Chapter 2

MATERIALS AND METHODS
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Materials

All the chemicals used in the media for the growth of Achromobacter fisheri were of analytical grade. Bacto peptone and agar (Difco) were obtained from Difco Laboratories, U.S.A. and beef extract from Oxoid, England. Ferric chloride and glycerol were supplied by B.D.H., England.

Crystalline bovine serum albumin, crystalline ovalbumin, catalase, yeast alcohol dehydrogenase, deoxyribonuclease I, lysozyme (three times crystallized), dansyl chloride, dansyl amino acids kit, p-hydroxymercuribenzoate, p-chloromercuribenzenzene sulfonic acid, 5,5'-dithiobis (2-nitrobenzoic acid), 5,5'-dithiobis (2-nitrobenzoic acid), reduced glutathione, protamine sulfate (Salmine) were obtained from Sigma Chemical Company, U.S.A. Acrylamide, N,N'-methylenebisacrylamide, and N,N,N',N'-tetramethylethlenediamine were the products of Eastman Organic Chemicals, U.S.A. Benzyl viologen, methyl viologen, tyrosine, tryptophan, ethylenediaminetetracetic acid and p-dimethylaminobenzaldehyde were purchased from British Drug Houses Ltd., England. Sephadex G-200, and Blue Dextran 2000 were obtained from Pharmacia, Sweden and Bio-gel P-150 from Bio-Rad Laboratories, U.S.A.

The following chemicals were purchased from the suppliers indicated: L-nitroso-2-naphthol (Hopkins and Williams); sodium borohydride (Koch and Light Laboratories Ltd.); 2-mercapto-ethanol (Fluka, Switzerland); sodium dodecyl sulfate (HICO
Products Pvt. Ltd., Bombay); sulfosalicylic acid (Riedel, Germany).

All other chemicals were of analytical grade and were obtained from commercial sources. Prior to use, p-hydroxymercuribenzoate was crystallized by the procedure of Boyer (250). Sodium dodecyl sulfate was crystallized twice from ethanol before use.

Guanidine hydrochloride was prepared from guanidine carbonate (Analar, B.D.H.) according to the procedure of Kawahara and Tanford (251). The carbonate was recrystallized from aqueous solution by the addition of ethanol at 4°C, the crystals dried in vacuum and mixed with water to make a paste and converted to hydrochloride by the addition of cold concentrated HCl. The resulting solution was adjusted to pH 5.4, filtered and concentrated in vacuo below 40°C and the residual mass recrystallized from methanol. The crystals were stored dry in a vacuum desiccator over P₂O₅ and solutions prepared and used fresh.

The analytical grade urea (B.D.H.) was recrystallized from aqueous ethanol and the crystals stored dry over P₂O₅ in vacuum. Solutions were made fresh before use.

Hydroxylapatite gel was prepared according to the procedure of Tiselius, Hjerten and Levin (252). The gel was equilibrated with 0.001 M potassium phosphate buffer (pH 6.8). Nitrogen gas was obtained from Indian Oxygen Ltd., Bombay and was made O₂-free by passing over heated copper at 700-800°C and then through methylene blue solution reduced by hydrogen and
palladised asbestos (Danpha Chemicals, India).

Methods

Organism: The salt-water luminous bacterium Achromobacter fischeri used in the present investigation was kindly supplied by Dr. W.D. McElroy (McCollum-Pratt Institute, Johns Hopkins University, Baltimore, U.S.A.). The culture appeared as Gram-negative rods, sometimes slightly curved.

Maintenance and propagation of culture: A. fischeri was propagated on nutrient agar slant of the following composition:

- Peptone 0.5 g
- Sodium chloride 3.0 g
- Beef extract 0.3 g
- Glycerol 0.3 ml
- Agar 2.0 g
- Calcium carbonate 0.3 g
- Distilled water to make 100 ml

The first four constituents were dissolved in water and the pH was adjusted to 7.4 with 4N KOH. The final volume was then made to 100 ml. Agar and calcium carbonate were then added and the mixture steamed for one hour. For preparation of slants, 7-8 ml aliquots were distributed into 19 x 150 mm Pyrex test tubes and autoclaved at 15 psi (120°C) for 20 min. The tubes were shaken while hot in order to distribute calcium carbonate uniformly, immediately slanted and allowed to solidify. These slants were inoculated from the stock culture.
and incubated at 28°C for 24 hrs.

