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Preparation of Crystalline *Pseudomonas* Cytochrome *c*-551 and its General Properties

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A simple method has already been devised for extensive purification of four respiratory components from *Pseudomonas aeruginosa* in a true water-soluble state without the aid of detergents (Horio, 1958*a*). These components were called *Pseudomonas* (*P*)-cytochrome-554, *P*-cytochrome-551, *P*-blue protein and *P*-cytochrome oxidase (Horio, 1958*b*; Horio, Higashi, Matsubara *et al.* 1958). The same micro-organism also contains another cytochrome, *P*-cytochrome-560, which is similar to the so-called cytochrome *b*₁ and which has not yet been solubilized without the aid of sodium cholate (Horio, 1958*a*). From studies on purified respiratory components, cellular fragments and whole cells, Horio (1958*b*) and Yamanaka (1959) have concluded that in the electron-transferring system of *P. aeruginosa* *P*-cytochrome-551 and *P*-blue protein should be functional at a site corresponding to cytochrome *c* of animal mitochondria. Preliminary studies on chemical and physical properties of the purified components showed that *P*-cytochrome-551 bears a closer resemblance to a typical cytochrome *c* than it does to *P*-blue protein (Horio, 1958*a, b*).

The first crystallization of cytochrome *c* was accomplished by Bodo (1955), who isolated the

pigment from king penguin (*Aptenodytes patagonica*). It was extracted by the method of Keilin & Hartree (1937, 1945) and further purified with the use of the ion-exchange resin Amberlite IRC-50 (Margoliash, 1954*a, b*). By very similar methods, crystalline cytochrome *c* has since been obtained from a variety of sources, such as baker's yeast (Hagihara, Horio, Yamashita, Nozaki & Okunuki, 1956), ox heart (Kuby, Paléus, Paul & Theorell, 1956), horse heart (Nozaki, Yamanaka, Horio & Okunuki, 1957), fish heart (Hagihara *et al.* 1957) and wheat germ (Hagihara, Tagawa, Morikawa, Shin & Okunuki, 1958). In all cases, cytochrome *c* was purified from the crude to the purest state by chromatography on a weak cationic ion-exchange resin by procedures established by Paléus & Neilands (1950) and by Margoliash (1954*a, b*).

Despite the many similarities of *P*-cytochrome-551 to other types of cytochrome *c*, the standard procedure for the purification of the types of cytochrome *c* is inefficient for the purification of *P*-cytochrome-551 (Horio, 1958*a, b*). The main reason appears to be the acidic isoelectric point of *P*-cytochrome-551, which decreases the adsorptive power of a weak cationic resin such as Amberlite IRC-50 within its effective pH range.

In this paper, a suitable method for the preparation of crystalline *P*-cytochrome-551 from *P. aeruginosa* is presented together with a description of its general properties. A preliminary report on this work has been given by Horio, Higashi, Nakai, Kusai & Okunuki (1958).

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MATERIALS AND METHODS

Organism. A strain of *P. aeruginosa* was used which produces large amounts of cytochromes when it is grown as submerged cultures.

Media. Two types of media were employed: bouillon-peptone medium and nitrate medium. The former was composed of 5 g. of Ehrlich meat extract (Kyokuto Pharmaceutical Ind. Co. Ltd., Tokyo), 10 g. of Polypepton (Daigo Nutritional Chemicals Co. Ltd., Osaka), 2.5 g. of NaCl and 1 l. of water. The pH was adjusted to 7.0 with 5*N*-NaOH. The nitrate medium was composed of 1 kg. of Ehrlich meat extract, 1 kg. of Polypepton, 2 kg. of KNO₃, 0.64 kg. of KH₂PO₄, 0.36 kg. of Na₂HPO₄ and 100 l. of tap water (pH 6.6).

