-100 micelles from the detergent-enzyme complex. Purified formate dehydrogenase (1.8 mg) was adsorbed to a column (0.5 X 30 cm) of DEAE Bio-Gel A equilibrated with 0.1% Triton X-100, 10 mM Tris-HCl, pH 8. After washing with 2 column volumes of the equilibration buffer the formate dehydrogenase was eluted with 1 M NaCl in the equilibration buffer. Fractions were assayed for Triton X-100 and protein, and milligrams of bound Triton X-100 per mg of protein were calculated.

The partial specific volume of Triton X-100 was measured at 23° as described by Tanford et al. (23). The viscosity of 0.1% Triton X-100/1 mM NaCl/10 mM Tris-HCl, pH 8, was determined in an Ostwald viscometer at 5°C using distilled water and acetone as standards.

**Stokes Radius**

The Stokes radius of each enzyme-Triton X-100 complex was determined by gel filtration according to the method of Tanford et al. (23). A column (0.9 X 140 cm) of Bio-Gel A-1.5m was equilibrated with 0.1% Triton X-100/1 mM NaCl/10 mM Tris-HCl, pH 8. A sample (0.4 ml) of purified formate dehydrogenase or nitrate reductase was applied and the column was operated at a flow rate of 5.5 ml/hour (4°) 0.9-ml fractions were collected. The column was calibrated in the same manner using standard proteins eluting Triton X-100 from the buffer. The void volume and the total volume were determined using tobacco mosaic virus and sucrose, respectively. Published values of the Stokes radii of standard proteins were used (24).

**Enzyme Assays Methods**

**Subunit Molecular Weight by Gel Filtration**

A column (0.9 X 62 cm) of Bio-Gel A 1.5m was equilibrated with 0.1% sodium dodecyl sulfate/50 mM sodium phosphate, pH 7.2. Samples of 1% sodium dodecyl sulfate/1% mercaptoethanol/100 mM glycerol/50 mM sodium phosphate, pH 7.2, were heated at 100°C for 1 hour and 0.2 ml was layered on the column. The column was operated at room temperature and 0.8-ml fractions were collected. The distribution coefficient, Kav, equals Vv - V0/Vt - V0 (19). The void volume, Vv, and the total volume, Vt, were measured with blue dextran and sucrose, respectively. For measurement of Vv, the elution volume of blue dextran was determined (20). Published values of the molecular weights of standard proteins were used (17, 21).

**Separation of Polypeptides of Formate Dehydrogenase by Gel Electrophoresis in Presence of Sodium Dodecyl Sulfate**

Purified formate dehydrogenase (1.9 mg) was adsorbed to a column of DEAE-Bio-Gel A, washed with 2 column volumes of 0.1% sodium dodecyl sulfate/250 mM sodium phosphate, pH 8, to remove the Triton, and then eluted with 1 mM NaCl/10 mM Tris-HCl, pH 8. Fractions containing enzyme activity (all of the activity was recovered) were pooled and ammonium sulfate was added to 60% saturation. After centrifugation the precipitate was dissolved in 20 mM Tris/HCl, pH 7.2, and this preparation was heated at 100°C for 15 min. Iodoacetamide was added to a final concentration of 0.4 mM and the mixture was incubated for 4 hours at 30°C. The sample then was dialyzed against 1000 volumes of 0.1% sodium dodecyl sulfate/55 mM sodium phosphate, pH 7.2, for 48 hours at 4°C. Solid sucrose was added (27%) and 0.45 ml of this sample (0.79 mg of protein) was layered on a column (0.9 X 175 cm) of Bio-Gel A 1.5m equilibrated with 0.1% sodium dodecyl sulfate/25 mM sodium phosphate, pH 7.2. The column was operated at room temperature with a flow rate of 2.9 ml/hour.
Nitrile reductase was assayed at 30° by the method of Showe and DeMoco (0). One unit of enzyme activity is defined as that which reduces 1 μmol of nitrate/min.

Other Assays Methods

Since Triton X-100 interfered with the measurement of protein by the method of Lowry et al. (27), protein was measured by the method of Hirs (20) using bovine serum albumin (Pentex) as a standard.

Heme was estimated as pyridine hemochromes (28). Acid extractable and non-extractable FAD and FMN were assayed fluorometrically by the method of Wilson and King (29). In the determination of acid-extractable flavin, heat-denatured protein was digested either with 0.5 mg of Pronase (Calbiochem)/mg of protein for 30 min at 60° or with 1 mg of trypsin/mg of protein for 2 hours at 37°. Quinone content was measured after extraction with n-hexane. The extract was taken to dryness and the residue was resuspended in absolute ethanol. The concentration of coenzyme Q and vitamin K₃ was determined from the decrease in absorbance at 275 and 288 nm, respectively, after reduction by sodium borohydride (30).

Molybdenum was estimated by the method of Johnson and Arkley (31). Samples (1 ml) were mixed with 0.5 ml of perchloric acid and digested at 200° for 2 hours, then 0.1 ml of nitric acid was added and digestion was continued for 2 more hours. Selenium and molybdenum also were determined by neutron activation analysis (General Activation Analysis, Inc., San Diego, Calif.). Non-heme iron was assayed with bathophenanthroline (32) and acid-labile sulfide was assayed with dimethylphenyleneidineamine (33).

The phospholipid content of formate dehydrogenase was measured by phosphorus analysis (34) of the chloroform/methanol extract (35) of the perchloric acid-precipitated enzyme. Triton X-100 was assayed by the method of Carew (36) using benzene to extract the detergent-cobalt-thiocyanate adduct instead of ethylene dichloride. RNA was measured as hot trichloroacetic acid-soluble pentose by the procedure of Schneider (37) using ribose as a standard. Carbohydrate was measured by the phenol-sulfuric acid method of Ashwell (38). Radioactivity of ¹¹⁵Se and ¹⁴C were measured by scintillation counting in Triton-toluene counting fluid (90) unless otherwise noted.

Growth Conditions

_Escherichia coli_ (HfrH thi⁻) was grown anaerobically on a basal anaerobic growth medium (3) containing 0.1 μM sodium selenite, 0.1 μM sodium molybdate, and 1% potassium nitrate and harvested at the end of exponential growth.

Purification of Formate Dehydrogenase and Nitrate Reductase

Step 1—Cells were grown in 45-liter batches, rapidly chilled to 0–4°, and harvested by centrifugation. The cells were washed twice with 0.9% NaCl/50 mM sodium phosphate, pH 7.2, and the cell paste was stored frozen at -15°. The thawed cell paste was stored frozen at -15°. The thawed cell paste was stored frozen at -15°. The thawed cell paste from four liters of culture were resuspended in absolute ethanol. The concentration of coenzyme Q and vitamin K₃ was determined from the decrease in absorbance at 275 and 288 nm, respectively, after reduction by sodium borohydride (30). The reddish brown precipitate was resuspended in 0.1 M Tris-HCl, pH 7.2, and dialyzed for 24 hours against 20 volumes of this buffer and then for an additional 24 hours against 20 volumes of 0.1 M Tris-HCl/1 M NaCl, pH 7.2. The dialyzed solution was centrifuged at 15,000 × g for 1 hour and the supernatant was mixed with enough solid ammonium sulfate to give a final concentration of 4 to 6 mg of protein/ml.

