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PURIFICATION AND PROPERTIES OF CYTOCHROMES *c* OF *AZOTOBACTER VINELANDII*

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SUMMARY

1. An improved method for extraction and purification of *Azotobacter vinelandii* cytochromes *c* by chromatography with DEAE-cellulose and Sephadex and by isoelectric focusing is described. Yields of purified cytochromes c_4 and c_5 were approximately double those obtained with previous methods. Cytochromes c_4 and c_5 were homogeneous as evidenced by their purity index values and electrophoretic patterns on acrylamide gel. Both were slowly autoxidizable; cytochrome c_4 did not combine with CO, whereas cytochrome c_5 showed 10% combination.

2. A third cytochrome *c* from *A. vinelandii*, designated as minor cytochrome c_4, was separated on DEAE-cellulose columns. Though spectrally identical, its molecular weight is half that of cytochrome c_4.

3. Cytochrome c_4 is a monomer with 2 hemes per molecule and a molecular weight of 24000 $\pm$ 2000. Cytochrome c_5 was isolated as a dimer of 24400 $\pm$ 1000 molecular weight, but under denaturing conditions it is split into monomers half this size, each containing 1 heme group.

4. Cytochrome c_4 has 4 histidines per molecule; cytochrome c_5 has 1 histidine per monomer. Tryptophan is absent from cytochrome c_4.

5. Neither the relative cytochrome *c* content of cells grown on ammonia nor functional tests supported a direct role for *A. vinelandii* cytochromes *c* in nitrogen fixation. Large differences in rates of oxidation among the bacterial cytochromes *c* were noted with oxidases from *A. vinelandii* and from beef heart. Cytochrome *c* oxidation by *A. vinelandii* was always highly sensitive to KCN; this supports the concept that all bacterial cytochromes *c* are on one branch of a split terminal oxidase chain.

INTRODUCTION

Bacterial cytochromes *c* are a heterogeneous group of heme proteins with unique properties[1], but their evolutionary relationships have been much less intensively studied than those of the mammalian type cytochromes *c* (refs. 2, 3). The fact that relatively few bacterial cytochromes *c* have been obtained in the quantity and degree of purity required for such studies has been a major difficulty.

Spectrophotometric observations have shown that the aerobic, nitrogen-fixing

organism *Azotobacter vinelandii* has comparatively high cytochrome *c* levels[4]. Extraction and purification revealed 2 cytochromes *c* having different visible spectra; cytochrome c_4 with α, β and γ peaks at 551, 522, and 416 nm, respectively, and cytochrome c_5 with corresponding peaks shifted about 4 nm to the red[5]. Both cytochromes *c* have high redox potentials with $E_0' = 0.30$–0.32 V, are acidic and were reported to be nonoxidizable by beef heart cytochrome *c* oxidase[5]. NEUMANN AND BURRIS[6] described the use of a CM-cellulose column for separation of cytochromes c_4 and c_5 and from iron analysis they calculated a minimum molecular weight of 11200 for c_4 and 11600 for c_5.

Both purification procedures[5,6] have deficiencies; purification time is increased by inclusion of several dialysis steps, and an empirical basic lead acetate precipitation is sensitive to the gum level of the culture. In addition, yields are relatively low, and the acidic chromatography apparently is detrimental to the cytochromes *c*.

Functional studies of *A. vinelandii* cytochromes *c* have been limited. Aided by a sensitive spectrophotometric analysis of electron transport particles from *A. vinelandii*, JONES AND REDFEARN[7] concluded that terminal electron transport is branched. The majority of electrons appeared to be transported through the branch containing cytochromes b_1 and a_2, whereas cytochromes *c*, a_1 and *o* appeared to function through an alternate path sensitive to very low levels of KCN (10^{-5} M). They[7] were unable to study the separate *A. vinelandii* cytochromes *c* because of the proximity of their absorption peaks.

Other investigators[8] have cited inhibitor studies to support a direct role for *A. vinelandii* cytochromes *c* in electron transport in nitrogen fixation, but consideration of the potentials of the cytochromes makes this most unlikely.

This paper presents an improved method for separating and purifying the individual cytochromes *c* of *A. vinelandii* and describes chemical and physical properties which emphasize the recognized diversity of bacterial cytochromes *c*. The possible role of these heme proteins as electron carriers in nitrogen fixation and their relative reactivity in cytochrome *c* oxidase assays has been investigated.

MATERIALS AND METHODS

Growth of bacteria

A. vinelandii strain OP was grown on a nitrogen-free modified Burk's medium[9]. Transfers of about 5 % inoculum were made to a 150-l fermentor in which growth was continued for 8–10 h until absorbance at 660 nm reached 1.0 (Coleman Junior spectrophotometer with 18-mm test tube). The cells were harvested while still growing exponentially; the yield was 200–220 g dry wt. per 150 l and the cell paste was stored at −20°. When cells were cultured in the 150-l fermentor on ammonia rather than N_2 as a nitrogen source, 0.5 g NH_4Cl/l was included, and the pH was maintained at 7.0 with a pH stat which controlled the addition of anhydrous ammonia.

Preparation of oxidase particles

Thirty g thawed cell paste were mixed with 120 ml of 0.02 M sodium phosphate (pH 7.4), at 4°, and the cells were disrupted in a French pressure cell driven with a power press (8000–12000 lb/inch^2). Sediments from successive centrifugations for 15 min at 12000 × *g* and 30 min at 34000 × *g* were discarded. The small particle

fraction, sedimented by 60 min centrifugation at 117000 × *g*, is enriched in terminal electron transport components; it was resuspended once in the initial pH 7.4 buffer with an homogenizer and was recentrifuged. When particles were prepared for measurement of cytochrome *c* oxidase activity, phosphate buffer was replaced with 0.05 M Tris–HCl (pH 7.5) because phosphate is inhibitory in the assay[10].

