Identification of Flavin Adenine Dinucleotide and Heme in a Homogeneous Spermidine Dehydrogenase from *Serratia marcescens*

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SUMMARY

Spermidine dehydrogenase has been purified 5,000-fold to homogeneity from extracts of *Serratia marcescens*. The molecular weight is 76,000; no evidence has been obtained for subunits. The spectrum of the enzyme is characteristic of a heme protein. Both iron-protoporphyrin IX and flavin adenine dinucleotide have been identified in the enzyme in a molar ratio of heme to FAD to enzyme protein of approximately 0.8:0.9:1.0.

*Serratia marcescens* contains an enzyme which oxidizes spermidine to 1,3-diaminopropane and \( \Delta^1 \)-pyrroline (Equation 1)

\[
\text{Spermidine} \quad \begin{array}{c}
\text{NH}_2\text{CH}_2\text{CH}_2\text{NHCH}_3\text{CH}_2\text{CH}_2\text{NH}_2 \\
\text{NH}_2\text{CH}_2\text{NH}_2 + \text{H}_2\text{C}--\text{CH}_3
\end{array} \\
\text{1,3-Diaminopropane} \quad \Delta^1\text{-Pyrroline}
\]

Earlier work in this laboratory showed that this enzyme was largely particulate in crude extracts (5); a method for solubilization and partial purification was described, and a requirement for an electron acceptor was demonstrated. The finding that the enzyme was inactivated by acid ammonium sulfate precipitation and was partially reactivated by prolonged preliminary incubation with flavin adenine dinucleotide indicated that FAD was a cofactor (5). In the present studies the enzyme has been purified to homogeneity. Its molecular weight is 76,000. We have found that both FAD and the heme, iron-protoporphyrin IX, are present in the pure protein. The molar ratios of enzyme to heme to flavin are approximately 1:0.8:0.9.

* A preliminary report of this work was presented at the meeting of the American Society of Biological Chemists in April 1968 (1).
The heme does not significantly affect this value (15, 16) is supported by the published value of 1.9415 for the specific refractive increment of hemochromogen derivative was determined with the Cary model 11 recording spectrophotometer. The molar extinction coefficient of the dichloroindophenol at pH 6.5 is $1.84 \times 10^4$ (10).1

**D. Phenazine Methosulfate-Nitro Blue Tetrazolium Assay**—This assay for qualitative determinations of enzyme activity on analytical disc gels was a modification of the method described by Katzen and Schimke (11) for dehydrogenase assays on starch gels. A mixture consisting of 0.1 m potassium phosphate (pH 7.2), 1 mm phenazine methosulfate, 1 mm spermine, and 200 \(\mu\)g per ml of nitro blue tetrazolium was prepared immediately before use. After electrophoresis of the protein on an analytical gel (see below), the gel was incubated in the dark in a test tube containing the assay mixture for 15 to 30 min at 25°. Excess dye was removed by repeated washing with water in the dark; the enzyme bands retained a deep purple stain. The gels were stored in 7.5% acetic acid after the staining procedure.

**Protein Determinations**—Protein was routinely determined by the Lowry procedure (19), using bovine serum albumin as the standard. With the purified enzyme, the protein value obtained by the Lowry procedure agreed within 10% with the value determined by a refractometric method with the use of Rayleigh interference optics.2 For the calculations of the protein concentration by the latter method (13), we used the value of 1.875 \(\times\) 10² for the specific refractive increment of crystalline catalase, and for many helpful discussions.

**Analytical Ultracentrifugation**2—Studies were carried out in a Spinco model E analytical ultracentrifuge, equipped with either Rayleigh interference optics, or with a photoelectric scanner (23). Enzyme solutions were dialyzed for 18 hours against 0.1 m potassium phosphate buffer, pH 7.2.

**Determination of Molecular Weight**—The molecular weight of the native enzyme was determined according to the method of Yphantis (25). Protein solutions contained approximately 0.5 mg per ml, and column heights of 3 mm were used. The solution was centrifuged in a double-sector cell at 24,000 rpm at 4° to 7° until equilibrium was reached (21 to 28 hours); Rayleigh interference optics were used. The molecular weight of enzyme which had been denatured by dialysis for 18 hours at 25° against 6 m guanidine

1 Bachrach and Oser (4) have developed a quantitative assay for spermidine, which depends on the oxidation of spermidine to \(\Delta^1\)-pyrroline by a lyophilized culture of S. marcescens, and reaction of the \(\Delta^1\)-pyrroline with o-aminobenzaldehyde. By using a moderately purified enzyme, and dichloroindophenol as the electron acceptor, the sensitivity of the assay can be increased 10-fold.