The organism was maintained at 4°C and subcultured routinely every month.

**Basal liquid medium for growth:** To obtain large amounts of cells, the organism was grown in the following basal liquid medium:

- Sodium chloride, (NaCl) 30 g.
- Ammonium phosphate, (NH₄)₂HPO₄ 0.5 g
- Potassium dihydrogen phosphate, (KH₂PO₄) 2.1 g
- Disodium hydrogen phosphate, (Na₂HPO₄·12H₂O) 7.06 g
- Magnesium sulfate (MgSO₄) 0.1 g
- Ferric chloride (FeCl₃·6H₂O) 0.01 g
- Glycerol 3 ml
- Peptone 10 g.
- Distilled water to make one liter
- pH was adjusted to 7.4 by 4N KOH

All the constituents were dissolved separately, then mixed and made to the final volume. The media were autoclaved at 15 psi for 20 min. When nitrite was required to be incorporated, this alone was sterilised by passing through a Seltz filter.

**Growth conditions and collection of cells:** Inocula from 24 hr cultures grown on agar slopes were transferred into 500-ml conical flasks containing 100 ml liquid medium and grown for 20 hr at 28°C on a rotary shaker, 210 rpm. The organism was subcultured through two transfers in liquid media (without nitrate) under aerobic conditions. The inoculum was then transferred
to 15 liters of the basal liquid medium in glass carboys containing 0.1% potassium nitrate. Antifoam (0.2 to 0.3 ml, Alkaterge C, Commercial Solvents Corporation, U.S.A.; one part antifoam mixed with four parts of liquid paraffin) was added to each carboy in order to prevent excess frothing. The culture was kept at 22 to 30°C and purified air was continuously forced through the cultures from sintered glass units at 550 ml/min. After growing for 18-20 hr, the cells were harvested in a refrigerated Sharples centrifuge (2,000 rpm) at a flow rate of about 10 liters per hour. The bacteria were washed free of nitrite by suspending in 3% sodium chloride and centrifuging. The cells were stored as a paste at 15°C until used.

**Definition of unit of activity and specific activity:**

The unit of nitrite reductase activity is defined as the amount of enzyme required to cause disappearance of 1 μmole of nitrite in 10 minutes at 30°C and pH 7.5 using reduced benzyl viologen as the electron donor under the experimental conditions given in the text. The specific activity of the enzyme is defined as the activity per mg of protein.

**Estimation of nitrite reductase activity:**

Nitrite reductase was measured in Thunberg tubes after evacuation and refilling with O₂-free nitrogen with chemically reduced benzyl viologen as electron donor. The rate of reduction was measured by determining the decrease of nitrite concentration in the reaction mixture by the diazo-coupling procedure of Snell and Snell (253). The details of the assay
procedure are as follows.

The incubation mixture contained, in a final volume of 1.5 ml, 200 μmoles of potassium phosphate (pH 7.5), 0.67 μmoles of NaN₃ and enzyme protein. 0.5 ml of benzyl viologen (10 mg/ml in water) and 1 ml of freshly prepared dithionite (1 mg/ml) in 0.2 M potassium phosphate (pH 7.5) were placed in the side arm of the Thunberg tube and the tubes were evacuated immediately. The reaction was started by addition of reduced benzyl viologen. The final pH of the reaction mixture was 7.5. After 4-6 min of incubation at room temperature, the reaction was terminated by opening the Thunberg tubes and shaking for few seconds to oxidize all the reduced benzyl viologen. To 1 ml of reaction mixture was then added 1 ml of sulfanilamide reagent (1% w/v in 1 M HCl) followed by 1 ml of N-(1-naphthyl)-ethylenediamine dihydrochloride (0.02% w/v in water). The resulting red color was read at 540 nm after 10 minute after making the volume to 9.5 ml. The amount of enzyme used was adjusted so that the nitrite utilized was between 0.2 - 0.3 μmoles. An optical density of 0.5 for 10 mm light path was taken as equivalent to 0.1 μmole of nitrite reduced. A blank with all the assay constituents except enzyme was always run.

Estimation of catalase activity: Catalase was assayed according to Beers and Sizer (254). 2.9 ml of buffered solution of hydrogen peroxide (0.2 ml of 30% H₂O₂ in 50 ml of 0.05 M potassium phosphate buffer, pH 7.0) was taken in 3 ml capacity silica cuvette. 0.1 ml of enzyme sample was then added to the substrate solution and the decrease in optical density per 1-2 min,
used as the standard.