Enzyme assays

The activity of cytochrome *c* oxidase was measured at 23–24° and at pH 7.5 in a 1-cm light path cuvette in a Cary 15 recording spectrophotometer. The 0.55-ml reaction mixture contained 28 μmoles Tris and small particles containing about 0.3 mg protein. Cytochrome *c* levels were varied from 0.006 to 0.07 mM. Oxidized samples were reduced with the minimum amount of sodium dithionite required for complete reduction, and decomposition products of dithionite were removed immediately by passage through a column of Sephadex G-25. The reaction was started by the addition with stirring of the small particle fraction, and the decrease in absorbance was followed at the appropriate α peak (cytochrome c_4 and minor cytochrome $c_4 = 551$ nm, cytochrome $c_5 = 554$ nm and horse heart cytochrome $c = 550$ nm). The reaction was terminated by the addition of a few crystals of potassium ferricyanide to completely oxidize the cytochrome.

The first-order rate constant was calculated by the method of SMITH[11] after all absorbance values had been corrected for nonspecific absorbance contributed by the oxidase particles.

Reciprocal plots were constructed using velocities expressed as μmoles/min calculated from an extrapolation of the initial reaction velocities. The μmoles of cytochrome *c* oxidized per min were calculated by dividing the extrapolated absorbance change per min by the $(\alpha_{red} - \alpha_{ox})$ value for each cytochrome; α_{red} is the millimolar extinction coefficient of the reduced cytochrome at the α peak, and α_{ox} is the millimolar extinction coefficient of the oxidized cytochrome at the α peak. The $(\alpha_{red} - \alpha_{ox})$ value for cytochromes c_4 and c_5 is 15.9 (ref. 5) and for horse heart cytochrome *c* is 18.5 (ref. 12).

Nitrogen fixation by crude extracts of *A. vinelandii* was measured by the method of KELLY *et al.*[13] with the following modifications: $MgCl_2$ was reduced from 10 to 5 mM, ATP was reduced from 5 to 2.1 mM, dithionite was reduced from 20 to 15 mM and Tris–HCl buffer was increased from 25 to 50 mM. About 5 mg crude extract protein were used per assay.

Molecular weight determinations

Molecular weights were estimated with Sephadex G-100 superfine according to the method of ANDREWS[14]. Centers of peaks were judged to the nearest ml from plotted curves.

The sodium dodecyl sulfate–acrylamide gel technique[15] for measuring molecular weights of proteins under denaturing conditions was utilized to determine molecular weights of cytochrome *c* subunits. Protein samples, 1 mg/ml, were made to 1% mercaptoethanol and 1% sodium dodecyl sulfate in 0.1 M sodium phosphate buffer (pH 7.0). Gels used for electrophoresis contained 10% acrylamide and 0.1% detergent. Buffer reservoirs contained 0.1 M sodium phosphate (pH 7.0) and 0.1% sodium dodecyl sulfate detergent. A split gel technique[16], in which the sample is layered on one side of a strip of 1-mm-thick polyethylene dividing the volume above

the gel and a standard protein (myoglobin) is layered on the other side, insured that both samples and standards were subjected to the same electrophoretic conditions. About 0.02 ml (20 μg) of sample was separated electrophoretically at 8 mA per tube for about 5 h at room temperature. Extruded gels were soaked in several changes of 20 % sulfosalicylic acid to remove residual detergent before staining with Coomassie brilliant blue.

Isoelectric focusing

Isoelectric focusing was conducted with the LKB 8101 Ampholine column[17]. The column was used both as a preparative tool to purify 15-mg quantities of cytochrome c_5 and as an analytical device to determine isoelectric points.

Amino acid analyses

From 1.3 to 2.5 mg of *A. vinelandii* cytochromes *c* after dialysis against 0.001 M sodium phosphate buffer (pH 7.4) were made to 6 M HCl and hydrolyzed at 110° under vacuum as described by MOORE *et al.*[18,19]. A humin precipitate, probably derived from tryptophan and/or porphyrin was removed by centrifuging for 10 min at 34000 × *g*. The few peaks giving less than 0.05 absorbance units were analyzed by the absorbance method (instruction manual, Beckman–Spinco Model 120 amino acid analyzer) rather than by the height × width method.

Cysteine and/or cystine were analyzed by the performic acid oxidation procedure of MOORE[20]. Because the cytochromes were insoluble in the performic acid reagent, the oxidation time was lengthened to overnight at 0°.

Tryptophan in cytochrome c_4 was analyzed by the $Ba(OH)_2$ hydrolysis method of DREZE[21]. Later an improved method* for tryptophan analysis with NaOH hydrolysis was used for both cytochromes c_4 and c_5. Fifty mg hydrolyzed starch (Connaught starch for gel electrophoresis) and 0.78 g NaOH (final concentration about 6 M) were added to 3 ml of a solution containing 1.5–4 mg cytochrome *c*. The solution was placed in a sealed chamber and evacuated and flushed 3 times with N_2 before sealing under vacuum and hydrolyzing 20 h at 120°. The hydrolysate was adjusted to pH 5 with concentrated HCl in an ice bath, filtered through a sintered-glass filter and made to 5 ml with citrate buffer (pH 5.28). Four ml were added immediately to a starch column on a Beckman–Spinco Model 120 amino acid analyzer.

Acrylamide gel analysis

Acrylamide gel analyses of various fractions obtained during purification of the cytochromes *c* from *A. vinelandii* were made by the technique of DAVIS[22] for anionic electrophoresis and by that of REISFELD *et al.*[23] for cationic electrophoresis. Staining was effected with Coomassie brilliant blue[24]. When a more specific staining of heme proteins was desired, a benzidine peroxidase stain was used[25].

Protein

Cytochromes *c* from *A. vinelandii* and horse heart were measured quantitatively with their appropriate α peak extinction coefficients[5,26]. Protein of crude extracts was estimated by a microbiuret method[27] with bovine serum albumin as the standard.

* F. OELSHLEGEL, Jr., personal communication.

Chemicals

Standard proteins used in Sephadex and acrylamide gel molecular weight estimations include Sigma Type III horse heart cytochrome *c*, Grade I lysozyme, Type II chymotrypsinogen A (bovine pancreas), Grade V ovalbumin and crystallized and lyophilized bovine serum albumin, and Mann crystallized and lyophilized sperm whale myoglobin. NADH was Sigma Grade III, disodium salt. A beef heart electron transport particle fraction[28] and a purified cytochrome *c* oxidase fraction[29] were gifts of Dr. R. Harris.