The supernatant was brought to 50% saturation by adding more saturated ammonium sulfate solution. The mixture was stirred for 1 hour and the precipitate was collected by centrifugation at 15,000 × g for 1 hour. The reddish brown precipitate was resuspended in 0.1 M Tris-HCl, pH 7.2, and dialyzed for 24 hours against 20 volumes of this buffer and then for an additional 24 hours against 20 volumes of 0.1 M Tris-HCl/1 M NaCl, pH 7.2. The dialyzed solution was centrifuged at 15,000 × g for 1 hour and the supernatant was mixed with enough 10% Triton X-100 to give a final concentration of 0.5%, detergent. All detergent concentrations are expressed as a weight per volume per cent.

Step 2—After 1 hour this solution was layered on a column (2.5 × 42 cm) of DEAE-saturated (Bio-Gel A-5m) eluted with anaerobic 0.5% Triton X-100/0.1 M NaCl/0.1 M Tris-HCl, pH 7.2. The column was eluted with the same solution at a flow rate of 30 ml/hour and fractions were collected in the presence of air (Fig. 1). The relevant fractions were assayed and pooled within 3 hours of elution and then dialyzed against two changes each of 15 volumes of 0.1% Triton X-100/0.1 M NaCl in the same Triton-Tris buffer. The column was developed with a 2-liter linear gradient from 0 to 0.2 M NaCl in the same Triton-Tris buffer (Fig. 2). Formate dehydrogenase and nitrate reductase were separated at this step and both enzymes were stored at 4–8° under an atmosphere of N₂.

**RESULTS**

Solubilization studies

Preliminary experiments showed that all of the formate dehydrogenase could be recovered in the membrane-cell envelope fraction after cell lysis with lysozyme-EDTA (4). Formate dehydrogenase could be solubilized (i.e. released in a form not denatured by centrifugation at 100,000 × g for 1 hour) by treatment of the membrane-cell envelope fraction with the following detergents: Triton X-100, Brij 30T, Brij 30 (0.2%), or deoxycholate plus ammonium sulfate at 0°. Less than 50% of the original formate dehydrogenase activity was found to be soluble after treatment with Brij 30T or Brij 30 due to incomplete solubilization and loss of activity. Triton X-100 was less

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**Fig. 1.** Purification of formate dehydrogenase and nitrate reductase by gel filtration on Bio-Gel A-1.5m. Chromatography was performed as described under "Experimental Procedure," Step 5. A sample of 50 ml (140 mg of protein) was applied; 9.2-ml fractions were collected. Formate dehydrogenase activity was measured by the nitroblue tetrazolium method. The black bar denotes the fractions pooled for Step 6.
selective than deoxycholate, yielding more than twice as much total soluble protein. No formate dehydrogenase activity was solubilized by treatment with: Brij 35; Brij 56, Brij 96, or Brij 99 (0.2%) at 20°C; or the chaotropic agents (40) sodium per-chlorate, sodium thiocyanate, or guanidine HCl (0.5 to 1.0 M) at 0 or 30°C. Quantitative release of formate dehydrogenase could be obtained with deoxycholate plus ammonium sulfate, although somewhat less solubilization was usually noted in large scale preparations (for example, see Table I). The solubilization of formate dehydrogenase from membranes by deoxycholate-ammonium sulfate was adapted from the procedure of Linnane and Wrigley (6). We noted that the amount of formate dehydrogenase and nitrate reductase solubilized was dependent on the concentration of ammonium sulfate. Formate dehydrogenase was maximally solubilized between 25 and 35% saturated ammonium sulfate, while nitrate reductase was maximally solubilized at less than 20% saturated ammonium sulfate.

Enzyme Purification

The method adopted for the solubilization and purification of formate dehydrogenase and nitrate reductase from Escherichia coli grown anaerobically in the presence of nitrate is described under “Experimental Procedure.” After solubilization and ammonium sulfate fractionation, formate dehydrogenase and nitrate reductase were not separated by chromatography on Bio-Gel A-15m, DEAE-cellulose, or hydroxylapatite when these steps were carried out in the absence of Triton X-100. When the preparation from Step 4 (Table I) was subjected to gel filtration in the absence of detergent (Fig. 3), both formate dehydrogenase and nitrate reductase eluted in a broad skewed peak, indicating size heterogeneity (average molecular weight greater than one million). Formate dehydrogenase and nitrate reductase activities also co-sedimented during sucrose density gradient centrifugation with an $s_{20,w}$ of about 27 S. However, when gel filtration was carried out in the presence of Triton X-100, formate dehydrogenase and most of the nitrate reductase then eluted at a sharp peak with a reduced molecular weight (Fig. 3). Thus, the presence of Triton X-100 reduces the particle size of both formate dehydrogenase and nitrate reductase. The $s_{20,w}$ of formate dehydrogenase, Step 4, determined by sucrose density gradient centrifugation in the presence of Triton X-100 was 16 S. After the gel filtration step, formate dehydrogenase and nitrate reductase were separated by chromatography on DEAE-agarose in the presence of Triton X-100 (Fig. 2, Table I). It can be seen in Table I that the pooled formate dehydrogenase fractions (Step 6a) have no detectable nitrate reductase activity and the

Table I

<table>
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<th>Step</th>
<th>Total protein</th>
<th>Formate dehydrogenase</th>
<th>Nitrate reductase</th>
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<tr>
<td></td>
<td>mg</td>
<td>total activity</td>
<td>specific activity</td>
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<tr>
<td></td>
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<td>µmol/min X 10⁻³</td>
<td>µmol/min/mg</td>
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<tr>
<td></td>
<td></td>
<td>total activity</td>
<td>specific activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>µmol/min X 10⁻³</td>
<td>µmol/min/mg</td>
</tr>
<tr>
<td>1. Cells</td>
<td>35,400</td>
<td>45.8</td>
<td>1.3</td>
</tr>
<tr>
<td>2. Crude membranes</td>
<td>13,000</td>
<td>45.3</td>
<td>3.5</td>
</tr>
<tr>
<td>3. Deoxycholate extract</td>
<td>5,880</td>
<td>33.5</td>
<td>5.7</td>
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<tr>
<td>4. Ammonium sulfate fractionation, 40 to 50%</td>
<td>1,700</td>
<td>27.9</td>
<td>16.4</td>
</tr>
<tr>
<td>5. Bio-Gel A-1.5m</td>
<td>492</td>
<td>21.6</td>
<td>44</td>
</tr>
<tr>
<td>6. DEAE Bio-Gel</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>80</td>
<td>15.7</td>
<td>196</td>
</tr>
<tr>
<td>b</td>
<td>266</td>
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pooled nitrate reductase fractions (Step 6b) had no detectable formate dehydrogenase activity.