2 We wish to thank Dr. William Carroll and Mr. Ellis Mitchell for performing most of the studies in the ultracentrifuge. We wish to thank Dr. Werner Klee for the use of the ultracentrifuge equipped with the photoelectric scanner for the sedimentation velocity experiments. We wish to thank Dr. Carroll for determining the diffusion coefficient of the enzyme, for determining the specific refractive increment of crystalline catalase, and for many helpful discussions.
RESULTS

Growth of Organism and Preparation of Extract

S. marcescens (ATCC 25179) has been used as the source of the enzyme. Cells were grown at 30° for 18 hours, with vigorous aeration in a salts-citrate medium (39). No additional carbohydrate was added, since the cells used the citrate in the medium as a carbon source. Glucose, succinate, or a rich medium decreased the total enzyme yield. The addition of N-(3-aminopropyl)-1,3-propanediamine to the minimal medium increased the yield of enzyme 3-fold. Spermidine could replace N-(3-aminopropyl)-1,3-propanediamine, but was not used routinely because of the expense. Attempts to increase the yield of enzyme by the use of spermidine (10^{-4} M) as the sole carbon source resulted in a 6-fold increase in the specific activity of the enzyme over that obtained with minimal medium, but growth of the cells was very slow. Use of spermidine as the sole nitrogen source resulted in good growth of the culture with only a 3-fold increase in specific activity. N,N'- Bis(3-aminopropyl)-1,3-propanediamine tetrahydrochloride (10^{-4} M) did not support growth when used as either the sole carbon or the sole nitrogen source. The addition of 1,3-diaminopropane had no effect on the yield of enzyme.

1 We wish to thank Mr. George Poy for these determinations.

2 We wish to thank Dr. Uriel Bachrach for providing us with the strain of S. marcescens used in this study and Dr. Robert P. Williams for his S. marcescens strains (Nima, WF, and 933).
Under our standard conditions, the cells were a deep red (3, 5). Growth without aeration or growth at 37°C resulted in cells which contained 1/4 to 1/4 as much enzyme activity as our standard cultures; these cultures were essentially colorless.

We also tested another strain of S. marcescens (Nima), and two mutant strains of Nima (WF, 983), which cannot make the characteristic red pigment, prodigiosin (40). The enzyme activity of the crude extracts of all these strains was approximately the same as in our standard strain.

Cells were grown in a vat containing 320 liters of Vogel-Boumer (39) medium, 30 ml of N-(3-aminopropyl)-1,3-propanediamine, and 57 ml of concentrated HCl; 4 liters of a stationary culture were used as the inoculum. The culture was incubated for 17 to 20 hours at 30°C with vigorous aeration; antifoam was added as needed. The culture was then cooled, and the cells were harvested in a refrigerated Sharples centrifuge. The wet paste was applied to the column at the rate of 300 ml per hour. The precipitate was collected by centrifugation at 10,000 × g for 1 hour. The homogenate was then refrigerated until needed. The culture was homogenized three times; care was taken to cool the homogenate to 0-4°C after each treatment. The extract was then centrifuged in a refrigerated Sharples centrifuge. At this stage in most preparations, 70 to 80% of the total cellular activity was soluble with a linear gradient formed from 2 liters of 0.1 M KCl in 0.005 M potassium phosphate, pH 7.2, in the mixing flask, and 2 liters of 0.5 M KCl in 0.005 M potassium phosphate in the reservoir. The flow rate was approximately 300 ml per hour. The enzyme usually was eluted after 1500 ml of buffer had passed through the column. Fractions containing enzyme with a specific activity greater than 0.25 unit per mg of protein (approximately 1000 ml) were pooled. The active fractions from four columns were combined and used for Step 2.

**Step 3.** Microgranular DEAE-cellulose Chromatography—Calcium phosphate cellulose gel was prepared by the method of Price and Greenfield (43). A pad, 2.5 cm high x 16.5 cm in diameter, was prepared by pouring the calcium phosphate cellulose suspension onto a 16.5-cm sintered glass funnel; suction from a water pump was applied until all of the supernatant fluid was removed. The pooled DEAE eluates from Step 1 were poured onto the pad, and suction from a water pump was applied to remove the liquid rapidly. The filtrate, which contained approximately 80% of the activity, was collected in a suction flask immersed in ice slush. The pad was then washed with 500 ml of cold 0.01 M potassium phosphate, pH 7.2. Essentially all of the enzyme was recovered in the combined filtrate and wash. These combined filtrates were adsorbed to a second calcium phosphate cellulose pad prepared in the same way; the pad was washed with 500 ml of cold 0.01 M potassium phosphate buffer, pH 7.2. Approximately 80% of the enzyme was recovered in the combined filtrates. The protein was then precipitated by the addition of 51.6 g of solid ammonium sulfate (Mann’s special enzyme grade) per 100 ml (to 80% saturation). The precipitate was collected by centrifugation at 10,000 × g at 4°C for 20 min and dissolved in 400 ml of 0.01 M potassium phosphate, pH 7.2. This solution was dialyzed against 10 volumes of 0.005 M potassium phosphate, pH 7.2, for 5 hours, and then overnight against 10 volumes of fresh buffer.