RESULTS

Extraction and purification

French pressure cell treatment, sonication and high salt treatments give incomplete solubilization of the cytochromes *c* of *A. vinelandii*. However, butanol extraction[5] not only solubilizes cytochromes *c*, but also fails to solubilize or coagulates many contaminating proteins which are readily removed by centrifugation.

All operations were performed at 0–4° unless stated otherwise. Thawed cell paste (250 g) was mixed with an equal volume of *n*-butanol (technical) and was mixed 2 min in a Waring blendor. An additional 2 min of blending followed addition of 250 ml distilled water. Several butanol extracts were combined and centrifuged for 40 min at 3900 × *g* in a No. 845 head of an International centrifuge. Four layers were visible in the following order from the bottom to the top of the tube; cell debris, a red aqueous layer containing cytochromes *c*, a lipid layer and a butanol layer. After the aqueous layer was removed with a syringe, 250 ml water per 250 g original cell paste were added, and the previously described blending, centrifugation and removal of the aqueous layer was repeated. A third extraction was performed with 125 ml water per 250 g of original cell paste. All aqueous extracts were combined and stored overnight at —20°. The coagulated debris in the thawed extract was removed by centrifugation for 15 min at 3900 × *g*.

Solid $(NH_4)_2SO_4$ was added to the supernatant from 2 to 4 separate 250-g extractions to bring the concentration to 1.2 M. After 15 min the solution was centrifuged for 15 min at 3900 × *g*, and the floating lipoidal aggregate was removed with a spatula. The solution then was adjusted to 2.2 M $(NH_4)_2SO_4$ and after 15 min was centrifuged 15 min at 3900 × *g*. The floating pink solid was removed with a spatula, dissolved in a minimum of distilled water and transferred directly into Visking dialysis tubing. Throughout the $(NH_4)_2SO_4$ precipitation steps the pH was maintained at 8.0 by addition of 15 M NaOH. Control of pH is essential with acid-labile cytochromes *c* from *A. vinelandii*.

The cytochrome- and lipid-containing solution was dialyzed overnight against running tap water in 3.2-cm (flat width) Visking tubing and then against 2 or 3 successive 6-l volumes of 0.005 M Tris–HCl buffer (pH 7.6) until analysis with Nessler's reagent showed the diffusate had less than 3 μg N/ml.

The dialyzed solution was centrifuged free of denatured proteins and added as a narrow band onto a Whatman DEAE-52 cellulose column previously equilibrated with 0.005 M Tris–HCl buffer (pH 7.6) at 15°. Fifty ml of this same buffer were added to the column; the column was developed with a linear gradient of 500 ml of 0.03 M Tris (pH 7.6) and 500 ml of 0.12 M Tris (pH 7.6). A typical elution profile is illustrated

in Fig. 1. In addition to a large 270 nm peak appearing immediately after passage of the column holdup volume, 2 major cytochromes *c* (ref. 5), c_4 and c_5, *plus* a third previously unidentified cytochrome *c* of *A. vinelandii*, here designated as minor c_4, appeared. The visible spectrum of minor cytochrome c_4 is identical with that of cytochrome c_4 (ref. 5), but the minor cytochrome differs in other important physical properties from cytochrome c_4, as will be detailed later. Minor cytochrome c_4 is not an artifact of the butanol extraction, because cells that were simply sonicated, dialyzed and chromatographed on DEAE-cellulose revealed the same 3 cytochrome bands. The percentages of cytochromes c_4, c_5 and minor c_4 after butanol extraction and chromatography were 65, 30 and 5%, respectively.

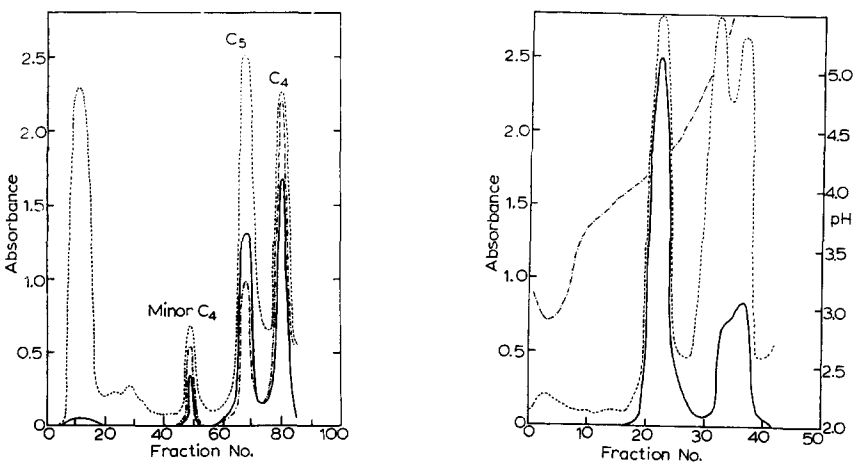


Fig. 1. Chromatography of *A. vinelandii* cytochromes *c* on DEAE-cellulose. 90 ml of a dialyzed $(NH_4)_2SO_4$ precipitate containing 93 mg cytochromes *c* were added to a column of DEAE-cellulose 3.6 cm in diameter and 13 cm in height preequilibrated with 0.005 M Tris–HCl buffer (pH 7.6). The column, maintained at 15°, was developed with a linear gradient of 500 ml of 0.03 M Tris–HCl (pH 7.6) and 500 ml of 0.12 M Tris (pH 7.6). The flow rate was 1.0–1.5 ml/min and 10-ml fractions were collected. – – – –, $A_{270\ nm}$; ———, $A_{554\ nm}$; –·–·–·–, $A_{550\ nm}$.

Fig. 2. Isoelectric focusing of cytochrome c_5. Four ml (19 mg) of cytochrome c_5 purified on DEAE-cellulose were added in place of the 'light' ampholyte solution (pH range 3–5) at the middle of the stepwise sucrose gradient. Isoelectric focusing was conducted at 15° and at 2.0–2.3 W for 24 h. The column was drained at 2 ml/min and 2-ml fractions were measured with a pH meter at 15°. Absorbances were recorded after dilution to 3.0 ml with 0.02 M potassium phosphate buffer (pH 7.4). –·–·–·–, pH; – – – –, $A_{270\ nm}$; ———, $A_{554\ nm}$.