(b) Method of Lowry et al.:

Protein determinations in the subsequent purification steps were carried out with the Folin-Ciocalteu reagent as described by Lowry et al. (257). Crystalline bovine serum albumin was used as the standard and the final solution was read at 750 nm, the absorption peak. Samples free of ammonium sulfate and Tris and containing only low concentrations of phosphate were used to avoid interference from these substances.

(c) Optical method:

Protein determination by the optical method of Warburg and Christian (258) was done by using the following empirical equation (259) to correct for light absorption due to nucleic acids, the light path being 10 mm:

\[
\frac{4}{7} (2.3 \times (O.D._{280}\text{nm} - O.D._{340}\text{nm})) - (O.D._{260}\text{nm} - O.D._{340}\text{nm})
\]

This method was used, although it was somewhat inaccurate, to obtain rapid comparative estimates of protein content. The concentrations of serum albumin and mammalian cytochrome c in solutions were calculated from their extinction coefficients at 280 nm (260) and 550 nm (261) respectively.

(d) Micro-Kjeldahl method:

This method was used to determine the protein of electrophoretically homogeneous sample of enzyme to compare the final specific activities obtained by this method and those based on protein determinations by optical and Lowry's methods. The protein was calculated from total nitrogen on the assumption
that the protein contained 16% N, 1 - 1.5 mg enzyme protein, dialyzed against distilled water, was digested with 2 ml of conc. H₂SO₄ and about 1 gm of digestion mixture of composition, 5 mg CuSO₄·5H₂O, 0.5 mg powdered selenium and 250 mg KHSO₄ (262), until completely clear. The ammonia liberated from the digest by steam distillation under alkaline conditions was absorbed in N/70 H₂SO₄ and nitrogen was calculated by titrating against standard NaOH solution. The accuracy of the method was judged by using standard ammonium sulfate solution. For comparison, protein estimations on standard solution of cytochrome c and bovine serum albumin were also carried out by this method.

**Ammonium sulfate fractionation:**

Ammonium sulfate saturation refer to 0°C and the quantity required for changing the degree of saturation was calculated according to Jagannathan et al. (259) from the following equations:

For solid ammonium sulfate: \[ X = \frac{50(S₂-S₁)}{1.028 S₂} \]

For saturated ammonium sulfate: \[ Y = \frac{100(S₂-S₁)}{1-S₂} \]

where \( X \) equals gm of solid ammonium sulfate to be added to 100 ml of solutions of saturation \( S₁ \) in order to change it to saturation \( S₂ \), and \( Y \) equals ml of saturated solution to be added to 100 ml of solution to change its saturation from \( S₁ \) to \( S₂ \), \( S₁ \) and \( S₂ \) being expressed in fractions of saturation at 0°C. Ammonium sulfate, solid or solution, was added slowly
with gentle stirring to avoid frothing and the liquid was allowed to stand for 30-40 min, then centrifuged at 4,000 x g for 45 min.

**Ultracentrifugation:**

The ultracentrifugal studies were carried out in the Beckman Spinco model E ultracentrifuge equipped with a phase plate-schlieren optics and a rotor temperature indicator and control device capable of maintaining a constant temperature during the run. All the determinations in aqueous system were carried out at 3-8°C, and in denaturing systems at 20-25°C, using red-sensitive I-N spectroscopic plates (Kodak) and a 660 nm red filter for recording the sedimentation profile of the pink colored protein. A counter balance with the usual reference holes was used to provide reference points for determining radical distances from the axis of rotation.

**Homogeneity and sedimentation coefficient:**

Homogeneity and $s_{20,w}$ were routinely determined from sedimentation velocity runs using a 4° sector, 12 mm standard cell at a speed of 59,780 rpm. Some of the runs were also carried out in a Beckman valve-type synthetic boundary cell of 12 mm thickness and 4° sector. Photographs taken at different time intervals were read either on a Hilger (L-50) or a Haertner (model M 2060) microcomparator. Correction for the stretching of the analytical rotor (0.02 cm) was determined according to the method of Kegeles and Gutter (263). Sedimentation coefficient was calculated in the usual manner from the plots
of the logarithm of distance of sedimenting boundary from
the axis of rotation versus time (264). The sedimentation
coefficients ($s_{\text{obs}}$) were normalized to water at $20^\circ \text{C}$ ($s_{20,\text{w}}$)
after making density and viscosity corrections (264). A value
of 0.73 ml/g calculated from amino acid composition as
in text described below was used after correction for the temperature.