**Step 4.** DEAE-cellulose Chromatography—A typical purification is presented (Table I). In some preparations, however, when an early step failed to yield the expected purification, an additional DEAE-cellulose or calcium phosphate column was included in the procedure. All of the steps were carried out at 0-4°C, except where otherwise noted.

**Step 1.** DEAE-cellulose Chromatography (DEAE I)—Whatman DE-22 was prepared by washing alternately with 0.5 N HCl, H2O, 0.5 N NaOH, and H2O (42). The pH was then adjusted to 7 with 1 N KH2PO4, and the DEAE-cellulose was washed thoroughly with water. A column (4.5 × 50 cm) was packed under 1.5 pounds of pressure and was washed with 4 liters of 0.002 M potassium phosphate buffer, pH 7.2. One-fourth of the total crude extract (approximately 1000 units, 1000 to 2000 ml) was applied to the column at the rate of 300 ml per hour. The column was then washed with 1 liter of 0.1 M KCl in 0.005 M potassium phosphate buffer, pH 7.2. The enzyme was eluted with a linear gradient formed from 2 liters of 0.1 M KCl in 0.005 M potassium phosphate, pH 7.2, in the mixing flask, and 2 liters of 0.5 M KCl in 0.005 M potassium phosphate in the reservoir. The flow rate was approximately 300 ml per hour. The enzyme usually was eluted after 1500 ml of buffer had passed through the column. Fractions containing enzyme with a specific activity greater than 0.25 unit per mg of protein (approximately 1000 ml) were pooled. The active fractions from four columns were combined and used for Step 2.

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**Table I**

| Step               | Volume | Total Protein | Total Activity | Recovery % | Specific Activity |
|--------------------|--------|---------------|----------------|------------|------------------|
| Extract            | 6,225  | 48,100        | 4,400          | 100        | 0.09             |
| DEAE I chromatography | 3,900 | 4,960         | 1,850          | 42         | 0.37             |
| Calcium phosphate cellulose adsorption | 500 | 725          | 1,330          | 30         | 1.84             |
| DEAE II chromatography | 8     | 76            | 680            | 15         | 9.0              |
| Hydroxylapatite chromatography | 10 | 6.4           | 450            | 10         | 70.0             |
| Acrylamide gel electrophoresis | 1.6 | 0.6           | 250            | 6          | 420.0            |

A unit is the amount of enzyme that oxidizes 1 μ mole of spermidine in 1 min, using Assay A.

Protein content was assayed by the Lowry procedure (12) in all steps except the last; after gel electrophoresis it was assayed by the extinction at 280 μm (see "Experimental Procedure").
Step 3 was adsorbed onto the column; the column was eluted first with 50 ml of 0.005 M potassium phosphate, pH 7.2, and packed in a column (1.8 X 25 cm). The dialyzed fraction from Step 2 was applied directly to the column. Stepwise elution was carried out with potassium phosphate buffers, pH 7.2; 5-ml fractions were collected. The column was washed first with 100 ml of 0.07 M buffer, and then was eluted successively with 100 ml of 0.075 M, 100 ml of 0.08 M, 200 ml of 0.095 M, and 100 ml of 0.09 M buffer. The fractions with the best specific activity (usually eluted with 0.085 M buffer) were pooled and concentrated by ultrafiltration in a collodion bag apparatus. This ultrafiltration sometimes resulted in an additional 2-fold increase in specific activity. The enzyme was dialyzed against 100 volumes of 0.001 M potassium phosphate, pH 7.2, for 12 hours.

**Step 4. Hydroxylapatite Chromatography**—Hydroxylapatite, prepared by the method of Levin (44), was gently packed into a column (2.5 X 16 cm) and was washed with 0.001 M potassium phosphate, pH 7.2. The concentrated dialyzed enzyme from Step 3 was adsorbed onto the column; the column was eluted first with 50 ml of 0.005 M potassium phosphate, pH 7.2, followed by a linear gradient formed from 100 ml of 0.005 M potassium phosphate, pH 7.2, in the mixing vessel, and 100 ml of 0.05 M buffer in the reservoir. Fractions of 1 ml were collected; the activity was eluted after 48 ml of the gradient had been collected.