Cytochrome c_4, already about 80% pure, was brought to a homogeneous state by passage at 15° through a Sephadex G-75 superfine column (2.2 cm in diameter and 43 cm in height) which had previously been equilibrated with 0.05 M sodium phosphate buffer (pH 7.4). Pure fractions were pooled and used for further chemical and physical studies.

Molecular sieving by Sephadex G-75, however, was only partially successful with cytochrome c_5, as 2 or 3 successive columns were required for complete purification. The isoelectric focusing technique was much more successful; the results are presented in Fig. 2. Here cytochrome c_5 (Peak a) is completely separated from impurity Peaks b and c. Peak c, pink in color and having considerable absorbance at 554 nm, is not another *A. vinelandii* cytochrome *c*; rather it is identical spectrally with

the recently isolated Azotobacter iron-sulfur protein II (ref. 30). The presence of this protein, which exhibits changes in spectral absorbance at 554 nm opposite to those of cytochrome c_5, explains the anomalous decrease in absorbance when cytochrome c_5, from the DEAE-cellulose column is reduced with dithionite. The cytochrome c_5 purified by isoelectric focusing was concentrated in an Amicon Diaflo cell and was injected onto a column of Sephadex G-75 superfine similar to that used to purify cytochrome c_4. This column completed the purification of cytochrome c_5 and removed low molecular weight ampholytes.

TABLE I

SUMMARY OF PURIFICATIONS AND YIELDS OF CYTOCHROMES *c*

	mg cytochrome c per 100 g dry wt. cells	*Recovery* (%)	*Purity ratio* (R^*)
Butanol extract	166	100	
Dialyzed $(NH_4)_2SO_4$ ppt.	95	58	0.0027
Cytochrome c_4			
DEAE-cellulose column	57	34	1.02
Sephadex G-75	47	28	1.30
Cytochrome c_5			
DEAE-cellulose column	26	16	0.42
Isoelectric focusing	19	11	1.05
Sephadex G-75	14	9	1.16
Minor cytochrome c_4			
DEAE-cellulose column	4.6	2.8	0.68

$^* R = \frac{\text{absorbance at } \alpha \text{ peak (reduced)}}{\text{absorbance at 270 nm (oxidized)}}$.

A summary of the purification and yields of *A. vinelandii* cytochromes *c* is given in Table I. The butanol extract values are approximate because of the difficulty in measuring a small change in absorbance against a highly turbid background. The yields of pure cytochromes *c* from cells grown with air as a nitrogen source were 32–52 mg cytochrome c_4 per 100 g dry weight and 13–16 mg cytochrome c_5 per 100 g dry weight as compared with 15–25 mg c_4 per 100 g dry weight and 8–13 mg c_5 per 100 g dry weight reported for an earlier method[6]. Under the usual conditions of purification, 4 or 5 days are required to obtain pure cytochromes c_4 and c_5 from 1 kg (200 g dry weight) of cell paste.

The purity ratios of 1.30 for cytochrome c_4 and 1.16 for cytochrome c_5 are similar to the values of 1.27 and 1.19 previously reported for crystalline preparations of the same cytochromes[6]. Purity also was monitored by acrylamide gel analysis; Fig. 3 illustrates the protein species present at various steps in the purification of cytochrome c_4. Though not readily visible in Fig. 3, a faint leading band was present in purified c_4 preparations on anionic electrophoresis. If very high concentrations of cytochrome were loaded on the gel, the band had the pink color of cytochromes *c*. Also, it reacted strongly with benzidine-peroxide stain, a further indication of a heme protein. It

is perhaps an altered form of cytochrome c_4, possibly deamidated. A more striking example of altered cytochrome forms arising during purification is seen in Fig. 4 which depicts purified cytochrome c_5 stained with both Coomassie brilliant blue and benzidine-peroxide after anionic electrophoresis. Further evidence that the minor bands are altered forms of cytochrome c_5 comes from the fact that their number and intensity increased with lengthy storage at —20° or upon exposure to acidic conditions (< pH 5). Chromatography on Sephadex G-75 of *A. vinelandii* cytochromes *c* stored 2 weeks at —20° indicated that partial (5 %) polymerization had occurred.

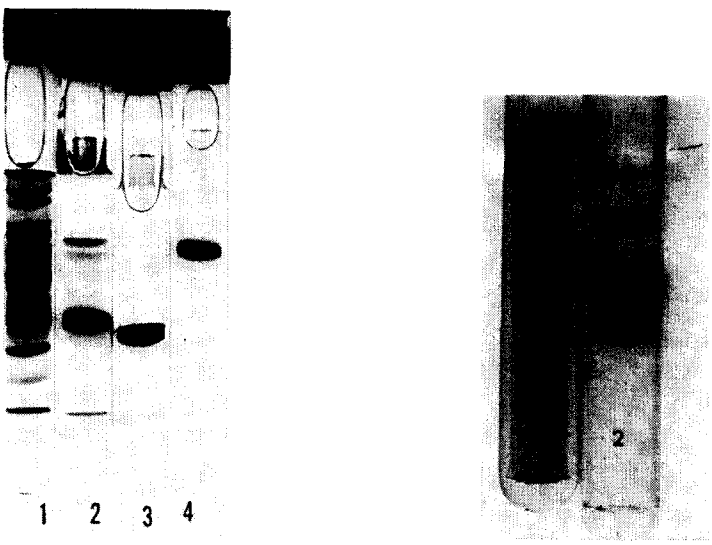


Fig. 3. Separations on acrylamide gel of fractions in the purification of cytochrome c_4. Gels 1–3, anionic electrophoresis; Gel 4, cationic electrophoresis; Gel 1, dialyzed $(NH_4)_2SO_4$ fraction; 14 μg cytochrome c_4; Gel 2, cytochrome c_4 (120 μg) purified on DEAE-cellulose; Gels 3 and 4, cytochrome c_4 (120 μg) purified on Sephadex G-75 superfine.

Fig. 4. Electrophoretically separable forms of purified cytochrome c_5. Gel 1, 120 μg c_5, stained with benzidine–H_2O_2; Gel 2, 120 μg c_5, stained with Coomassie brilliant blue.