Growth of the organism. The organism was maintained on the bouillon-peptone medium solidified with agar (2%, w/v). Bulk growth of the organism for the purpose of *P*-cytochrome-551 purification was carried out in three stages. The bouillon-peptone medium (15 ml.) was inoculated and incubated at 37° for 24 hr. with vigorous shaking. The resulting culture was added to 6 l. of bouillon-peptone medium and incubated at 37° for 24 hr. Finally, this bouillon-peptone culture was added to 90 l. of the nitrate medium in a deep steel tank and incubated at 37° without shaking until the pH of the medium rose to approx. 8 (usually 20 hr.). During the incubation a Kotaki photoelectric colorimeter, type 5-D, was used with the filter no. 11 (610–750 mμ) to estimate turbidity of the medium. The extinction began to increase when the pH rose above 7 (approx. 9 hr. after the inoculation) and became maximal when the pH reached approx. 8. Prolonged incubation, which leads to a further rise in pH, should be avoided since the cells may then autolyse and liberate the cytochromes into the culture medium. At the end of incubation the medium usually contained approx. 8 mg. (wet wt.) of the organisms/ml.

Preparation of resin. The small beads of Duolite CS-101 (polymethacrylic acid resin, supplied by Chemical Process Co., Redwoods City, Calif., U.S.A.) were ground and the resin powder from 150 to 200 mesh was collected. The mesh size of the resin is not critical, but the size selected combines ease in pretreating the resin with a high adsorbing capacity. The resin was heated with *N*-NaOH at approx. 80° for 15–20 min. and then washed completely with water. The alkaline treatment and washing were repeated. The resulting resin was suspended in *N*-HCl, stirred for 15–20 min. and washed with water until the pH of the resin suspension rose to about 5. The product will be called the H-form resin. The H-form resin was further suspended in 0.1 *M*-ammonium phosphate buffer, pH 6.0, and then washed with water. This treatment with buffer was repeated until the pH of the resin suspension became 6.0. This resin will be called the pH 6 resin. Amberlite CG-50 can be used in place of Duolite CS-101, although its efficiency is slightly lower.

Preparation of activated aluminium oxide. From activated aluminium oxide for chromatography (Takeda Pharmaceutical Co. Ltd., Osaka, Japan), a powder from 200 to 300 mesh was collected and treated with alkali and acid in the same way as the resin. After a complete washing with water, the aluminium oxide was immersed in 0.1 *M*-ammonium phosphate buffer, pH 6.0, and then washed with water. This treatment with buffer and the subsequent wash-

ing were repeated until the pH of the suspension became 6.0. The product will be called the pH 6 aluminium oxide.

Absorption spectra. A Shimadzu photoelectric spectrophotometer, type QB-50, with silica cuvettes (1 cm. optical path) was used. The purity of *P*-cytochrome-551 during fractionation is expressed as the ratio $(E_{551}^{\text{red}} - E_{570}^{\text{red}})/E_{280}$, where E_{551}^{red} and E_{570}^{red} are extinctions of the dithionite-reduced sample at 551 and 570 mμ respectively, and E_{280} is the extinction at 280 mμ of a sample not treated with reducing or oxidizing agents. The absorption spectra of crystalline *P*-cytochrome-551 were measured with a Cary recording spectrophotometer, model 14.

Zone electrophoresis on a vertical starch column. Zone electrophoresis was carried out on a vertical column (30 cm. × 3 cm. diam.) packed with raw-potato-starch powder, under the following experimental conditions: 0.1 *M*-sodium phosphate buffer, pH 7.0; approx. 20 mA at 350–400 v with anode at the bottom of the column; temp., 4–5°. The starch (Wako Pure Chemical Industries, Co. Ltd., Osaka, Japan) was washed three times with a large amount of *N*-HCl, then sufficiently with water. The powder was washed with a large amount of buffer, and only the buffered powder able to sediment on standing within half an hour was used.