**Step 5. Preparative Acrylamide Disc Gel Electrophoresis**—The material obtained in Step 4 was subjected to preparative disc gel electrophoresis in a Canalco jacketed column PD-2/70 in a 4°C cold room, with the water jacket filled, but without additional cooling. The concentrated enzyme from Step 4 was brought to 10% sucrose concentration by adding solid sucrose; a drop of indicator dye (bromphenol blue) was added. This solution was layered on a column consisting of a 1.5-cm stacking gel and a 3-cm separating gel, pH 8.8, prepared according to the Canalco prep-disc formulation (21). A current of 8 ma was applied until the protein moved into the separating gel. The current was then increased to 10 to 15 ma. Current was applied for approximately 5 hours. Elution of the column was carried out continuously with 5% sucrose-0.38 M Tris-HCl, pH 8.8, while the current was applied at a flow rate of 2 to 3 ml per min. The deep red band formed by the enzyme was eluted approximately 1 hour after the dye. The 10 fractions with the highest ratios of units to 280 nm absorbance were pooled and then concentrated by dialysis against 0.1 M potassium phosphate, pH 7.2, in an ultrafiltration collodion sac (Table I).

### Criteria of Purity

All of the recoverable activity moved as one sharp red band on preparative disc gel electrophoresis. Analytical disc gel electrophoresis at pH 8.8 (20) showed that this enzyme is homogeneous, when freshly prepared, had only one band of activity, determined by the nitro blue tetrazolium assay, with an Rf of 0.67; only one band of protein with the same Rf was found on a parallel gel. Data obtained in the analysis of the enzyme from the linear character of the plots of the data from the sedimentation equilibrium and sedimentation velocity experiments presented below (Figs. 2, 3, and 4).

### Stability of Enzyme

The pure enzyme could be stored at -20°C in 0.1 M potassium phosphate buffer, pH 7.2, for over 6 months with less than 10% loss of activity. Storage of the enzyme for 3 or 4 months at -20°C resulted in the appearance of multiple bands of activity which migrated more slowly than the major band. Analytical disc gel electrophoresis of this material showed that these additional bands usually contained less than 5% of the protein. The enzyme was stable when heated for 5 min at 58°C. However,
The partial specific volume, determined as described in "Experimental Procedure" (29), was 0.738 (Fig. 3). Determination of the \( \bar{\nu} \) from the amino acid content (see below) (26) gave a value of 0.736.

The molecular weight determinations, calculated from the data obtained from sedimentation equilibrium centrifugations of Fig. 2 and Fig. 3, Curve A, were 78,000 and 76,000. The value obtained from the sedimentation velocity centrifugation and the diffusion coefficient (see below) was 79,000 (24, 26).

The molecular weight calculated from the data obtained from centrifugation in a sucrose density gradient (35), using catalase, glutathione reductase, and hexokinase as markers, was 76,000.

The sedimentation velocity of the enzyme was determined by scanning at 280 \( \text{m}_{\lambda} \) to follow the protein and at 404 \( \text{m}_{\lambda} \) to follow the 414-\( \text{m}_{\lambda} \) absorbing material (23). A straight line of identical slope was obtained from both sets of data, indicating that the sedimentation velocity was identical at both wave lengths. Thus, the 404-\( \text{m}_{\lambda} \) absorbing moiety and the protein sedimented as a unit (Fig. 4).

\[
D_{280} = 7.45 \times 10^{-7} \text{ cm}^2 \text{ per sec.}
\]

In addition to the studies on native enzyme described above, the molecular weight was determined for enzyme that had been denatured by dialysis against 6 M guanidine-0.1 M 2-mercaptoethanol for 24 hours at 25°. Sedimentation equilibrium centrifugation was then carried out at 25° and 24,000 rpm in 6 M guanidine. The molecular weight was found to be 70,000.

When the sample which had been denatured with guanidine was examined spectrally, almost all of the 414-\( \text{m}_{\lambda} \) absorbance was absent, suggesting that the heme had been lost during the denaturing process. To study whether or not the removal of cofactors was associated with a small decrease in the molecular weight, we used other procedures which removed some or all of the heme and flavin. One sample of enzyme was dialyzed against 1 M potassium phosphate, pH 7.2, for 2 hours and then overnight against 0.1 M phosphate buffer. About 50% of the 414-\( \text{m}_{\lambda} \) absorbance was removed by this dialysis. High speed sedimentation equilibrium centrifugation was carried out on this preparation; calculation of the molecular weight led to a value of 65,500.