Purified cytochromes c_4 and c_5 undergo autoxidation very slowly. For example, cytochrome c_4 was 10 % oxidized after 13 days storage at —20°. Purified cytochrome c_4 did not combine with CO, whereas the α peaks of pure reduced cytochrome c_5 decreased about 10 % in intensity when purged with CO, probably because of alteration by exposure to acidic conditions during isoelectric focusing. Nevertheless, pure c_5 was as active as c_5 purified only to the DEAE-cellulose stage when tested for cytochrome *c* oxidase activity with *A. vinelandii* electron transport particles.

Molecular weights

Fig. 5 presents the results of molecular weight estimations on Sephadex G-100. Four separate determinations for cytochrome c_4 and 3 for cytochrome c_5 gave values of 25100 ± 900 for c_4 and 24400 ± 1000 for c_5. There was enough material for only one determination of molecular weight on minor cytochrome c_4, and this gave a value of 12200. The cytochrome c_4 and c_5 values were surprisingly high, approximately double the minimum molecular weights estimated by iron analysis[6]. However, the

same values were found on Sephadex columns saturated with 0.1 M mercaptoethanol and when samples were run at either the DEAE-cellulose or Sephadex G-75 stage of purification.

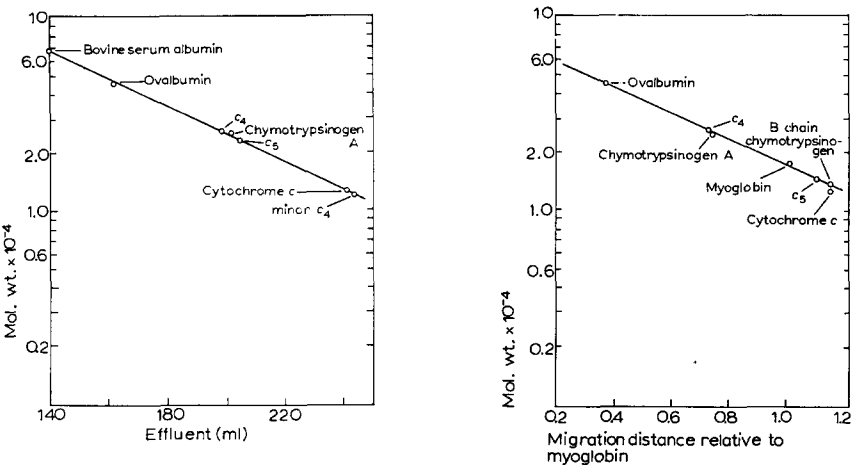


Fig. 5. Molecular weight determinations on Sephadex G-100. Five mg of each protein in 2 ml 0.05 M sodium phosphate buffer (pH 7.4) were injected into the bottom of a Sephadex G-100 superfine column 2.5 cm in diameter and 70 cm in height preequilibrated with the same buffer. Temperature was 15°, and the upward flow rate was 0.3 ml/min. After 110 ml had been eluted, 3-ml fractions were collected and assayed for protein at 280 nm or for cytochrome *c* at the wavelength of the respective α peak.

Fig. 6. Molecular weight determinations on acrylamide gels with sodium dodecyl sulfate. Proteins, 1 mg/ml, were dissolved in 1% sodium dodecyl sulfate *plus* 1% mercaptoethanol and 5–10% sucrose. Twenty μg of each was layered on one side of the 'split' gel and the same amount of standard myoglobin on the other. Electrophoresis was conducted at 8 mA per tube for 5–6 h. Mercaptoethanol was omitted from the chymotrypsinogen A sample.

To determine whether the cytochromes were dimers, samples treated with 1% mercaptoethanol and 1% sodium dodecyl sulfate were separated electrophoretically on polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Whereas cytochrome c_4 and cytochrome c_5 migrated together under nondenaturing conditions on Sephadex G-100, they migrated markedly different distances under the denaturing conditions induced by sodium dodecyl sulfate (Fig. 6). In two experiments the calculated molecular weight of cytochrome c_4 was 22800 $\pm$ 800, and in three determinations the molecular weight of cytochrome c_5 was 14000 $\pm$ 100, a value approximately half that found under nondenaturing conditions with Sephadex G-100.

The relative molecular weights of possible cytochrome c_4 and c_5 subunits also were estimated by centrifugation with 8 M urea and 0.1 M mercaptoethanol in sucrose density gradients. The relatively short distances migrated by the low molecular weight cytochromes in the highly viscous solvent prevented an accurate estimation of molecular weights. However, cytochrome c_4 consistently migrated farther than c_5. The combined results of experiments on Sephadex and with detergent polyacrylamide gels indicate that cytochrome c_4 is a monomer of 24300 $\pm$ 2300 molecular weight, whereas cytochrome c_5 is a dimer with subunits of 13100 $\pm$ 1500 molecular weight.

TABLE II

AMINO ACID ANALYSIS OF CYTOCHROME c_4

Amino acid	*μmoles per 100 mg protein*			*μmoles per 1 μmole His*	*Residues per molecule c_4*
	11 h	*21 h*	*70 h*		
Lys		46.0	46.4	2.91	12 (11.6)
Amide-NH_2		89.1	97.0	5.86	23 (23.4)
His		15.9	15.9	1.00	4 (4.0)
Arg		29.9	30.6	1.91	8 (7.64)
Asp + Asn	96.5	104	89.0	6.07	24 (24.3)
Thr	40.3	41.8	41.3	2.59	10 (10.4)
Ser	47.2	46.2	43.6	3.01	12 (12.0)
Glu + Gln	88.1	88.2	82.5	5.57	22 (22.3)
Pro	52.4	53.0	47.6	3.33	13 (13.3)
Gly	110	109	106	6.83	27 (27.3)
Ala	151	149	145	9.33	37 (37.3)
Val	17.0	22.4	21.4	1.39	6 (5.56)
Met	31.0	27.4	25.2	2.02	8 (8.08)
Ile	26.6	28.9	27.2	1.74	7 (6.96)
Leu	64.5	65.5	62.9	4.05	16 (16.2)
Tyr	23.6	24.8	22.2	1.49	6 (5.96)
Phe	19.8	18.0	17.0	1.18	5 (4.72)
Cys*				0.86	4 (3.46)
Trp**				0	0
					221 Residues (*minus* amide-NH_2)

* Determined by performic acid oxidation.
** Determined by NaOH hydrolysis.