Oxidation-reduction potentials. The method of Henderson & Rawlinson (1956) for measuring E_0' of heart-muscle cytochrome *c* was used. The solution of crystalline *P*-cytochrome-551 was dialysed against 0.2 *M*-sodium phosphate buffer, pH 6.50, for 3 days in a refrigerator. The dialysed sample was reduced by addition of a suitable amount of sodium dithionite just before assay. The reduced sample was assayed by titration with 3 *mm*-potassium ferricyanide dissolved in the phosphate buffer. The concentration of *P*-cytochrome-551 (E_{551}^{red} 0.66, 3.00 ml.) was previously adjusted so that the assay could be accomplished with 15–20 drops (0.15–0.20 ml.) of the ferricyanide solution.

Electrophoretic analysis. A Tiselius apparatus was used. The cells contained equilibrated buffer (0.03 *M*-sodium phosphate buffer or sodium acetate buffer, adjusted with NaCl so that *I* was 0.10) as the overlying solution. A sample of *P*-cytochrome-551 recrystallized three times, was dialysed at 4–5° against the buffer for 3 days. Runs to examine the homogeneity of *P*-cytochrome-551 were carried out in an ice-water bath, and runs to calculate electrophoretic mobilities in a water bath regulated at 20 ± 0.05°. Calculations were based upon the ascending boundaries.

Ultracentrifugal analysis. Sedimentation measurements were made with a Spinco model E ultracentrifuge (rotor type AN-A). The runs were carried out at 259 700 *g* at room temperature (approx. 20°). The average of initial and final rotor temperatures was used for calculating $S_{20,w}$ by the method of Svedberg & Pedersen (1940).

Diffusion analysis. Diffusion was studied by the cylindrical-lens schlieren method with the use of cells of the Neurath type. The temperature of the thermostat was regulated within ± 0.01° according to the technique of Moriguchi (1932) and measurements were carried out at 20°. $D_{20,w}$ was calculated by the inflexion-point method (Neurath, 1942).

Partial specific volume. The densities of the solution and of the solvent were measured at 20 ± 0.05° with an Ostwald-type pycnometer (3 ml. capacity).

Analysis of iron and nitrogen. A sample of *P*-cytochrome-551 was dialysed with stirring against glass-distilled water

for 72 hr. at 4–5° with several changes of the outside water. The dialysed solution was dried at 60° over P_2O_5 until the residue attained constant weight. Iron was estimated by the o-phenanthroline method (Fortune & Mellon, 1938; Thiel, Heinrich & Hengel, 1938) after the residue had been subjected to the wet oxidation described by Sandell (1950). Nitrogen was assayed by a micro-Kjeldahl method.

EXPERIMENTAL AND RESULTS

Purification of P-cytochrome-551

Step 1. Acetone-dried powder of cells. The cells grown in approx. 100 l. of nitrate medium were collected by filtration on a Büchner funnel containing filter paper and a layer of Celite (Hyflo Super-cel). About 760 g. (wet wt.) of cells was collected. The cells and Celite were mixed with 5 l. of solid CO_2 -acetone in a large, wide-mouth Thermos flask. The temperature was maintained below 5° during this acetone treatment. Five minutes were required to complete the mixing. The mixture was allowed to stand for 10 min., rapidly filtered on a Büchner funnel and the collected cells were rapidly washed with 2 l. of cold acetone on the same funnel. The resulting powder was dried overnight at room temp. (10–20°) in a current of air.

Step 2. Extraction of respiratory components from the acetone-dried cells. The acetone-dried cells with Celite were well mixed with 10 l. of 0.1 M-sodium citrate adjusted to pH 7.6 with N-NaOH. The mixture was heated at 40° for 10 min. with continuous stirring and was filtered at room temperature through a layer of Celite on a Büchner funnel. The filtration took 1 day and yielded 8.3 l. of dark-brown filtrate, pH 6.2. A second extraction was performed with 10 l. of the sodium citrate either with or without heat treatment. The second extract contained one-tenth of the amount of P-cytochrome-551 of the first extract, and the purity of the second extract was much lower than that of the first extract.