Another sample was subjected to acid ammonium sulfate precipitation to remove the FAD, as described in the legend to Fig. 6, and then dialyzed overnight against 0.1 M potassium phosphate buffer, pH 7.2. A molecular weight of 68,000 was determined from sedimentation equilibrium data. The molecular weight of the holoenzyme from which this preparation was derived was 74,000.

In all of these determinations, the experimentally determined \( \bar{\nu} \) of 0.738 was used, with the corrections necessary for guanidine concentration or temperature.

Similar results were obtained when the molecular weight of the protein was determined by electrophoresis on sodium dodecyl sulfate gels. The enzyme, denatured by dialysis against sodium dodecyl sulfate (as described in "Experimental Procedure"),
The amino acid composition of the spermidine dehydrogenase is summarized in Table II.

### Amino Acid Analysis

| Amino Acid | Residues per Mole of Spermidine Dehydrogenase |
|------------|---------------------------------------------|
| Lysine     | 29.7                                        |
| Histidine  | 16.1                                        |
| Arginine   | 32.9                                        |
| Aspartic acid | 79.9                                    |
| Threonine  | 29.4                                        |
| Serine     | 36.8                                        |
| Glutamic acid | 75.7                                    |
| Glycine    | 64.9                                        |
| Glutamine  | 42.0                                        |
| Methionine | 16.1                                        |
| Proline    | 42.0                                        |
| Cysteic acid | 2.7                                     |
| Alanine    | 61.4                                        |
| Tyrosine   | 24.3                                        |
| Phenylalanine | 28.4                                    |
| Valine     | 45.0                                        |
| Methionine | 16.1                                        |
| Isoleucine | 31.0                                        |
| Leucine    | 65.5                                        |
| Isoleucine | 31.0                                        |
| Leucine    | 65.5                                        |
| Tyrosine   | 24.3                                        |
| Phenylalanine | 28.4                                    |
| Cysteic acid | 2.7                                     |

The values given here are the average of three analyses on two independent preparations of pure enzyme recalculated for a molecular weight of 75,000. Two of the assays were performed on aliquots from the same preparation; one of these was hydrolyzed in the presence of 4% (v/v) thioglycollate, as described by Matsubara and Sasaki (45). In this analysis the value for proline was approximately 25% greater than in the other runs. The third analysis was performed on another preparation after precipitation with 5% trichloroacetic acid. There was no significant difference between the values obtained for the two independent preparations of enzyme. Although the Matsubara and Sasaki procedure was satisfactory for the determination of tryptophan in chymotrypsin, we were not able to use it for the determination of the tryptophan content of spermidine dehydrogenase. With this enzyme, other products developed during the hydrolysis which chromatographed with tryptophan on the amino acid analyzer column, and which resulted in an atypical absorption after reaction with ninhydrin.

### Isoelectric Point

The isolectric point of the enzyme, determined by electrofocusing according to the method described in “Experimental Procedure,” was at pH 4.5.

### Identification of Flavin Adenine Dinucleotide in Spermidine Dehydrogenase

In earlier studies, Campello, Tabor, and Tabor (5) had indicated that impure enzyme which had been inactivated by acid ammonium sulfate precipitation could be partially reactivated by 0.1 mM FAD. In this study, we have been able to demonstrate similar results with pure enzyme by adding carrier bovine serum albumin and using the conditions described by Strittmatter (16). Reactivation of 13% was observed within 2 min, and of 25% within 1 h after the addition of FAD (Fig. 5, Curve A).

Additional evidence for the presence of FAD was obtained from the spectrum of the soluble cofactor in the supernatant fraction after acid ammonium sulfate precipitation of the pure protein. The spectrum (Fig. 6, Curve A) closely resembles that of FAD (Fig. 6, Curve B). The spectrum of the apoenzyme (Fig. 6, Curve C) resembles that of an oxidized heme (see below) (17). In the spectrum of the native enzyme (Fig. 1, Curve A) one cannot find spectral evidence for the flavin since the flavin absorbance is masked by the prominent 414-nm peak.

The FAD content of four independent preparations of the pure enzyme was also determined fluorometrically, as described under “Experimental Procedure.” The average value found was 0.86 mol of FAD per mol of enzyme (using the observed molecular weight of 70,000) (Table III). No FMN was present.