Amino acid analyses

The results of amino acid analyses conducted on purified cytochromes c_4 and c_5 are listed in Tables II and III. For cytochrome c_4, serine, methionine and phenylalanine values were corrected for destruction by extrapolation to zero time, and methionine values were corrected for small conversion to methionine sulfoxide. When obvious destruction had occurred during 70 h hydrolysis (aspartic acid, glutamic acid and proline) these values were omitted from the average. A recovery of 99% of the amino acids was obtained from the cytochrome c_4 after hydrolysis for 21 h, but destruction of amino acids during the 70-h hydrolyses decreased this value to 89%.

The number of μmoles were calculated relative to histidine to obtain a minimum molecular weight. This value for cytochrome c_4 is 5800 while the cytochrome c_5 value is 11500. The contribution of heme is not included in these figures. Based upon previously described physical determinations of molecular weight, the true number of residues of each amino acid per molecule of cytochrome c_4 must be 4 times the μmoles/μmole histidine (see the final column of Table II). The cytochrome c_5 minimum molecular weight calculated from amino acid analysis, on the other hand, is similar to the values found by physical methods. Therefore, the number of μmoles amino acid per μmole histidine is identical with the residues per molecule value.

From the measured absorbance of the cytochrome c_5 solution before hydrolysis and the calculated minimum molecular weight of 12100 (including heme) one can calculate, assuming 100% recovery on the amino acid analyzer, that the millimolar

TABLE III

AMINO ACID ANALYSIS OF CYTOCHROME c_5

Amino acid	*μmoles per 100 mg protein*		*Mean*	*μmoles per μmole His*	*Residues per molecule* c_5
	1	2			
Lys	64.3	68.6	66.5	8.82	9 (8.82)
Amide-NH_2	67.8	71.6	69.7	9.12	9 (9.12)
His	7.8	7.3	7.5	1.00	1 (1.00)
Arg	21.5	19.5	20.5	2.72	3 (2.72)
Asp	96.0	102.0	99.0	13.10	13 (13.10)
Thr	37.5	36.7	37.1	4.82	5 (4.82)
Ser	41.7	42.1	41.9	5.56	6 (5.56)
Glu + Gln	60.2	60.2	60.2	7.99	8 (7.99)
Pro	24.7	24.0	24.4	3.24	3 (3.24)
Gly	134	135	135	17.9	18 (17.9)
Ala	183	180	182	24.1	24 (24.1)
Val	54.4	48.7	52.1	6.90	7 (6.90)
Met	10.8	13.0	11.9	1.58	2 (1.58)
Ile	17.5	18.2	17.9	2.37	2 (2.37)
Leu	83.8	84.1	84.0	11.1	11 (11.1)
Tyr	6.4	6.4	6.4	0.854	1 (0.854)
Phe	0	0	0	0	0
Cys*				4.07	4 (4.07)
Trp**				0.86	1 (0.86)
					118 residues (*minus* amide-NH_2)

* Determined by performic acid oxidation.
** Determined by NaOH hydrolysis.

extinction coefficient of cytochrome c_5 at 554 nm (reduced) = 26.2 and at 554 nm (oxidized) = 9.6. These values are somewhat higher than the 23.8 and 7.9 values found for cytochrome c_4 at 550 nm (ref. 5).

The amino acid composition of the 2 cytochromes is similar in many respects; both have a high ratio of acidic to basic amino acids, and glycine and alanine contribute a large number of residues in each. Tryptophan, however, is absent from cytochrome c_4, and phenylalanine is absent from cytochrome c_5. Cytochrome c_5 has only 1 histidine per heme group, whereas cytochrome c_4 has 2 histidines per heme as inferred from minimum molecular weights of 11200 for cytochrome c_4 and 11600 for cytochrome c_5 determined by iron analysis[6].

Isoelectric points, as determined by isoelectric focusing at 15° were 4.40 and 4.69 ± 0.02 for reduced and oxidized cytochrome c_4 and 4.20 ± 0.08 and 4.44 ± 0.09 for reduced and oxidized cytochrome c_5. The reduced form was clearly resolved from the oxidized form even in the wide-range pH 3–10 ampholyte system.

Relation of cytochromes c to nitrogen fixation

The possibility that the *A. vinelandii* cytochromes *c* might be involved in nitrogen fixation was investigated by comparing the cytochrome *c* content of cells grown on ammonia with that of cells actively fixing nitrogen. When cytochrome *c* was extracted from ammonia-grown cells, as previously described for cells actively fixing nitrogen, the cytochrome *c* level in butanol extracts was 2–3 times lower on a dry

weight basis in cells grown on ammonia. When purification of cytochromes *c* from these same cells was continued through the DEAE-cellulose step, an elution pattern identical to that of N_2-grown cells (Fig. 1) was obtained. The peaks eluted at the same relative positions and were in the same proportions in both types of cells. Cytochromes c_4, c_5, and minor c_4 of ammonia-grown cells had visible spectra identical with their counter parts from nitrogen-fixing cells. Likewise, cytochromes c_5 and c_4 had the same apparent molecular weight on Sephadex G-100, and the isoelectric point of cytochrome c_4 was identical in ammonia-grown and in nitrogen-fixing cells.

TABLE IV

TESTS OF NADH AND SUCCINATE AS DONORS IN CELL-FREE NITROGEN FIXATION

Expt. No.	*Electron donor*	*Electron donor concn. (mM)*	*N_2 fixed (nmoles)*
1	$Na_2S_2O_4$	15	1050
	NADH	15	18
	Succinate	15	0
2	$Na_2S_2O_4$	15	880
	NADH	15	22
3	$Na_2S_2O_4$	15	1010
	NADH	30	8

If the *A. vinelandii* cytochromes *c* were involved as intermediate carriers in nitrogen fixation, one would expect that electron donors able to reduce them would also be capable of effecting fixation in the standard cell-free system containing an ATP-generating system. However, as shown in Table IV, neither NADH nor succinate serves as an electron donor in these extracts which show good fixation with dithionite. The trace of fixation apparent with NADH was not increased when NADH (1 mM) was generated from an ethanol dehydrogenase system or when 3 mM dithiothreitol was added or when crude extracts were dialyzed before assay.