A 150-fold purification of formate dehydrogenase was obtained (Table I) with yields ranging from 34 to 68%. For good recoveries of formate dehydrogenase activity it was essential that the entire purification be carried out in an anaerobic atmosphere. When Steps 1 to 5 (Table I) were carried out anaerobically 100% of the formate dehydrogenase activity was accounted for (including discarded fractions), while in the presence of air, the recovery of formate dehydrogenase at Step 5 varied from 1 to 20%, due to irreversible inactivation of the formate dehydrogenase activity. When Step 6 was performed aerobically 90% of the formate dehydrogenase activity was lost.

**Purity of Formate Dehydrogenase**

The formate dehydrogenase obtained at the final step (Step 6a) of the purification in most preparations contained a number of aggregated forms of the enzyme. The conditions required to prevent the aggregation of the enzyme have not yet been found. A major problem in the assessment of the purity of formate dehydrogenase is the difficulty in obtaining the enzyme as a single disaggregated species.

Two types of results were obtained at the final step (Step 6) of the purification. During DEAE-agarose chromatography formate dehydrogenase was eluted as a single peak (most preparations, Fig. 4) or as two peaks (Fig. 2) of activity, indicating the presence of multiple forms of the enzyme. We investigated the possibility that these two peaks represent different aggregation states of the enzyme. Electrophoresis was performed on Fractions a to f (Fig. 2), and the gels were stained for protein (Fig. 5A). Fractions d to f appeared to contain a single form of the enzyme while Fractions a to c contained a number of different migrating forms of the enzyme. The protein band observed in Fractions d to f was absent in Fractions a and b. With Fractions a and b another protein band was observed which barely penetrated the gels indicating the presence of a very large protein. Similar gels were run on Fractions b and e and stained for formate dehydrogenase activity (b' and e' of Fig. 5A) and bands were observed which corresponded exactly to the protein-stained bands. Sodium dodecyl sulfate gel electrophoresis experiments showed that Fractions a to f did not differ in their polypeptide composition. The same three polypeptides, designated α, β, and γ, are observed in each fraction tested (Fig. 5B).

In other preparations formate dehydrogenase eluted from the DEAE-agarose column in a single peak (Fig. 4). Analysis of these single peaks by Triton gel electrophoresis showed a single form of the enzyme in some preparations, while in other preparations multiple aggregation forms of the enzyme were observed. The cause of aggregation is not known; however, it appears to occur after the gel filtration step and could possibly be initiated under the low ionic strength conditions of Step 6 (Table I). Throughout the remainder of this report the operational term “homogeneous formate dehydrogenase” will be used to denote that enzyme at Step 6a which showed a single band of enzyme activity and protein after Triton gel electrophoresis. Although we cannot rigorously exclude that multiple forms of formate dehydrogenase could arise in the course of purification.
purification via limited deamination or other hydrolytic reactions, the facile aggregation of isolated enzyme leads us to retain the simple notion that the multiple forms have arisen via aggregation. Aggregation of the homogeneous formate dehydrogenase occurred when gel electrophoresis was carried out in the absence of Triton X-100 as judged by the appearance of multiple forms of slower migrating enzyme (detected by both protein and enzyme stains, not shown).

Formate dehydrogenase appears to be very nearly pure as judged by Triton gel electrophoresis; however, a small amount of protein (1% to 2%) was consistently detected as two or three bands near the α polypeptide after sodium dodecyl sulfate gel electrophoresis (Fig. 5B). These bands could represent incompletely dissociated polypeptides since they are observed in elevated amounts when the sample is not heated during sodium dodecyl sulfate dissociation. In any case, the enzyme is at least 99% pure if α, β, and γ are all considered to be subunits of formate dehydrogenase. It can be seen in Fig. 5B that, although each fraction contains the γ polypeptide, there was some variation in the density of staining of this polypeptide. This difference could be caused by the difficulty in fixing and staining small proteins in gels (41). It is also possible that the difference could reflect the loss of some of the γ polypeptide during purification. On the other hand, the γ polypeptide could be a tightly associated impurity in formate dehydrogenase amounting to about 7% of the total protein. Sedimentation velocity studies with homogeneous formate dehydrogenase confirming to exist as a Triton X-100-polypeptide complex. Detergent binding with Triton X-100-protein complex. Detergent binding interactions do not involve disulfide bonds. After gel electrophoresis in the presence of sodium dodecyl sulfate, the molecular weight of the three polypeptides of formate dehydrogenase, α, β, and γ, were estimated to be 110,000, 32,000, and 20,000, respectively (Fig. 6). Similar molecular weights (120,000, 35,000, and 20,000) were observed after gel filtration in the presence of sodium dodecyl sulfate (Fig. 7).

In one experiment the polypeptides of formate dehydrogenase were separated by gel filtration in the presence of sodium dodecyl sulfate (Fig. 8). The three protein peaks corresponded to pure α, β, and γ polypeptides, as judged by sodium dodecyl sulfate gel electrophoresis (not shown). In this experiment the amounts of α (501 µg), β (175 µg), and γ (50.7 µg) polypeptides recovered correspond to 4.55, 5.36, and 2.5 nmol, respectively. Thus, the molar ratio of α:β:γ is 1.1:1.2:0.55. A number of determinations of the relative staining of polypeptide subunits after sodium dodecyl sulfate gel electrophoresis indicated a 1:1 ± 0.1 molar ratio of α:β. In these experiments the molar ratio of α:γ varied from 1:0.2 to 1:1. Further experiments are required to ascertain whether this variation reflects the partial loss of a formate dehydrogenase subunit or the presence of a variable amount of impurity.

**Physical Properties of Formate Dehydrogenase**

**Subunit Studies**—The dissociation of formate dehydrogenase by sodium dodecyl sulfate (as judged after gel electrophoresis in the presence of this detergent) required incubation at 100° for several minutes or at 20° in the presence of 8 M urea for several hours. Reducing agent was not required, indicating that the subunit interactions do not involve disulfide bonds. After gel electrophoresis in the presence of sodium dodecyl sulfate, the molecular weight of the three polypeptides of formate dehydrogenase, α, β, and γ, were estimated to be 110,000, 32,000, and 20,000, respectively (Fig. 6). Similar molecular weights (120,000, 35,000, and 20,000) were observed after gel filtration in the presence of sodium dodecyl sulfate (Fig. 7).