Step 3. Treatment of the extract with rivanol and precipitation of the respiratory components by ammonium sulphate. To 8.3 l. of the first filtrate in step 2, 1 l. of aqueous 4% rivanol (2:5-diamino-7-ethoxyacridinium lactate monohydrate, supplied by Fujisawa Pharmaceutical Ind. Co. Ltd., Osaka) was added. On mixing, a bulky fibrous precipitate was produced which was removed by filtration through gauze. To the filtrate was added 9.6 kg. of ammonium sulphate (95% saturation). After being kept overnight in a refrigerator, the resulting precipitate was collected on a Büchner funnel containing Celite. The precipitate plus Celite was dialysed against running tap water for 3 days in a cold room, with some changes of dialysis tubing. During this dialysis, brown impurities were precipitated. The volume of the dialysed and filtered solution was approx. 1.3 l.

Step 4. Treatment of the dialysed solution with pH 6 resin. The dialysed solution was passed through a column (50 cm. × 3 cm. diam.) packed with pH 6 resin. The rivanol, not removed by dialysis in step 3, was adsorbed at the top of the column. Just below the yellow zone of the adsorbed rivanol was a second zone consisting of all the P-cytochrome oxidase, some of the P-cytochrome-554 and a large amount of impurities. The P-cytochrome-551 together with P-blue protein passed through the column. The P-cytochrome oxidase was subsequently removed from the column by elution with M-ammonium phosphate buffer, pH 7.0, and stored for further purification (Horio, Higashi, Matsubara *et al.* 1958).

Step 5. Adsorption of P-cytochrome-551 on H-form resin. The solution which passed through the pH 6 resin in step 4 was passed through a column (50 cm. × 3 cm. diam.) packed with H-form resin. The P-cytochrome-551 together with P-blue protein and P-cytochrome-554 were completely adsorbed. The charged column was washed with water and then eluted with M-ammonium phosphate buffer, pH 7.0. The eluate was kept neutralized with frequent addition of dilute sodium hydroxide. The part of the eluate which showed an absorption band around 550 m μ (156 ml.) was collected. Up to this step, the content of P-cytochrome-551 could not be precisely assayed spectrophotometrically because of coloured impurities. In one case the final yield of cytochrome was calculated on the basis of the eluate at this stage containing 100 arbitrary units (Table 1).

Step 6. Chromatography of P-cytochrome-551 on pH 6 aluminium oxide. The reddish brown eluate from the H-form resin was dialysed against running tap water in a cold room for 1 day and then passed through a column (20 cm. × 3.5 cm. diam.) of pH 6 aluminium oxide. Brown impurities were adsorbed at the top of the column and P-blue protein and P-cytochrome-551 were adsorbed just below the brown band. P-blue protein has a higher affinity for the aluminium oxide than P-cytochrome-551. However, in most cases it was difficult to observe it, since it was partly in the colourless reduced form and the oxidized form was largely masked by brown impurities and the red colour of P-cytochrome-551. The brown, top portion of the aluminium oxide was scraped away and the rest of the column was washed with water. (All attempts to fractionate the adsorbed substances by means of eluting buffers before removal of the brown impurities led to a serious decrease in yield.) The adsorbed P-cytochrome-551 slowly moved down the column during washing. When a sample of eluate, treated with dithionite and examined with a microspectroscope, showed an absorption band around 550 m μ the washing was stopped. The

Table 1. Summary of purification of *Pseudomonas* cytochrome-551

Main steps	Volume (ml.)	Purity ($E_{551}^{red.} - E_{570}^{red.}/E_{280}$)	Yield (%)
1. Extract of acetone-dried powder of cells	8300	—	—
2. Eluate from H-form Duolite CS-101 column	156	0.028	(100)
3. Eluate from pH 6-aluminium oxide column	200	0.090	59
4. Fractionation by ammonium sulphate (60–90% saturation)	30	0.12	52
5. Freeze-dried powder	(708 mg.)	0.14	52
6. Zone-electrophoresis			
(a) First run	5	0.82	46
(b) Second run	5	1.00	41
7. Crystallization			
(a) Cryst. ppt.	2.9	1.18	28
(b) First crystals	2.5	1.26	22
(c) Second crystals	1.7	1.27	15
(d) Third crystals	1.2	1.27	9

washed column was then eluted with *m*-ammonium phosphate buffer, pH 7.0, and the fractions containing *P*-cytochrome-551 were collected (200 ml.). This eluate, which also contained *P*-blue protein, was brownish red. If it was reduced by sodium dithionite, its colour changed to pink, characteristic of reduced *P*-cytochrome-551, since the *P*-blue protein present in the eluate was simultaneously bleached by the reducing agent. The eluate also contained a trace of *P*-cytochrome-554.