**Molecular weight:**

Molecular weight determinations were made by the
approach-to-equilibrium method of Archibald (265) as described
by Schachman (264) in a synthetic boundary cell. The phase
plate was used at an angle of 80° and the approximate speed of
the centrifugation for linear extrapolation of the gradient
curve was calculated according to Labar (266). The protein
solution was dialyzed overnight at 0-4°C against 100 volumes of
0.05 M phosphate buffer (pH 6.8) with two changes and then
spun at 10,000 x g for 15 min before filling in the cell.

0.6 ml solution (0.5 - 1.0% protein) was used directly in the
sector of the synthetic boundary cell with the cup empty
for determination of changes in concentration. Only readings
at the meniscus were taken. Initial protein concentrations
were determined by layering the solvent buffer system (0.2 ml)
from the cup at a speed of about 8,000-10,000 rpm over the
protein solution (0.4 ml) and immediately adjusting rotor
speed to that used in the corresponding run for determining
concentration depletion at the meniscus. Photographic plates
were read at 0.1 mm intervals either on a Hilger L-50 two-way
measuring micrometer with a sensitivity of one micron or a
model M 2060Aertner microcomparator. Areas were determined by trapezoidal analysis.

For determining the molecular weight in denaturing systems, the enzyme was precipitated with solid ammonium sulfate and the protein precipitate dissolved in appropriate denaturing buffer system. The enzyme was dialyzed against the denaturing buffer for 70-80 hr with at least four changes of the buffer. The densities and viscosities for guanidine HCl and urea solutions were taken from tables of Kawahara and Tanford (251).

As a check on the accuracy of the method, the molecular weight of crystalline bovine plasma albumin was determined. Consistent values were obtained and were on the average 68,000 in both dilute aqueous buffer solution and in 8M urea. This is in good agreement with the reported molecular weight values in the literature (267,268). The method of Archibald was preferred over that of Yphantis (269) because of difficulty in obtaining a stabilized supply of current over a long period of time.

**Gel filtration studies:**

A column (1.6 x 55 cm) of Sephadex G-200 (40-120 μ) or Bio-Gel P-150 was equilibrated at 4°C with 50 mM potassium phosphate buffer, pH 6.8. Hydrated gel and buffer were routinely degassed under vacuum prior to use. When the bed had settled to a constant height the sample solution (0.5 - 1.0 ml) containing 10% sucrose was carefully layered under the buffer solution above the gel. Eluate fractions of 1 ml were collected at a flow rate of 10-12 ml/hr and assayed for protein and/or
enzymic activity. Dextran Blue 2000 (Pharmacia) was used to
determine the void volume ($V_0$) and phenylalanine to measure the
inner volume ($V_1$). The total volume ($V_t$) was determined directly
with water. The elution volume ($V_e$) of a given solute zone
was taken in all cases as the effluent peak position of the
solute. The column was calibrated with proteins of known
molecular weights (270) or Stokes' radii (271). Gel filtration
data are presented in terms of $V_e/V_0$, $K_d$ and $K_{av}$, the parameters
involved in several mathematical correlations of elution volume
with Stokes' radius and molecular weight (271-273). The
parameters, $K_d$ and $K_{av}$ are calculated as defined by the
following equations (274):

$$K_d = \frac{V_e - V_0}{V_1} = \frac{V_e - V_0}{V_t - V_{g} - V_0}$$

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

where $V_e$, $V_0$, $V_t$ and $V_1$ have the same meaning as described
above. $V_g$, the volume occupied by the gel grains is estimated
from the following relation

$$V_g = \frac{V_t}{B \cdot d}$$

where $B$ = bed volume per gram of dry Sephadex G-200 (approx
35 ml/g) and $d$ is the density of dry Sephadex G-200 (1.65 g/ml)
(273). For the columns used in the present work
$V_g = 1.73$ and $K_{av} = 0.97 K_d$. 
Polyacrylamide gel electrophoresis:

Analytical disc gel electrophoresis was performed according to Davis (275) using 7.5% acrylamide gel polymerized with 0.07% persulfate. The discontinuous buffer system of Davis was used in which separation gel contains a Tris-HCl buffer of pH 6.9. Sample and stacking gels were omitted. After 2 hr of preliminary electrophoresis to eliminate persulfate ions, 50-200 µl sample (made dense with 20% sucrose) was applied through the upper buffer onto the surface of the gels. Electrophoresis was carried out in the cold room (4°C) at 3 mA per tube for 3-4 hr until the dye, bromophenol blue, reached the bottom of the gel. The electrode buffer was Tris-glycine, pH 8.3. After the electrophoresis the protein bands were stained with 1% Amido Schwarz in 7% acetic acid. The destaining of the gel was either performed electrophoretically or by diffusion in 7% acetic acid for about 24 hr.

The molecular weight of native nitrite reductase using gel electrophoresis was determined according to the method described by Hedrick and Smith (276). Separation gels with various concentrations of acrylamide (6-18%) were prepared according to Ornstein and Davis (277) except that the ratio of acrylamide to bis (N,N'-methylenebisacrylamide) was 30:1 which was maintained constant in all the gels. The use of spacer gel was found unnecessary. Samples (100 µl) in 5 mM Tris-glycine buffer, pH 8.2 containing 50% glycerol and 0.05% bromophenol blue were layered on top of the gels. Electro-
phoresis was carried out at 2 mA for 30 min and 4 mA for 2 hr. in a cold room (4°C). At the end of the run the dye front was marked by inserting 25 gauge copper wire. The staining and destaining were performed as already described. Migration of dye and protein bands was measured on an illuminated box using a magnifying glass mounted on the top of the light box. Measurements were accurate to ± 0.5 mm.

SDS-gel electrophoresis containing 0.1% SDS was carried out as described by Shapiro at al. (278) except that samples after treatment with 1% SDS and 1% 2-ME at pH 7.0 were incubated at 37°C for about 4 hr and were not dialyzed prior to electrophoresis. The electrophoresis was carried out at 8 mA per tube for 3 hr.

N-terminal analyses:

N-terminal amino acid residue was determined as dansyl derivative using the technique described by Gros and LaBrousse (279). Protein (about 1 mg) was dansylated in 60 mM phosphate buffer pH 8.5 containing 4 M urea for 30 min at 30°C. The dansylated protein was precipitated with 10% TCA; the precipitate was recovered by centrifugation and washed twice with 1 M HCl. The dansyl-enzyme was hydrolyzed with 5.7 M HCl at 110°C for 4 hr; the hydrolysate was taken to dryness, and the residue was suspended in 0.1 ml of a mixture of acetone-HCl (9:1, v/v). Thin layer chromatography on silica gel plates was used for identification of dansylamino acids using solvent system A of Morse and Morecker (280) and the solvent system b of Deyl and Rosmus (281). Migrations were compared with
standard dansylamino acids (Sigma) as necessary. N-terminal residues of glutathione and lysozyme were determined as a check on the accuracy of the method and were found to be the same as reported in the literature. The quantitative determination of N-terminal amino acid residue was performed according to the procedure of Gros and Labouesse (279) with slight changes. The dansylation of the enzyme (10 to 12 nmoles) and its hydrolysis was carried out as already described for qualitative experiments. Chromatography was performed in solvent system A of Morse and Horecker. The fluorescent spot was drawn up from the plate and the dansylmethionine was eluted three times with 0.5 ml of chloroform-methanol-acetic acid (7:2:2) mixture. The eluates were combined, evaporated to dryness and dissolved in 5 ml of absolute ethanol. The fluorescence of the samples was measured in a Beckman DU Spectrophotometer equipped with a fluorescence attachment provided with a 365 nm entrance filter. A standard dansylmethionine solution was used as a reference. Control experiments with methionine gave dansylmethionine in about 50-60% of the theoretical yield.

**Amino acid analyses:**

The amino acid analyses of nitrite reductase were performed without prior removal of heme groups. To prepare acid hydrolysates, lyophilised samples of the enzyme (approximately 1-2 mg protein) were heated with three-times glass-distilled constant boiling HCl at 110°C for 18, 24, 30 and 48 hr in evacuated and sealed Pyrex tubes in a block heater as described by Moore and Stein (282). The hydrolysates were evaporated to dryness
in a rotary flash evaporator at 45°C. The residual HCl in
the hydrolysates was removed by dissolving the residue in
about 2 ml of deionized water and taken to dryness by flash
evaporation. This was repeated two times. The residues were
taken up in citrate buffer, pH 3.8, and aliquots were analyzed
in a Spinco model 120-3 automatic amino acid analyzer by the
method of Spackman et al. (283).