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**Molecular Weight**—Purified formate dehydrogenase appears to exist as a Triton X-100-protein complex. Detergent binding measurements (Fig. 9) showed that homogeneous formate dehydrogenase contains 0.20 ± 0.3 mg of Triton X-100/mg of protein. The molecular weight of formate dehydrogenase was estimated by the procedure of Tanford et al. (23) who showed that the molecular weight (M) of a protein in a protein-detergent complex may be determined by the relation

$$M = \frac{\text{e}_{\text{protein}} - \text{e}_{\text{detergent}}}{\text{M} \cdot \text{M}_{\text{detergent}} - \text{M} \cdot \text{M}_{\text{protein}}}$$

![Fig. 6 (left). Subunit molecular weights of formate dehydrogenase and nitrate reductase as determined by sodium dodecyl sulfate gel electrophoresis. Samples were treated as described under Fig. 5B. The upper line and lower line refer to determinations made on separating gels of 5 and 8% acrylamide, respectively. Electrophoresis and protein staining were carried out as described under "Experimental Procedure." The standard proteins and their molecular weights are thyroglobulin (335,000), β-galactosidase (130,000), conalbumin (78,000), bovine serum albumin (66,000), catalase (60,000), ovalbumin (43,000), aldolase (40,000), glyceraldehyde-3-phosphate dehydrogenase (30,000), trypsin (23,000), and hemoglobin (15,500). α, β, and γ refer to the subunits of formate dehydrogenase; α, β, and γ refer to the subunits of nitrate reductase.](image)

![Fig. 7 (right). Subunit molecular weights of formate dehydrogenase as determined by gel filtration on Bio-Gel A-1.5m in the presence of 0.1% sodium dodecyl sulfate. Sample preparation and chromatography are described under "Experimental Procedure." The standard proteins and their molecular weights are bovine serum albumin (68,000), hexokinase (27,500), and lysozyme (14,300). α, β, and γ refer to the subunits of formate dehydrogenase.](image)
of the formate dehydrogenase-Triton X-100 complex determined large because of the uncertainty of tip. An error of 3% in $, indicates that we are looking at a single molecular species of cytochrome c (17 Å).

The sedimentation velocity at 5° in the presence of 0.1% Triton X-100 and 1 M NaCl was 7.95 S (s~2w = 18.1 S). The Stokes radius of homogeneous formate dehydrogenase determined by sedimentation velocity at 5° in the presence of 0.1% Triton X-100 and 1 M NaCl was 76 Å (Fig. 10). Only one sharp peak of formate dehydrogenase activity was observed in these experiments, which for IYD of Triton X-100 in 1 M NaCl. The sedimentation coefficient, R, is the Stokes radius of the protein-detergent complex, tip is the partial specific volume of the protein. Nitrate reductase from the same preparation contained less than 0.1 nmol of selenium/mg of protein, indicating that little selenium is nonspecifically incorporated into protein (for example, as the selenium analogs of the sulfur-containing amino acids) under these conditions. Somewhat higher levels of selenium (6.2 nmol/mg of protein or 0.95 mol/mol of heme) were detected by neutron activation analysis in another preparation of formate dehydrogenase.

The 75Se of formate dehydrogenase was not removed by dialysis at pH 7 or by precipitation with 10% trichloroacetic acid or 95% acetone. Jenkins (42) has shown that selenite can be nonenzymatically incorporated into protein. He suggested that the selenium was covalently bound between the sulfurs of half-cystine residues and showed that this form of selenium could be removed by dialysis of the selenoprotein at pH 11.5 or by treatment with 0.5 M mercaptoethanol, 50 mM sodium sulfite, or 5 mM KCN at 30° for 30 min. None of these treatments resulted in the release of 75Se from formate dehydrogenase suggesting that the enzyme may not contain selenium as seleno-trisulfide. 75Se could be released (trichloroacetic acid-soluble) by treatment of formate dehydrogenase with 3% H2O2 for 1 hour at 30°.

Heme—After DEAE-agarose chromatography the formate dehydrogenase-containing fractions were colored (reddish brown) and were found to contain heme. The heme content was coincident with formate dehydrogenase activity which was eluted from the DEAE-agarose column with 75Se radioactivity and protein (Fig. 4). From the specific radioactivity of selenium in the growth medium (114 cpm/μmol of selenium) and in formate dehydrogenase (496 cpm/μg of protein) the selenium content of formate dehydrogenase was estimated to be 4.4 nmol of selenium/mg of protein. Nitrate reductase from the same preparation contained less than 0.1 nmol of selenium/mg of protein, indicating that little selenium is nonspecifically incorporated into protein (for example, as the selenium analogs of the sulfur-containing amino acids) under these conditions. Somewhat higher levels of selenium (6.2 nmol/mg of protein or 0.95 mol/mol of heme) were detected by neutron activation analysis in another preparation of formate dehydrogenase.

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nearly constant (107 ± 13 pmol/min/mg of protein) in Fractions 31 to 34 (Fig. 12) which represent the bulk of the total activity of formate dehydrogenase. Purified formate dehydrogenase was adsorbed to a DEAE-agarose column, washed with 10 volumes of 10 mM Tris-HCl, pH 8, to remove Triton X-100, and then eluted with 0.2 M NaCl/10 mM Tris-HCl, pH 8. The spectra were recorded at 20° under anaerobic conditions in a cuvette of 1-cm path length. The sample contained 0.5 mg of protein/ml. The reduced spectrum was obtained 5 min after the addition of 50 mM formate and was identical (visible portion) to that obtained by the addition of oxidized enzyme at 414 nm) was again coincident with formate dehydrogenase activity and protein and a constant ratio of heme to protein (milligrams per ml) was observed, 5.8 ± 0.4. The specific activity of formate dehydrogenase was nearly constant (107 ± 13 pmol/min/mg of protein) in Fractions 31 to 34 (Fig. 12) which represent the bulk of the total activity (90%). The decreased specific activity in the later fractions could be due to the presence of some inactive formate dehydrogenase or other impurity.

The absorbance spectra of oxidized and reduced formate dehydrogenase were shown in Fig. 13. The spectra were obtained after the removal of Triton X-100 because this detergent absorbs strongly in the ultraviolet region (ε_max = 24). The oxidized and reduced absorbance maxima are those of a b-type cytochrome and are essentially the same as those previously found in partially purified preparations of formate dehydrogenase (5, 6). The cytochrome of formate dehydrogenase became completely reduced in the presence of formate (i.e. compared to dithionite reduction). Under anaerobic conditions this reduction was rapid: half of the cytochrome was reduced immediately (<2 s) and 95% reduction was obtained in less than 1 min at 20°. The heme content of purified formate dehydrogenase is 6.5 nmol/mg of protein. This is 4.2-fold higher than that reported for partially purified formate dehydrogenase obtained from aerobically grown E. coli (6).