### Identification of Heme in Spermidine Dehydrogenase

FAD content of FAD. Pure spermidine dehydrogenase (0.1 ml containing 8 μg of protein with a specific activity of 400 units per mg) was added to 0.15 ml of a solution of bovine serum albumin (1.7 mg per ml). To this was added 2.0 ml of 5 mM KCl, followed by 5 ml of 4 M (NH₄)₂SO₄, mixed with 0.027 ml of 1 M H₂SO₄. The precipitate which formed was centrifuged rapidly (1 min at 10,000 rpm at 0°) and was dissolved immediately in 0.1 ml of 1 M potassium phosphate, pH 7.2. A portion, 0.05 ml, was added to 0.05 ml of 1 M KH₂PO₄, to bring the pH to 8.3, and was stored at room temperature for 2 h. After this preliminary incubation, 0.05 ml (Sample A) was cooled at 0°, and sufficient FAD was added to give a final FAD concentration of 0.1 mM. A second portion (Sample B) was cooled and used for the assay without further treatment. The activities of the enzyme solutions with FAD (Sample A) and without FAD (Sample B) were assayed at various time intervals to determine the enzymatic activity. A, enzyme previously incubated with FAD (O--O); B, enzyme without added FAD (O--O).
violet shifted from 272 to 276 mμ (Fig. 6C). In order to demonstrate that the heme remains in a constant ratio to the active protein during purification, we determined the ratio of the 414-mμ absorption peak to enzymatic activity over an 11-fold increase in purification. The ratio was 2.75 in a 450-fold purified enzyme and 1.62 after the maximum purification (5000-fold purification). The ratios of absorbance at 414 mμ to enzyme activity for two independent preparations of pure enzyme were 1.62 and 1.70.

To identify the type of porphyrin present, and to determine the amount present, we formed the pyridine hemochromogen derivative of the pure enzyme (see “Experimental Procedure”) (18). The spectrum of this derivative (Fig. 7) was identical to that formed with iron-protoporphyrin IX (48). From the molar extinction coefficient of the pyridine hemochromogen derivative of iron-protoporphyrin IX (18, 48), we determined the number of moles of heme per mole of enzyme. In three of the preparations used for the flavin determination there was an average of 0.78 mole of heme per mole of enzyme (Table III).

### Characteristics of Enzyme Reaction

The enzyme reaction rate, carried out with a pure preparation, was linear with respect to time and enzyme concentration. The rate of oxidation did not vary significantly over a range of spermidine concentrations from 0.0005 to 0.1 mM. Thus, the $K_m$ for spermidine was too low to determine (less than 0.0005 mM); the pH optimum was at 7.2. Two equivalents of ferricyanide were reduced per mole of spermidine. The $K_m$ for spermine in the standard incubation mixture (pH 7.2) was 0.02 mM. The pH optimum was 8.8; at this pH the $K_m$ was approximately 0.05 mM. Two equivalents of ferricyanide were reduced per mole of spermine, suggesting that spermine was oxidized at only one of the secondary amine groups. Monoacetylspermidine B, $N,N'$-bis(3-aminopropyl)-1,3-propanediamine, and $N$-(3-aminopropyl)-1,3-propanediamine, which are similar to spermidine in structure, are oxidized more slowly than spermidine, even when present in much higher concentrations (Table IV) and under saturating conditions. However, the following secondary amines were not oxidized at all when present in the concentration range from 0.5 mM to 5 mM: dibutylamine, monoacetylspermi...
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**Table IV**

Relative rates of oxidation

| Substrate | % of rate with spermidine |
|-----------|--------------------------|
| Spermidine | 100 |
| 10^{-4} M | 100 |
| 5 \times 10^{-4} M | 60 |
| N,N'-bis(3-Aminopropyl)-1,3-diaminopropane | 48 |
| N-5-Aminopropyl)-1,3-propanediamine, 10^{-4} M | 36 |
| Spermine, 5 \times 10^{-3} M | 17 |
| N-(3-Hydroxypropyl)-1,4-diaminobutane, 5 \times 10^{-4} M | 7 |
| Monoaoyethyl spermidine A, 5 \times 10^{-3} M | 0 |

Quinacrine, added to a reaction mixture at 0.2 mm final concentration, resulted in 80% inhibition. Metal binders (EDTA at 1 mm, \(\alpha\)-phenanthroline at 0.07 mm, cuprizone at 0.1 mm, neocuproine at 1 mm, cupferron at 1 mm, and diethylthiocarbamate at 1 mm) had no effect on activity. Isoniazid, iproniazid, cyanide, sodium azide (each at 1 mm), and borohydride reduction did not inhibit the enzyme.