The amide content of the enzyme was determined from the
amount of ammonia liberated during acid hydrolysis of the enzyme.
No separate determination were performed because of insufficient
amount of the enzyme available.

determination of sulfhydryl groups:

The free thiol groups of nitrite reductase were determined
by titration of the enzyme in the presence and absence of
denaturing agents (8M urea, and 8M urea plus 1% SDS) with
p-HMB and DTNB. When denaturing agents were used, the protein
was initially incubated with these solutions for 60 min prior
to the addition of DTNB or p-HMB.

p-HMB titration

Titrations of the enzyme with p-HMB were carried out
essentially as described by Benesch and Benesch (284). 8-9 mg
of p-HMB (sodium salt) is dissolved in 1 ml of 0.04 M NaOH
and solution made to 25 ml. p-HMB solutions were standardized
both spectrophotometrically by direct optical density
determination at 232 nm ($\varepsilon_M = 1.69 \times 10^4$) (280) and by titration
against standard reduced glutathione solution as described by

T145G
Benesch and Benesch (284). The determinations by the two methods were in good agreement.

Titrations of nitrite reductase samples were carried out on accurately measured aliquots of the protein in 1 ml stopped silica cuvettes of 10 mm light path. The protein samples were taken in 50 mM potassium phosphate buffer, pH 7.0. Small aliquots of standard p-HMB solution were added to the experimental solution and the blank which contained equal volume of buffer. The contents were mixed and the optical density was measured at 255 nm after each addition till there was no further change. The observed optical densities were corrected for dilution and plotted against the volume of the p-HMB added. The end point is obtained from the intersection of the two lines as shown in Fig. 15.

DTNB titration:

The titrations of the enzyme with DTNB were carried out according to the procedure described by Thorner and Paulus (285). Appropriate dilutions of the enzyme were prepared in 1 ml volume in 1-ml capacity silica cuvettes of 10 mm light path and the titration was started by the addition of 0.02 ml of 10 mM DTNB in 0.05 M potassium phosphate buffer, pH 7.5. The reaction was monitored at 412 nm with a Beckman DU Spectrophotometer over a period of about 6-8 hr. The same procedure was used when the titrations were performed in the presence of the denaturing agents except that the reaction was complete within about an hour's time. An extinction coefficient of 13,600 (286) was used for reduced thionitrobenzoate for calculating the
free thiol groups. The accuracy of the method was checked with reduced glutathione.

**Determination of total sulfhydryls and disulfide bonds:**

Total -SH groups and -S-S- linkages in nitrite reductase were determined by two different procedures:

1) **Reduction of -S-S-linkages with NaBH₄ in 8M urea followed by DTNB titrations after removal of excess of NaBH₄.**

2) **Oxidation of -SH and -S-S- groups to cysteic acid by the standard procedure of performic acid oxidation followed by acid hydrolysis and cysteic acid estimation using an amino acid analyzer.**

1) **Reduction of by NaBH₄ followed by DTNB titration:**

The reduction and estimation were carried out by a slight modification of the procedure of Cavallini, Graziani and Dupre (287). Test tubes (18 x 110 mm) with marks at 3 ml and 6 ml were used for this experiment. The following were added to the tubes in the order shown: 1.44 g of solid urea, 0.1 ml of 0.1 M diNa-EDTA, 1 mg of the purified enzyme, 1 ml of 2.5% NaBH₄ prepared just before use and water to make upto 3 ml. The tubes were shaken in order to dissolve urea and incubated at 38°C for 45 min. 0.5 ml of 1 M KH₂PO₄ containing 0.2 M HCl was then added. The destruction of NaBH₄ was completed by adding 2 ml of acetone. The solution was shaken thoroughly and brought well into contact with the walls of the test tube. Nitrogen was passed through the solution and 0.1 ml of 0.1 M DTNB was added under nitrogen. After standing for 15 min the light
absorption at 412 nm was determined. The number of sulfhydryl groups (N) was calculated using the following formula.

\[ N = \frac{M_w \times A \times V}{12000 \times m} \]

where

- \( M_w \): molecular weight of the protein
- \( A \): absorbancy
- \( V \): volume of the final solution
- \( m \): weight in mg of the protein sample analyzed

The accuracy of the method was checked with bovine serum albumin and lysozyme as standard.