Other Components—Chemical analysis showed that homogeneous formate dehydrogenase contains molybdenum, non-heme iron, and acid-labile sulfide (Table II). No acid-releasable flavin was found in our purified preparations of formate dehydrogenase-bound flavin was not detected after proteolytic digestion of the enzyme. The fluorescence of added FAD and FMN were unchanged after being carried through the same analyses in the presence of formate dehydrogenase, indicating that our lack of flavin detection was not due to quenching of the fluorescence (e.g. by heme) or reduction of flavin by the enzyme. Formate dehydrogenase has long been considered to be a flavin-containing enzyme (43) since flavin was detected in partially purified preparations of this enzyme (5, 6). Itagaki et al. (5), however, did not report the amount of flavin in their preparation and Linnane and Wrigley (6) reported the presence of only 0.35 mol of flavin/mol of heme in their preparation. It would now appear that the E. coli formate dehydrogenase is not a flavoprotein.

Quinone was not detected in our preparation of formate dehydrogenase (Table II). The removal of Triton X-100 (which interfered with the quinone measurement) had no effect on the reduction of cytochrome b by formate.

The purified formate dehydrogenase (Step 6a) contains less than 0.2% phospholipid and less than 0.4% RNA. Total sugar analysis by the phenol-sulfuric acid method revealed the presence of 0.1% Triton X-100/10 mM Tris-HCl, pH 7.4. The column was developed with the same buffer at a flow rate of 1.8 ml/hour (4-5°); 2.0-ml fractions were collected.

![Fig. 12. Rechromatography of purified formate dehydrogenase on Bio Gel A-15m. A sample of Step 6 formate dehydrogenase (4 mg of protein in 0.5 ml) was layered on a column (1 X 143 cm) of Bio-Gel A-15m equilibrated with 0.1% Triton X-100/10 mM NaCl/0.1 mM Tris-HCl, pH 7.4. The column was developed with the same buffer at a flow rate of 1.8 ml/hour (4-5°); 2.0-ml fractions were collected.](image1)

![Fig. 13. The oxidized and reduced absorption spectra of formate dehydrogenase. Purified formate dehydrogenase was adsorbed to a DEAE-agarose column, washed with 10 volumes of 10 mM Tris-HCl, pH 8, to remove Triton X-100, and then eluted with 0.2 M NaCl/10 mM Tris-HCl, pH 8. The spectra were recorded at 20° under anaerobic conditions in a cuvette of 1-cm path length. The sample contained 0.5 mg of protein/ml. The reduced spectrum was obtained 5 min after the addition of 50 mM formate and was identical (visible portion) to that obtained by the addition of solid sodium dithionite.](image2)

**TABLE II**

Composition of formate dehydrogenase

Details of the analytical procedures are given under "Experimental Procedure" or in the text (for [35Se] selenium analysis).

<table>
<thead>
<tr>
<th>Component</th>
<th>nmol/mg of protein</th>
<th>Molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heme</td>
<td>6.5</td>
<td>1</td>
</tr>
<tr>
<td>Molybdenum</td>
<td>6.2*</td>
<td>0.95</td>
</tr>
<tr>
<td>Molybdenum</td>
<td>4.8*</td>
<td>0.74</td>
</tr>
<tr>
<td>Selenium</td>
<td>4.4*</td>
<td>0.68</td>
</tr>
<tr>
<td>Selenium</td>
<td>6.2</td>
<td>0.95</td>
</tr>
<tr>
<td>Non-heme iron</td>
<td>89</td>
<td>14</td>
</tr>
<tr>
<td>Acid-labile sulfide</td>
<td>82</td>
<td>13</td>
</tr>
<tr>
<td>Coenzyme Q&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Vitamin K&lt;sub&gt;d&lt;/sub&gt;</td>
<td>&lt;0.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Flavin</td>
<td>&lt;0.1</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>&lt;2</td>
<td>&lt;0.3</td>
</tr>
</tbody>
</table>

* Determined by chemical assay.
* Determined by neutron activation analysis.
* Determined by 35Se specific radioactivity.
* Determination was made on detergent-free enzyme. The purified formate dehydrogenase was adsorbed to a DEAE-Bio-Gel A column, washed with 10 volumes of 10 mM Tris-HCl, pH 8, and then eluted with 0.2 M NaCl/10 mM Tris-HCl, pH 8.
of 2% carbohydrate (as glucose equivalents). We have not yet attempted to verify this result by identification of specific sugar residues. The composition of formate dehydrogenase is summarized in Table II.

**Catalytic Characteristics of Purified Formate Dehydrogenase**

The highest specific activity of formate dehydrogenase which we have observed was 220 μmol of phenazine methosulfate-dichlorophenolindophenol reduced/min/mg of protein, yielding a turnover number of 33,800 mol/min/mol of heme. The Kₘ for formate was 0.12 mM. It does not seem relevant to compare the specific activity of this enzyme with less pure preparations of others due to differences in assay procedures and to the fact that activity is rapidly lost in the presence of oxygen (see below). We have observed homogeneous preparations of formate dehydrogenase having specific activities with phenazine methosulfate-dichlorophenolindophenol as low as 20 μmol/min/mg of protein due to less than rigorous exclusion of oxygen at Step 5 or Step 6 of the purification.

**Acceptor Specificity**

Of the electron acceptors tested formate dehydrogenase showed the highest activity with phenazine methosulfate plus dichlorophenolindophenol (Table III). Dichlorophenolindophenol alone is a good acceptor for the enzyme. Itagaki et al. (5) reported that partially purified formate dehydrogenase was inactive with dichlorophenolindophenol. Although the basis for this difference is not clear it should be noted that the cytochrome b of their preparation was not reduced by formate. The particulate and the soluble, homogeneous formate dehydrogenase have similar relative activities with dichlorophenolindophenol and nitroblue tetrazolium (Table III), indicating that certain catalytic properties of the enzyme have been maintained.

### TABLE III

**Electron acceptor specificity of formate dehydrogenase**

All reactions were carried out anaerobically at 30°C. The reduction of electron acceptors (except coenzyme Q₆) was monitored spectrophotometrically. The reaction mixtures (4 ml) contained formate dehydrogenase, acceptor, 20 mM formate, and 0.15 M sodium phosphate, pH 7.2. Coenzyme Q₆ reduction was measured as described under "Experimental Procedure." All activities are based on a two-electron transfer. PMS, phenazine methosulfate; DCI, dichlorophenolindophenol.