Assays of cytochrome c oxidase

To determine the relative rates of oxidation of the *A. vinelandii* cytochromes *c* when oxidized by cell-free particles of the organism, K_m and v_{max} values (Table V) were determined using cytochromes c_4 and c_5 purified through the DEAE-cellulose stage. A least-squares analysis showed the K_m for cytochrome c_5 to be 3-fold lower than

TABLE V

K_m AND v_{max} VALUES FOR OXIDATION OF CYTOCHROMES *c* BY *A. vinelandii* PARTICLES

Cytochrome	K_m *(mM)*	v_{max} *(μmoles/min per mg protein)*
Horse heart cytochrome *c*	0.27	0.76
Cytochrome c_4	0.11	0.29
Cytochrome c_5	0.039	0.43

the K_m for cytochrome c_4 and 7-fold lower than the K_m for horse heart cytochrome *c*. Likewise, the first-order rate constant, k ($sec^{-1} \cdot mg\ protein^{-1}$), did not vary over a 5-fold range of cytochrome *c* concentration for each cytochrome; for cytochrome c_5 the rate constant (0.12) was approximately 3 times that for either cytochrome c_4 (0.038) or horse heart cytochrome *c* (0.043). The rate of oxidation of horse heart cytochrome *c* by the *A. vinelandii* particles is noteworthy in view of the fact that very few bacterial oxidase preparations oxidize mammalian type cytochrome *c*.

TABLE VI

COMPARATIVE RATES OF CYTOCHROME OXIDATION

Cytochrome c	*Rates (nmoles per min)*		
	Azotobacter small particle	*Beef heart cytochrome oxidase*	*Beef heart electron transport particle*
Horse heart	7.45	13.8	18.3
Cytochrome c_4	9.80	2.8	2.1
Cytochrome c_5	17.3	0	0
Minor c_4	18.3	0	0

The rates of oxidation of the *A. vinelandii* and mammalian type cytochromes *c* were investigated further with oxidases from *A. vinelandii* and beef heart (Table VI). The results should be compared in the vertical columns only, because the protein concentration of the oxidase preparations was not measured; the same cytochrome concentration (0.010 mM) was used in all cases. Again it is apparent that the small particle fraction oxidized horse heart cytochrome *c* and cytochrome c_4 at approximately the same rate. However, 2–3 times this rate was obtained when either cytochrome c_5 or minor cytochrome c_4 was used as the donor for the bacterial oxidase. With the two types of beef heart oxidases there was no detectable oxidation of cytochrome c_5 or minor cytochrome c_4, whereas cytochrome c_4 was oxidized at rates up to 20% those of the mammalian type cytochrome *c*; evidently cytochrome c_4 is functionally different from cytochromes c_5 and minor c_4.

JONES AND REDFEARN[7] have proposed, from careful spectral analysis of the *A. vinelandii* electron transport particles and their oxidoreduction reactions, that the terminal electron transport pathway of *A. vinelandii* is branched. They suggested that most of the electron flow proceeds through cytochromes b_1 and a_2 on a pathway relatively insensitive to KCN, whereas minor electron flow passes through a cyanide-sensitive shunt including cytochromes c_4 and c_5 together with cytochromes a_1 and *o*

TABLE VII

KCN SENSITIVITY OF CYTOCHROME *c* OXIDATION BY *A. vinelandii* PARTICLES

Cytochrome	*% inhibition at KCN concn. (mM)*		
	10^{-4}	10^{-3}	10^{-2}
Horse heart			66
Cytochrome c_4	0	14	66
Cytochrome c_5	23	34	85
Minor c_4	26	26	73

(50 % inhibition with 10^{-5} M cyanide). This proposal was tested by using the *A. vinelandii* cytochromes *c* separated by DEAE-cellulose chromatography (Table VII). It is apparent that oxidation of all types of cytochromes *c* is very sensitive to KCN. The inhibition was immediate and all purified cytochromes tested had comparable sensitivity, although oxidation of cytochrome c_4 appeared somewhat more resistant to KCN than was oxidation of the other cytochromes. Similar extreme sensitivity of *A. vinelandii* small particles to KCN was observed in assays in which ascorbate *plus* *p*-phenylenediamine was oxidized; this has been reported by other authors[7,31]. Comparable inhibition of NADH or succinate oxidation requires approximately 100 times as concentrated KCN (10^{-3} M).

DISCUSSION

The purification procedure described has several advantages over older methods[5,6]. Several dialysis steps are omitted, and a single passage through DEAE-cellulose gives a 300-fold increase in the purity ratio of cytochrome c_4 and a 150-fold increase in that of cytochrome c_5. Production of gum by *A. vinelandii* causes no problems. Except for the isoelectric focusing step in the purification of cytochrome c_5, harmful acidic conditions are avoided. The yields of 32–52 mg pure cytochrome c_4 per 100 g dry weight and 13–16 mg pure cytochrome c_5 per 100 g dry weight are double those previously obtained; the Azotobacter compares favorably with such rich sources of cytochrome *c* as mammalian heart which yields 76–96 mg per 100 g dry weight (assuming 75 % water content)[32].

Various altered forms of the cytochromes *c* appeared such as those encountered with mammalian type cytochrome *c* (ref. 2). However, negligible alteration (less than 5 %) occurred when purification procedures were conducted with no intermediate storage of cytochromes. Lyophilization might reduce formation of altered cytochromes.

Optimal yields of cytochromes *c* were obtained when cells were grown on N_2 rather than ammonia as a nitrogen source. We also have found that nitrogen-fixing cells grown in continuous culture with oxygen as the limiting nutrient have twice the cytochrome *c* concentration of cells grown with normal aeration in batch culture. Similar results have been reported by other authors[33]. The decrease in cytochromes *c* when cells were grown on ammonia involves a proportional decrease in all 3 cytochromes *c*; coordinate repression of the nitrogenase enzyme complex and a single cytochrome *c* does not occur. This fact, *plus* the barely detectable nitrogen fixation observed when common cytochrome *c* reducing agents such as NADH and succinate were used in cell-free experiments (ATP-generating system included), do not support the theoretically unlikely suggestions of a direct role for cytochromes *c* in nitrogen fixation by *A. vinelandii*[8]. GVOZDEV *et al.*[34] likewise have reported negative evidence for a direct role for cytochromes of *A. vinelandii* in nitrogen fixation. The 2–3-fold increase in cytochrome *c* level in cells actively fixing nitrogen may reflect an increased demand for ATP production by oxidative phosphorylation rather than a direct carrier role for cytochrome *c* in nitrogen fixation. Other *A. vinelandii* cytochromes, especially a_2, are present in much higher levels in nitrogen-fixing cells than in cells grown on urea[35]. The lower yield of cytochromes *c* when cells were grown with high aeration may have resulted from decreased protein synthesis as a result of inhibition of ni-

trogen fixation by O_2 (refs. 36, 37). The effect of different aeration rates on synthesis of cytochrome *c* by cells grown on ammonia has not been tested.