2) Performic acid oxidation and cysteic acid estimation:

The total half-cystine content was determined as cysteic acid after oxidation with performic acid according to Moore (288) followed by hydrolysis and amino acid analysis as above. The performic acid treatment removes heme moieties of the heme protein (289). This method will, therefore, also estimate the cysteine residues which are bound to heme moieties of the protein.

Estimation of tyrosine and tryptophan:

Tyrosine and tryptophan were determined both spectrophotometrically by the method of Benese and Schmid (290) and Goodwin and Norton (291) and colorimetrically by the method described by Uehara et al. (292) for tyrosine and that of Spies and Chambers (293) for tryptophan. The values of tryptophan and tyrosine obtained by these methods were in good agreement.
Proteins show selective absorption in the ultraviolet region and the position of the absorption maximum varies with pH. The majority of the constituent amino acids do not show any absorption in the region 250-320 nm and it is known that phenylalanine, tyrosine and tryptophan are responsible for the observed ultraviolet absorption of protein solutions. In 0.1 M NaOH the absorption by tyrosine and tryptophan is much stronger and that by phenylalanine is negligible. Under these conditions the protein solutions may be treated as two-component system for spectrophotometric analysis. The intensity of absorption at the point where the curves for tyrosine and tryptophan intersect is a direct measure of the total molar solute concentration and will be the same however the proportions are varied. At other wave lengths the intensity of absorption will vary with the relative proportions of the components. Using 0.1 M NaOH as solvent the two absorption curves intersect at 294.4 nm ($\epsilon = 2375$) and 257.15 nm ($\epsilon = 2748$). By determining the absorption of the protein in 0.1 M NaOH at the above two wavelengths and at one other wavelength (e.g. at 280 nm) it is possible to determine the relative proportions of tyrosine and tryptophan in the protein.

Thus, if $x = \text{total mole/1 in solution}$

$y = \text{g mole of tyrosine}$

$x-y = \text{g mole of tryptophan}$

At any wavelength other than the point of intersection let
e tyrosine be $A$, and e tryptophan be $B$ and the observed intensity of absorption for a 10 mm cell $E$
then,

\[ E = yA + (x-y)B \]

or \[ Y = \frac{E - xB}{A - B} \]

$x$ = $E$ value at an intersection
$\epsilon_{tyr}$ at an intersection

(2) Benoce and Schmid’s method (graphical method):

This method is based upon measuring the absorbance of the protein in 0.1 M NaOH in the range between 278 and 294 nm at 2 nm intervals. The readings are plotted against the wavelength and a line is drawn tangentially to the two characteristic peaks. From the slope of the tangent, the maximum absorption between 270 and 290 nm, and the molecular weight of the protein the tyrosine and tryptophan content is determined.

Attempts to use heme-free enzyme in the determination of tyrosine and tryptophan by spectrophotometric methods were not successful as heme-free enzyme (heme split by Paul’s procedure) tends to precipitate in alkaline solutions. The heme of nitrite reductase absorbs more or less uniformly in the range 272 to 292 nm and is therefore not likely to interfere with the characteristics of the slope of the tangent used in Benoce and Schmidt’s method to compute the ratio between tyrosine and tryptophan. A correction for absorption due to heme was however applied to the value of absorption maximum which estimates the total $x$ tyrosine and tryptophan
content. A similar correction was applied in Goodwin and Morton's method.

(3) **Colorimetric determination of tyrosine**

Tyrosine content of nitrite reductase was also determined by the colorimetric method of Uehara, Mannen and Kishida (292). The method involves alkali-denaturation of protein in a boiling water bath, the color forming reaction between tyrosine and l-nitroso-2-naphthol in 19 N H₂SO₄, and measurement of the absorbance at 520 nm. The procedure is as follows:

Nitrite reductase (0.6 - 1.5 mg) in water (1 ml) was added to 1 ml of 0.15% (w/v) l-nitroso-2-naphthol in 0.1 N NaOH and 2 ml of a mixture of equal volumes of 0.025 N HNO₃ and 0.3 N NaOH. The mixture was heated in a boiling water bath for 10 min and then placed in a water bath at 50°C until equilibrium was reached. Concentrated H₂SO₄ (4 ml) was then added. The red color was measured after 15 min against a reagent blank at 520 nm. Tyrosine standards were run at the same time. The accuracy of the method was checked with ovalbumin and ribonuclease.