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>Acceptor concentration</th>
<th>Wavelength</th>
<th>Relative formate dehydrogenase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>600</td>
<td>Homogeneous enzyme</td>
</tr>
<tr>
<td></td>
<td></td>
<td>600</td>
<td>Particulate enzyme</td>
</tr>
<tr>
<td>PMS + DCI</td>
<td>0.24 ± 0.087</td>
<td>600</td>
<td>100a</td>
</tr>
<tr>
<td>DCI</td>
<td>0.087</td>
<td>600</td>
<td>23</td>
</tr>
<tr>
<td>Ferricyanide</td>
<td>2.0</td>
<td>420</td>
<td>20</td>
</tr>
<tr>
<td>Nitroblue tetrazolium</td>
<td>0.066</td>
<td>530</td>
<td>15</td>
</tr>
<tr>
<td>Methyl viologen</td>
<td>1.0</td>
<td>600</td>
<td>0.2</td>
</tr>
<tr>
<td>Benzyl viologen</td>
<td>1.0</td>
<td>600</td>
<td>2.4</td>
</tr>
<tr>
<td>Methylene blue</td>
<td>0.04</td>
<td>650</td>
<td>30</td>
</tr>
<tr>
<td>Coenzyme Q₆</td>
<td>1.0</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>FAD</td>
<td>0.072</td>
<td>450</td>
<td>0</td>
</tr>
<tr>
<td>FMN</td>
<td>0.1</td>
<td>450</td>
<td>0</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>1.0</td>
<td>340</td>
<td>0</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>1.0</td>
<td>340</td>
<td>0</td>
</tr>
</tbody>
</table>

* Step 2 enzyme (Table I).
* Specific activity was 142 μmol/min/mg of protein.
* Specific activity was 5.2 μmol/min/mg of protein.

The lack of ferricyanide reductase activity in membranes suggests that the preparation may consist of oriented vesicles which are not accessible to bulky anions. The activity with methylene blue and viologen dye was similar to that previously reported (5). The purified E. coli enzyme could not use NAD⁺ or NADP⁺ as electron acceptors (Table III). Neither FAD nor FMN were active as acceptors (Table III) and neither were stimulatory in the assay of formate dehydrogenase with phenazine methosulfate and dichlorophenolindophenol. Quinone has been implicated as an essential component of the formate-nitrate reductase in E. coli (44). Coenzyme Q₆ was shown to be an acceptor for a particulate preparation of formate dehydrogenase; however, the specific activity was only 1% of that with methylene blue (44). We found coenzyme Q₆ to be a very effective electron acceptor for the purified formate dehydrogenase (Table III).

### TABLE IV

**Effect of inhibitors on various formate dehydrogenase activities**

The reactions were carried out as described in Table III. Purified formate dehydrogenase, Step 6a (Table I), and inhibitor were incubated anaerobically for 5 min at 30°C in 0.15 M sodium phosphate, pH 7.2, and then the reaction was initiated by the addition of electron acceptor and formate (20 mM). The same conditions apply for oxygen inhibition except the incubation and reaction were conducted in the presence of air. PMS, phenazine methosulfate; DCI, dichlorophenolindophenol.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Relative formate dehydrogenase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PMS + DCI</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>NaCN, 5 mM</td>
<td>0.5</td>
</tr>
<tr>
<td>NaN₃, 5 mM</td>
<td>9.9</td>
</tr>
<tr>
<td>Iodoacetamide, 5 mM</td>
<td>55</td>
</tr>
<tr>
<td>N-Ethylmaleimide, 5 mM</td>
<td>99</td>
</tr>
<tr>
<td>n-Heptyl hydroxyquinoline-N-oxide, 0.1 mM</td>
<td>60</td>
</tr>
<tr>
<td>Oxygen (air), 0.25 mM</td>
<td>61</td>
</tr>
<tr>
<td>p-Hydroxymercuribenzoate, 0.1 mM</td>
<td>41</td>
</tr>
</tbody>
</table>
results obtained here suggest that the enzyme may contain a number of different catalytic sites for interaction with these artificial electron acceptors.

The enzyme activity measured with dichlorophenolindophenol, ferricyanide, and nitroblue tetrazolium was inhibited by oxygen (Table IV). This inhibition may be related to the oxygen inactivation of the enzyme. It should be noted, however, that the inhibition is immediate, requiring no prior incubation with oxygen, while the time course of inactivation is much slower. This suggests that oxygen may compete with other acceptors.

Only the activity with phenazine methosulfate-dichlorophenolindophenol was inhibited by n-heptyl hydroxyquinoline-N-oxide, which is a potent inhibitor of formate-nitrate reduction in the particulate fraction of E. coli (47) and also in the system reconstructed from purified formate dehydrogenase, nitrate reductase, and coenzyme Q_2 (12). The level of this inhibitor used in the experiment in Table IV causes a much greater inhibition of formate-nitrate reduction (100%, Ref. 48) than formate-phenazine methosulfate-dichlorophenolindophenol reduction. We also found that formate-coenzyme Q_2 reduction is not affected at the same level of inhibitor.

**Stability**—Formate dehydrogenase activity is lost when the enzyme is exposed to oxygen. This instability is enhanced in low ionic strength buffers, detergents, at temperatures above 0°C, and at pH greater than 7. The enzyme was remarkably stable in air in the presence of 1 M NaCl (in 10 mM Tris-HCl, pH 8) with less than 5% loss of activity after 24 hours (0°C). The same incubation in the absence of NaCl resulted in a 90% loss of activity. The purified enzyme (Step 6a) could be stored under oxygen, while the time course of inactivation is much slower.

The enzyme activity measured with dichlorophenolindophenol, methylene blue, nitroblue tetrazolium, was inhibited by oxygen, while the time course of inactivation is much slower. This suggests that oxygen may compete with other acceptors.

Formate dehydrogenase could not catalyze the reduction of CO_2 in the presence of reduced methyl viologen. The oxidation-reduction potential at pH 7 is -0.42 volts for CO_2/formate and -0.44 for oxidized/reduced methyl viologen. A reaction mixture of 4 ml containing 0.3 mM methyl viologen, 1 mM EDTA, and 0.15 M sodium phosphate, pH 7.2, was made anaerobic by bubbling with argon and then sodium dithionite was added to give a final concentration of 0.27 mM reduced methyl viologen. The non-enzymatic rate of oxidation of methyl viologen was monitored spectrophotometrically at 600 nm (less than 2 nmol/min). No change in the rate was observed following the addition of purified formate dehydrogenase, Step 6 (10 µg) and sodium bicarbonate (40 µmol). The rate was less than 0.01% of formate phenazine methosulfate-dichlorophenolindophenol reduction.