Step 7. Fractionation of *P*-cytochrome-551 with ammonium sulphate. To 200 ml. of the step 6 eluate, 76 g. of ammonium sulphate was added (approx. 60 % saturation), and the mixture was centrifuged at 10 000 *g* for 20 min. The brown precipitate was discarded, the brownish red supernatant was treated with a further 56 g. of ammonium sulphate (90 % saturation) and centrifuged again. The supernatant was yellowish green and contained *P*-blue protein and *P*-cytochrome-554 but very little *P*-cytochrome-551. The precipitate contained *P*-cytochrome-551 and *P*-blue protein but virtually no *P*-cytochrome-554. The *P*-blue protein in the supernatant fluid was precipitated by saturation with ammonium sulphate.

Step 8. Freeze-drying and zone electrophoresis of *P*-cytochrome-551. The sample which was precipitated between 60 and 90 % saturation of ammonium sulphate in step 7 was dissolved in a small amount of water, dialysed for 3 days in a cold room and freeze-dried. The resulting powder (708 mg.) could be stored at 4–5° for more than 3 months. It was dissolved in 4 ml. of 0.1 *M*-sodium phosphate buffer, pH 7.0, and a small amount of insoluble material was removed by centrifuging. The clarified supernatant was placed in the middle of a vertical column packed with starch and electrophoresis was

Fig. 1. Fractionation of *Pseudomonas* cytochrome-551 by zone electrophoresis on a starch column. The eluate was collected as 2 ml. fractions. If the sample were to be eluted from the position where it was originally placed, it would appear in the fractions shown by the arrow. $E_{630}^{ox.}$ was measured on a portion of the eluate fractions after oxidation with excess of potassium ferricyanide. The other extinctions were measured with portions of the eluate fractions previously reduced by excess of sodium dithionite. Detection of *P*-cytochrome-551, *P*-cytochrome-554 and *P*-blue protein contained in the eluate fractions was carried out microspectroscopically and their approximate quantities are expressed by $E_{551}^{red.} - E_{560}^{red.}$, $E_{554}^{red.} - E_{564}^{red.}$ and $E_{630}^{ox.} - E_{630}^{red.}$ respectively. —, *P*-cytochrome-551; - - -, *P*-blue protein; . . ., *P*-cytochrome-554.

carried out under the conditions described above. Brown impurities migrated downwards fairly rapidly, whereas *P*-blue protein and *P*-cytochrome-551 migrated very slowly in the same direction. When the electrophoresis was left running overnight, *P*-blue protein and *P*-cytochrome-551 became separately observable as blue and red zones

respectively. But electrophoresis for 3 days was necessary to complete the separation and to force most of the brown impurities out of the bottom of the column. At this stage, the column was eluted with 0.1M-sodium phosphate buffer, pH 7.0, with the aid of a 2 ml.-fraction collector. First of all, brown impurities at the bottom of the column were eluted and these were followed by *P*-cytochrome-551 as reddish pink fractions and *P*-blue protein, in its reduced form, as colourless fractions (Fig. 1). Although some of the *P*-cytochrome-554 had been adsorbed on the pH 6 resin, a little remained to be eluted after *P*-blue protein. All fractions that followed *P*-cytochrome-554 showed negligible extinction at 280 m μ . Those *P*-cytochrome-551 fractions whose $E_{551}^{\text{red.}}/E_{280}$ ratios were higher than 0.65 were combined and subjected to a second electrophoresis under the same experimental conditions as the first run. Fractions with $E_{551}^{\text{red.}}/E_{280}$ ratio greater than 0.9 were collected in a second run. When they were combined the ratio was 1.00, indicating that the purity of *P*-cytochrome-551 had risen approximately seven times as a result of electrophoresis (Table 1). Whenever the ratio was higher than 0.5–0.6, *P*-cytochrome-551 could be easily crystallized by the following procedure. However, in order to get the best yield of purest material, it was found advantageous to use samples purified by the second run. Fractions containing *P*-blue protein were nearly saturated with ammonium sulphate and stored in a refrigerator, together with the *P*-blue protein precipitated in step 7, for further study.