(4) **Colorimetric determination of tryptophan**

Tryptophan was determined by colorimetric method (procedure K) of Spies and Chambers (293). Eight milliliters of 23.7 N H₂SO₄ and 1 ml of 2 N H₂SO₄ containing 30 mg of p-dimethylaminobenzaldehyde were mixed and cooled to 25°C. To this solution is added 1 ml of aqueous enzyme sample. The solution was mixed, cooled to 25°C, and kept for 12 hr. To the solution was then added 0.1 ml of an 0.045% solution of
NaNO₂. After 30 min, absorbance is read and converted to weight of tryptophan from the standard curve prepared in a similar way. The accuracy of this method was checked with bovine serum albumin and ovalbumin.

**Determination of degree of hydrophobicity:**

The degree of hydrophobicity of *A. fischeri* nitrite reductase and nitrite reductases from *P. aeruginosa* and *Chlorella fusca* was calculated from their amino acid compositions. Three different methods were followed.

(1) **Fisher's method:**

According to Fisher (295) the degree of hydrophobicity is expressed in terms of a polarity ratio, $p$, which is defined by the following equation

$$ p = \frac{V_0}{V_1} $$

where $V_0$ and $V_1$ are the volumes occupied by polar and nonpolar residues, respectively. Arginine, histidine, lysine, aspartic acid, glutamic acid (and their amides), tyrosine, serine and threonine were considered by Fisher (295) as polar residues and all other amino acids as nonpolar residues.

(2) **Waugh's method:**

According to the method of Waugh (296) the hydrophobicity is measured in terms of MPS, the frequency of monopolar side chains. MPS is calculated by counting the tryptophan, isoleucine, tyrosine, phenylalanine, proline, leucine and valine residues and expressing the sum as a fraction of the total number of residues.
(3) Bigelow's method:

Bigelow's method (297) gives average hydrophobicity, $H_{av}$, which is based on Tanford's (298) free energies of transfer of amino acid side chains from an organic environment to an aqueous environment. $H_{av}$ is the total hydrophobicity divided by the total number of residues.

Determination of isoelectric point:

The isoelectric point of *A. fischeri* nitrite reductase was determined from the titration curve which was constructed on the basis of amino acid composition. Calculations for constructing the theoretical titration curve were carried out according to Cohn and Edsall (299) and were based on the assumption that there are no electrostatic interactions between ionizable groups and that each member of each species is identical, ionizing independently. The principle of calculation for theoretical titration curve is as follows.

If $n_1$ is the number of ionizable acid groups of class I and $\alpha_1$ the fraction of group in the ionized state at a particular pH, the number of negatively charged groups (class I) is given by the following expression.

$$n_1 = \alpha_1$$

$\alpha_1$ as a function of pH can be evaluated by the equation

$$pH = pK + \log \frac{\alpha_1}{1 - \alpha_1}$$

where $pK$ is the negative log of ionization constant of the respective ionizable group. In the case of basic groups,
however, the number of positively charged groups is given by

\[ n_2 (1 - \alpha_2) \]

where \( n_2 \) is the number of basic groups (class II) and \( \alpha_2 \) is the fraction of the groups that has ionized at a given pH.

Since proteins are polyvalent ampholytes containing a large number of acid and basic groups of more than one type, the total mean net charge (\( Z \)) at a particular pH is given by the difference between the sum of negatively charged and the sum of positively charged groups.

\[
Z = \left[ n_{\text{Arg}} (1 - \alpha_{\text{Arg}}) + n_{\text{Lys}} (1 - \alpha_{\text{Lys}}) + n_{\text{His}} (1 - \alpha_{\text{His}}) \right] - \\
\left[ n_{\text{carboxyl}} \alpha_{\text{carboxyl}} + n_{\text{Tyr}} \alpha_{\text{Tyr}} + n_{\text{Cys}} \alpha_{\text{Cys}} \right]
\]

\( \alpha \) and \( n \) have the same meaning as described above.

The net charge on the enzyme protein between pH 2 and pH 14 was calculated by the use of the above equations and the values were plotted against pH, resulting in a theoretical titration curve. The \( pK \) values for different acid and basic groups were taken from Mahowald, and Kuby (300).