**Properties of Purified Nitrate Reductase**

Our procedure for the purification of formate dehydrogenase also yielded a highly purified preparation of nitrate reductase. As shown in Table I nitrate reductase was purified 29-fold over whole cells with a 22% yield. In other preparations yields as high as 72% were obtained. The yields of nitrate reductase were variable and sometimes low because the purification procedure was adapted for maximal enrichment of formate dehydrogenase and each purification step was designed on that basis without regard to nitrate reductase. Only one-half of the nitrate reductase was solubilized in Step 3 (Table I); however, if 10% saturated ammonium sulfate was used, essentially all of the nitrate reductase could be solubilized along with 20 to 40% of the formate dehydrogenase.

The nitrate reductase activity which was eluted from DEAE-agarose at Step 6 showed a peak with a shoulder (Fig. 2). Fractions 132 to 174 (Fig. 2) had a constant specific activity (75 ± 3 µmol of nitrite produced/min/mg of protein). All nitrate reductase fractions were colored (reddish brown) and contained heme (Fig. 2); however, the heme to protein ratio is higher in the peak than in the shoulder. We have previously reported that this suggests that two forms of the enzyme exist which differ in their heme content (12). These two forms of nitrate reductase which are partially resolved by DEAE-agarose chromatography may be completely separated by gel electrophoresis in the presence of Triton X-100; the forms enriched in the peak (nitrate reductase I) and shoulder (nitrate reductase II) fractions have been estimated to contain 6.7 and 3.2 nmol of heme/mg of protein, respectively (12).

The purified preparation of nitrate reductase, Step 6b, contains three types of polypeptides (12) whose molecular weights were estimated by sodium dodecyl sulfate gel electrophoresis.
to be 155,000, 63,000, and 19,000 (Fig. 6). There is some evidence that the heme may be associated with the 19,000-dalton polypeptide.

The Stokes radius of nitrate reductase was estimated to be 75 Å when determined in the presence of Triton X-100 (Ref. 10). The $n_{20,w}$ of nitrate reductase b was estimated to be 16 S by sucrose density centrifugation in the presence of Triton X-100. The molecular weight of nitrate reductase was approximated using Equation 1 and ignoring any contribution made by bound detergent (i.e., omitting the $\delta_d(1 - \delta_d \beta) \rho$ term). Using a $\delta_d$ of 0.725 cm$^3$/g (49), the molecular weight was estimated to be 498,000 ± 10%.

**DISCUSSION**

The purified formate dehydrogenase appears to exist as a globular protein-detergent complex free of phospholipid. Our results indicate that the enzyme is composed of three types of polypeptide chains and has the subunit structure $\alpha_3\beta_4\gamma_2$. The molecular weight of this structure is 608,000 ± 60,000 based on the molecular weights of the polypeptides. This is in good agreement with the value calculated from the heme content (molecular weight = 615,000 based on 4 hemes/mol) and the value based on the sedimentation coefficient and Stokes radius (molecular weight = 590,000). However, in view of the possible variation in the amount of the $\gamma$ polypeptide it seems prudent to consider alternative structures. If some of the $\gamma$ polypeptide is lost in the course of purification, the actual structure may be $\alpha_2\beta_4\gamma_4$ (molecular weight = 648,000) or, if $\gamma$ represents a tightly associated impurity (of about 7%), a structure of $\alpha_2\beta_4\gamma_2$ (molecular weight = 588,000) is possible.

This work has directly established that *E. coli* formate dehydrogenase is a selenoenzyme. Selenium was recently shown to be a component of glutathione peroxidase of erythrocytes (50, 51) and a protein ("Protein A") of the glycine reductase system of Clostridium sticklandii (52). Glutathione peroxidase (molecular weight = 84,000) contains 4 mol of selenium/mol of enzyme. "Protein A" (molecular weight = 12,000) contains 1 mol of selenium/mol of protein (53). A selenium-containing protein has been isolated from lamb muscle (54, 55) and identified as a hemoprotein (molecular weight = 10,000).

In 1954, Pinsent (1) observed that the formation of formate dehydrogenase activity in *E. coli* required the presence of trace amounts of selenium, molybdenum, and iron in the culture medium. This was the first enzyme for which a selenium requirement had been demonstrated. Similar requirements for the formation of formate dehydrogenase activity in a number of bacteria (56–58) suggest that selenium may be a general constituent of all formate dehydrogenases. Earlier work on selenium-containing preparations of formate dehydrogenase was carried out with preparations of unestablished purity or of low activity (7, 56) and could therefore not definitively establish selenium as a component of the enzyme. Further uncertainty results from the fact that selenium can be randomly incorporated into protein as selenocystine (50–61) and as selenotrisulfide (42, 62). Andreessen and Ljungdahl (56) showed that a selenium-containing formate dehydrogenase can be obtained from *Clostridium thermoacetica* grown in the presence of $^{75}$Se selenite. However, the $^{75}$Se specific activity (counts per min per mg of protein) remained nearly constant during five purification steps (in one step enzyme specific activity was increased 515% while $^{75}$Se specific activity increased only 17%). Thus, the significance of selenium in this preparation is not clear. Shum and Murphy (7) obtained a partially purified preparation of formate dehydrogenase from *E. coli* grown in the presence of $^{75}$Se selenite and showed that enzyme activity cosedimented with $^{75}$Se during sucrose density gradient centrifugation. The selenium content of the enzyme from *E. coli* (7) and *C. thermoacetica* (56) was not determined.

We have shown that selenium incorporation into formate dehydrogenase is highly specific. The specific radioactivity of nitrate reductase was less than 3% of that of $^{75}$Se-labeled formate dehydrogenase obtained from the same preparation. The data of Table II indicate that the enzyme may contain equimolar amounts of selenium, molybdenum and heme (4 mol of each/mol of enzyme).

The selenium was shown to be associated with a polypeptide of molecular weight 110,000 and would appear to be covalently bound since rather drastic conditions were required to remove it. The enzyme does not contain acid labile selenium, nullifying any possible role of selenium as a replacement of sulfide in the non-heme iron centers. It is not yet known if selenium participates in the enzyme catalysis. The selenoenzymes, glutathione peroxidase (63), glycine reductase (52), and formate dehydrogenase (this work) are all inhibited by iodoacetamide. It is possible that a selenol rather than a thiol group is modified by the alkylating agent which could indicate a functional role of selenium in these enzymes.

No flavin was found in purified formate dehydrogenase and the lack of involvement of flavin in the reaction is further indicated by the inability of flavin to act as an electron acceptor or to stimulate the reduction of dichlorophenolindophenol and phenazine methosulfate. The soluble NAD$^+$-dependent formate dehydrogenase of *Pseudomonas azotica* has been reported to contain 1 mol of FMN as well as 5 to 8 mol of iron and 7 to 8 mol of labile sulfide/300,000 g of protein (64).