**DISCUSSION**

Spermidine dehydrogenase is found in S. marcescens cultures even when grown in the absence of added spermidine. The amount of enzyme activity can be increased several fold by the addition of spermidine or N-(3-aminopropyl)-1,3-diaminopropane to the growth medium. About 50% of the enzyme is found in a particulate form in water extracts of S. marcescens. The natural factors which couple this enzyme to molecular oxygen are lost upon solubilization, and the soluble enzyme has a requirement for an added electron acceptor. During purification, a heme and FAD remain with the enzyme; in a homogeneous preparation, 6000-fold purified, we find a molar ratio of heme to flavin of enzyme to 0.8:0.9:1. We have been able to resolve the flavoprotein into an inactive apoenzyme, which still contains the heme; this undergoes about 25% reactivation upon the addition of FAD. We have also been able to remove the heme by dialysis against 1 mM potassium phosphate, but have not yet been able to reconstitute an active enzyme from the apoenzyme lacking the heme.

The molecular weight determinations from sedimentation equilibrium centrifugation, sedimentation velocity studies, and sucrose density gradient centrifugation yield a value of 76,000 ± 3000. No conclusive evidence for subunits has been obtained. However, suggestive evidence for the loss of a small polypeptide has been obtained by the decreased molecular weight (65,000 to 70,000) observed after dialysis of the holoenzyme against 1 mM phosphate, guanidine, or sodium dodecyl sulfate, and after dialysis of the flavin apoenzyme against 0.1 mM phosphate.

The spectrum of the pure enzyme contains two major peaks, one at 272 nm and one at 414 nm, and two minor peaks at 530 nm and 300 nm. When substrate is added to the enzyme in the absence of an electron acceptor, the 414-, 500-, and 560-nm peaks (which are characteristic of a heme) show the shifts which occur upon reduction of a heme; the 272-nm peak also shifts to 265 nm. It is difficult to find evidence of the FAD in the spectrum because of the very strong absorbance at 414 nm. However, after resolution of the enzyme, a 276-nm peak is found instead of the 272-nm peak of the holoenzyme; this is consistent with the loss of a flavin moiety absorbing at 260 nm.

The presence of both a heme and a flavin has been described in two other pure enzymes: crystalline yeast cytochrome bs (18), which contains a heme and FMN, and sulfite reductase of Escherichia coli, which contains a heme and both FMN and FAD (50). Both cofactors have also been found in a partially purified nitrate reductase from Neorospora crassa (51). FAD has been shown to be a cofactor for tyrosine oxidase of Streptomyces luteus (52) and for putrescine oxidase of Micrococcus rubens (53), and has been implicated as a cofactor for tissue monoamine oxidase in mammals and histaminase from hog kidney by some authors (54, 55), but not by others (56–58). Spermidine dehydrogenase is the only amine oxidase that has been shown to contain both FAD and a heme, and to require an added electron acceptor for activity.

*This isomer of acetyl spermidine (monoaoyethyl spermidine A) has the acetyl group on the primary amine of the 4-carbon chain, (i.e., \(\text{NH}(\text{CH}_2)_2\text{NH}(\text{CH}_2)_2\text{NHCOCH}_3\)). The monoaoyethyl spermidine B (with the acetyl group on the primary amine of the 3-carbon chain) is active as a substrate.

*References* (54, 55), but not by others (56–58). Spermidine dehydrogenase is the only amine oxidase that has been shown to contain both FAD and a heme, and to require an added electron acceptor for activity.
The *Serratia* spermidine dehydrogenase differs in its action on spermidine from the other amine oxidases which oxidize this substrate. The *Serratia* enzyme splits the spermidine molecule at the secondary nitrogen to form 1,3-diaminopropane and a 3-carbon aminoaldehyde. The reaction carried out by the spermidine dehydrogenase of *S. marcescens* provides the only known pathway for the biosynthesis of 1,3-diaminopropane.