Other evidence, in addition to the distinct peaks eluted from DEAE-cellulose columns, suggests that *A. vinelandii* contains at least 3 species of cytochrome *c*. Minor cytochrome c_4, although spectrally identical to cytochrome c_4, had a molecular weight of 12200 as indicated by passage through calibrated columns of Sephadex G-100, whereas the molecular weight of cytochrome c_4 was approximately twice this even under the denaturing action of sodium dodecylsulfate on acrylamide gels. In cells that had been simply sonicated, dialyzed and applied directly to a DEAE-cellulose column, minor cytochrome c_4 comprised 30% of the total cytochromes recovered. Therefore, its actual cellular concentration may be higher than indicated by the butanol extraction procedure.

Molecular weight estimations of cytochrome c_4 were similar under nondenaturing conditions (Sephadex G-100, 25100) and denaturing conditions (detergent acrylamide gels, 22800); this indicates that the protein has no smaller subunits. These data, together with the observations that 4 times the minimum molecular weight found by amino acid analysis is 24400 including heme, and that 2 times the minimum molecular weight found by iron analysis is 22400 (ref. 6), indicate that cytochrome c_4 is a protein of 24000 ± 2000 molecular weight with 4 histidines and 2 hemes per molecule. It has recently been reported that cytochrome c_4 has a molecular weight of 23500 on Sephadex G-100 and has 2 hemes per molecule[38].

The molecular weight of cytochrome c_5 derived from chromatography on Sephadex G-100 is 24400, roughly twice the 14000 value found by the detergent acrylamide gel technique and the 12100 value found from amino acid analysis assuming 1 histidine per molecule. NEUMANN AND BURRIS[6] calculated a minimum molecular weight of 11600 for cytochrome c_5 based on iron analysis. Apparently cytochrome c_5 has a molecular weight of 12800 ± 1200 and has 1 heme and 1 histidine per molecule. It is unknown whether it exists in the cell as a dimer. The cytochrome c_5 dimer from DEAE-cellulose columns functioned well in the assay with *A. vinelandii* oxidase particles; it was oxidized at 2–3 times the rate of cytochrome c_4, but no monomer preparations of cytochrome c_5 were available for comparative assay.

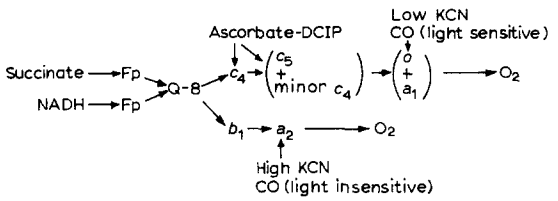
Apparently cytochrome c_4 is the only bacterial or mammalian type cytochrome *c* reported which has no tryptophan. This is significant, because the list of possible amino acids forming the second coordinating ligand to the heme group in cytochrome *c* has been reduced to a histidine, a methionine or a tryptophan residue[39].

Cytochrome c_5 has only 1 histidine per heme, and this characteristic places it in a class with the cytochromes *c* of *Pseudomonas fluorescens*[40] and Chromatium RHP[41]. This demonstrates that it is not necessary to have 2 histidines coordinated to heme in cytochromes *c*.

Demonstration that horse heart cytochrome *c* is oxidized at a relatively high rate by the bacterial oxidase particles places *A. vinelandii* among a group of 4–5 bacterial species known to be capable of oxidizing mammalian type cytochrome *c* (ref. 42). The relatively high rate of oxidation of the bacterial cytochrome c_4 by mammalian oxidase particles also is unusual[43]. The inactivity of reduced cytochromes c_5 and minor c_4 with these same oxidase particles provides further evidence, in addition to the detailed physical differences, of the diversity among the *A. vinelandii* cytochromes *c*.

Terminal electron transport in *A. vinelandii* can be diagrammed as in Scheme 1, the main features of the branched chain having been proposed by JONES AND REDFEARN[7].

The high sensitivity to KCN of the *A. vinelandii* particles when oxidizing all 3 cytochromes *c* indicates that all are oxidized by cytochromes $a_1 + o$ rather than by cytochrome a_2. The 2–3-fold higher rate of oxidation of cytochrome c_5 and minor cytochrome c_4 and the slightly higher redox potential of cytochrome c_5 (ref. 5) suggest that these 2 cytochromes may interact directly with the terminal oxidase.



Scheme 1. DCIP, dichlorophenolindophenol; Fp, flavoprotein.

Scheme 1 does not propose that the branch of the chain through cytochromes *c* carries only a minor share of the electrons from NADH and succinate as suggested by JONES AND REDFEARN[7]. The v_{max} values found for cytochrome c_4 oxidation (0.29 μmole·min^{-1}·mg protein^{-1}) and for cytochrome c_5 oxidation (0.43 μmole·min^{-1}·mg protein^{-1}) fall short of the 5.5 and 0.53 values found for NADH and succinate oxidation with similar oxidase particles under similar conditions[44]. However, we have found the relative rates of the NADH, succinate and ascorbate (cytochrome *c*) oxidases are greatly dependent upon pH; ascorbate oxidation actually is much more rapid than either NADH or succinate oxidation at pH 6.0. Likewise, the turnover rates of cytochrome *c* oxidase *in vitro* rarely approach values reported for the enzyme in intact cells[2]. Further information on the relative importance of the 2 branches of the electron transport chain might be obtained by measurements of oxidative phosphorylation associated with NADH and succinate oxidation in the presence and absence of low levels of KCN.

Ubiquinone-8 (Q-8) is included in this scheme because it has been shown to be an essential component of both succinate and NADH oxidase[45,46], although there is some question whether or not it turns over rapidly enough to account for maximal rates of electron transport[35].

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