Relatively few enzymes have been reported to contain molybdenum as a constituent. These are the xanthine oxidase (65), aldehyde oxidase (66), and sulfite oxidase (67) of mammalian tissues, xanthine dehydrogenase (68), and nitrate reductase (69, 70) of bacteria, and the nitrate reductase of plants, fungi, algae, and bacteria (71, 72). With the identification of formate dehydrogenase as a molybdoprotein it appears that of the six known molybdenum-containing enzymes two may be found together in the membrane-bound complex which catalyzes the reduction of nitrate by formate (i.e., formate dehydrogenase and nitrate reductase). This fact explains the pleiotropy of some chlorate-resistant mutants of *E. coli* (i.e., ChlD mutants) which are thought to have a defective molybdenum processing system (73). Neither formate dehydrogenase nor nitrate reductase activities can be detected in the ChlD mutant unless high levels of molybdate (0.1 mM) are provided in the growth medium. Formate dehydrogenase and nitrate reductase are inhibited by cyanide which may indicate a functional role of molybdenum in these enzymes since cyanide has been shown to bind stoichiometrically to the molybdenum of molybdoenzymes (74); b-type cytochromes do not typically bind cyanide. The oxidized and reduced cytochrome b spectra of formate dehydrogenase and nitrate reductase are indistinguishable on the basis of their absorbance maxima2 (12). The heme of nitrate reductase would appear to be in a more labile configuration since heating the enzyme at pH 8.5 for 5

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1 The minimum possible radius of a globular particle, $R_{\text{min}} = [3M(\delta_p + \delta_d \rho_d)/4\pi N]^{1/3}$. Ratios of $R_e/R_{\text{min}}$ up to about 1.25 define globular particles (23). $R_e/R_{\text{min}}$ for formate dehydrogenase is 1.26 (using $M = 608,000$).

2 The absorbance maxima of reduced nitrate reductase reported in Ref. 12 should be corrected to: 559, 531, and 428 nm.
min results in a complete loss of the cytochrome spectrum and heme (12), while formate dehydrogenase after the same treatment retained all of its heme in a form which was still reducible by formate. Nitrate reductase (12) and formate dehydrogenase (this work) both have complex subunit structures apparently composed of three nonidentical polypeptide chains. The enzymes do not share a common polypeptide.

Formate dehydrogenase has widespread occurrence in animals, plants, yeast, and many bacteria (75), however, highly purified enzyme has been obtained from only two sources: *P. aceticum* (64) and *C. thermoaceticum* (76). The soluble enzyme of plants (77) and animals (78) use NAD + as the physiological electron acceptor, while the soluble bacterial enzymes have been shown to be specific for NAD + (64), NADP + (76), or ferredoxin (79, 80). The particulate enzyme of *Desulfovibrio vulgaris* utilizes a c-type cytochrome as the natural acceptor (81).

In *E. coli* under conditions of nitrate respiration, nitrate is the ultimate acceptor of electrons from formate. Electron flow from formate to nitrate is probably mediated by a complex chain of electron carriers which are present in the two purified protein complexes, formate dehydrogenase and nitrate reductase, plus a lipid carrier (quinone):

\[
\text{HCOOH} \rightarrow \text{dehydrogenase} \rightarrow \text{quinone} \rightarrow \text{reductase} \rightarrow \text{NO}_3^- \\
(\text{Se,Mo,Fe,heme}) \rightarrow (\text{heme,Mo,Fe})
\]

A functional role for these carriers and the intramolecular sequence of electron flow remains to be shown for both of these enzymes. A number of lines of evidence indicate a role for the heme in this pathway. Maximal levels of formate dehydrogenase, cytochrome *b*, and nitrate reductase in anaerobically grown cells require the addition of molybdenum (3) and nitrate (3, 8) to the culture medium which implies a coordinate regulation of these components. Formate causes the reduction of cytochrome *b* in whole cells (48, 82), membrane preparations (83), and the reconstituted formate-nitrate reductase system (12). The reduced cytochrome *b* is reoxidized by the addition of excess nitrate in each case. Rapid kinetic studies would be helpful in determining if the rate of cytochrome *b* reduction is compatible with the rate of the over-all reaction (formate-nitrate reduction). Formate-nitrate reductase appears to require a quinone cofactor in addition to the protein-bound electron carriers.

Itagaki (44) has reported that formate-nitrate reductase activity is lost in quinone-depleted membrane particles and may be partially restored by the addition of coenzyme Q (vitamin *K* 2 was 38% as effective on a mole per mole basis). It was recently shown (12) that coenzyme Q 10 stimulated formate-nitrate reductase activity 33-fold in a system containing only purified formate dehydrogenase and nitrate reductase. The site(s) of quinone action remains to be established.

The key components of the formate-nitrate reductase system of *E. coli*, formate dehydrogenase and nitrate reductase, are associated with the cell membrane where they may exist as a discrete complex. Evidence for the existence of such a complex was presented by Itagaki et al. (5) who obtained a soluble preparation of formate dehydrogenase, cytochrome *b*, and nitrate reductase with each enriched 10-fold over membranes. Our evidence suggests that formate dehydrogenase and nitrate reductase may be tightly associated after their release from the membrane. During the purification of formate dehydrogenase we found that the pooled agarose eluates (Table 1) contains little protein that is not associated with either formate dehydrogenase or nitrate reductase (see Fig. 4). Formate dehydrogenase and nitrate reductase were not separated on the basis of size or charge by a number of purification steps carried out in the absence of detergent. It is possible that the association of these enzymes in *vitro* is fortuitous and does not reflect the nature of the in vivo interaction. It is not clear that formate dehydrogenase and nitrate reductase must interact in the membrane since electron transfer between the enzymes could be facilitated by a mobile quinone carrier.

Experiments with lysyloxime-EDTA (4) indicated that about 20% of the cellular protein of *E. coli* is associated with the cell envelope fraction. Using the purification factors obtained from Table I, one can calculate that formate dehydrogenase and nitrate reductase represent 3.3 and 17% of the protein, respectively, of the cell envelope. Both these enzymes are located on the cytoplasmic membrane which makes up about one-third of the protein of the cell envelope (84). Thus, 10% of the protein of the cytoplasmic membrane is present as formate dehydrogenase and 52% of the protein is present as nitrate reductase.8 The large amount of protein of this organelle associated with the formate-nitrate reductase system indicates the importance of this system under anaerobic growth conditions (in the presence of nitrate). Other donors can provide electrons for nitrate reduction in *E. coli*, for example, NADH, succinate, and lactate (82, 85) and these membrane-bound dehydrogenases may also form complexes with nitrate reductase. However, formate appears to be the major electron donor for nitrate reduction during anaerobic growth in the presence of nitrate (3, 48, 85)

The availability of purified formate dehydrogenase and nitrate reductase and the knowledge of their composition will now permit experiments designed to study the regulation of synthesis of subunits and their assembly into a functional membrane complex.

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8 MacGregor et al. (49) reported that 15% of the protein in the cytoplasmic membrane was nitrate reductase.
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