REFERENCES
1. TABOR, C. W., AND KELLOGG, P. D., Fed. Proc., 27, 639 (1968).
2. RAZIN, S., GERY, I., AND BACHRACH, U., Biochem. J., 71, 551 (1959).
3. BACHRACH, U., J. Biol. Chem., 237, 3443 (1962).
4. BACHRACH, U., AND OSER, I. S., J. Biol. Chem., 235, 2098 (1958).
5. CAMPBELL, A. P., TABOR, C. W., AND TADOR, H., Biochem. Biophys. Res. Commun., 19, 6 (1965).
6. SCHÖFF, C., KOMKA, A., BRAUN, F., AND JACOBI, E., Ann. Chem., 659, 1 (1948).
7. TABOR, H., J. Biol. Chem., 188, 125 (1951).
8. JAKOBY, W. B., AND FREDDERICKS, J., J. Biol. Chem., 234, 2145 (1959).
9. HOLMSTED, B., LARSSON, L., AND THAM, R., Biochim. Biophys. Acta, 48, 182 (1961).
10. ARMOOTONG, J., MoD., Biochim. Biophys. Acts, 14, 104 (1964).
11. KATZEN, H. M., AND SCHIMKE, R. T., Proc. Natl. Acad. Sci. U.S.A., 54, 1218 (1965).
12. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J., J. Biol. Chem., 193, 265 (1951).
13. RICHARDS, E. G., TELLER, D. C., AND SCHACHMAN, H. K., Biochemistry, 7, 1054 (1968).
14. PERLMANN, G. E., AND LONGSWORTH, L. G., J. Amer. Chem. Soc., 80, 755 (1958).
15. MONDOVA, B., ROTILIO, G., AND BACHRACH, U., Biochemistry, 3, 279 (1964).
16. RAZIN, S., AND KIM, K., Biochem. Biophys. Res. Commun., 19, 1 (1965).
17. LEVIN, E., in S. P. COLOWICK AND N. O. KAPLAN (Editors), Methods in enzymology, Vol. 6, Academic Press, New York, 1967, p. 197.
18. TABOR, C. W., AND BACHRACH, U., J. Biol. Chem., 239, 2194 (1964).
19. VOGEL, H. J., AND BONNER, D. M., J. Biol. Chem., 218, 97 (1966).
20. WASSERMAN, H. H., McKEON, J. E., SMITH, L., AND FORGIONE, R., J. Amer. Chem. Soc., 82, 506 (1960).
21. ADAMS, E., AND NEWBERRY, S. L., Biochim. Biophys. Res. Commun., 6, 1 (1961).
22. PETERTON, E. A., AND SOBER, H. A., in S. P. COLOWICK AND N. O. KAPLAN (Editors), Methods in enzymology, Vol. 5, Academic Press, New York, 1962, p. 3.
23. PRICE, V. E., AND GREENFIELD, E. J., J. Biol. Chem., 209, 303 (1954).
24. LEVIN, Ô, in S. P. COLOWICK AND N. O. KAPLAN (Editors), Methods in enzymology, Vol. 5, Academic Press, New York, 1962, p. 27.
25. MATSUBARA, H., AND Sasaki, R. M., Biochem. Biophys. Res. Commun., 35, 175 (1969).
26. STRITTmatter, P., J. Biol. Chem., 236, 2320 (1961).
27. STRITTmatter, P., AND VELICK, S. F., J. Biol. Chem., 221, 233 (1956).
28. FALK, J. E., Porphyins and metalloporphyrins, American Elsevier Publishing Company, New York, 1964.
29. MASSEY, V., Biochim. Biophys. Acts, 54, 255 (1959).
30. SEGEL, L. M., AND KAMIN, H., in K. YAGI (Editor), Proceedings of the 2nd Conference on Flavins and Flavoproteins, University Park Press, Baltimore, 1968, p. 15.
31. GARRIT, R. H., AND NABON, A., Proc. Natl. Acad. Sci., U.S.A., 56, 1603 (1967).
32. KUMAGAI, H., Matsu, H., OGATA, K., AND YAMADA, H., Biochim. Biophys. Acta, 171, 1 (1969).
33. YAMADA, H., Katsu, O., AND OGATA, K., Agr. Biol. Chem. (Tokyo), 29, 1148 (1965).
34. ERWIN, V. G., AND HELLERMAN, L., J. Biol. Chem., 242, 4230 (1967).
35. KAPELLER-ADLER, R., AND MacFarlane, H., Biochim. Biophys. Acta, 67, 542 (1963).
36. NARA, S., GOMES, D., AND YASUNOBU, K. T., J. Biol. Chem., 241, 2774 (1966).
37. YAMADA, H., KUMAGAI, H., KAWA, H., AND Matsu, H., Biochim. Biophys. Res. Commun., 29, 725 (1967).
38. MONDOVA, B., ROTILIO, G., COSTA, M. T., FINAZZI-AGRA, A., CHRAMBO, H., HAYES, R. E., AND BROWN, H., J. Biol. Chem., 242, 1160 (1967).
39. FELDMAN, R., AND KIM, K., Biochim. Biophys. Res. Commun., 19, 1 (1965).
Identification of Flavin Adenine Dinucleotide and Heme in a Homogeneous Spermidine Dehydrogenase from *Serratia marcescens*

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