Step 9. Precipitation of P-cytochrome-551 based upon differences in solubility of its oxidized and reduced forms. The *P*-cytochrome-551 collected in step 8 was completely oxidized by addition of a minimal amount of potassium ferricyanide, nearly saturated with ammonium sulphate, and centrifuged down to a compact pellet. This was dissolved in a volume of water, such that the value of $E_{1\text{cm}}^{561}$ after reduction with dithionite was ≤ 30 . If the concentration is too low, the yield of *P*-cytochrome-551 in the following procedure is greatly decreased. During all crystallization procedures the pH was adjusted to approx. 7.5 with ammonia. To the oxidized solution, finely powdered ammonium sulphate was added in small portions until a slight turbidity appeared (30–40% saturation). The concentration of ammonium sulphate required to produce slight turbidity varied, and was especially affected by temperature and protein concentration. The turbid solution was immediately centrifuged at the same or a slightly lower temperature. The precipitation of a small portion of the *P*-cytochrome-551 at this stage indicates that the concentration of ammonium sulphate is appropriate for the following procedure. The deep-red supernatant was reduced by the addi-

tion of a small amount of sodium dithionite, when it became lighter in colour and also very turbid. The turbid solution was allowed to stand overnight and then centrifuged. The precipitate consisted of fine-needle crystals of *P*-cytochrome-551, in a yield of about 70% from the mother solution. The brownish red supernatant contained the rest of the cytochrome and a small amount of *P*-blue protein. To the crystalline precipitate of *P*-cytochrome-551, water was added dropwise and with gentle stirring until the suspension exhibited a silken sheen. The crystals were again collected in a centrifuge. The purity of the *P*-cytochrome-551 at this stage was usually higher than 93%. The crystals were dissolved in a little water and reduced with a small amount of dithionite ($E_{1\text{cm}}^{561} \leq 30$). The solution was clarified by centrifuging and recrystallized as before by addition of ammonium sulphate. Needle crystals appeared in the turbid solution after storage for a few hours at or just below room temperature (Plate 1). After three recrystallizations the iron content was 0.69% and the ratio, $E_{551}^{\text{red.}} - E_{570}^{\text{red.}}/E_{280}$ was 1.27, and these values were unchanged by further recrystallizations. Allowing a solution of suitable turbidity to stand at a temperature much below that at which the turbidity has been obtained should be avoided, since the solubility of *P*-cytochrome-551 in ammonium sulphate solution increases as the temperature is lowered. The final product represents a 45-fold purification of the eluate from H-form resin (step 2), and a 1500-fold purification of the extract of acetone-dried cells if it is assumed that all of the *P*-cytochrome-551 contained in this extract was collected on the column of H-form resin.

The *P*-cytochrome-551 could also be easily crystallized in its oxidized form if, after the oxidation with ferricyanide, the solution was treated with ammonium sulphate until it became fairly turbid and then allowed to stand for a few days. Oxidized *P*-cytochrome-551 crystallized both as needles and as octahedra: the yield, however, was lower than when the pigment was crystallized in its reduced form.

General properties of *P*-cytochrome-551

Iron and nitrogen content. A three-times recrystallized sample of *P*-cytochrome-551 contained $0.69 \pm 0.005\%$ (3) of iron and $16.2 \pm 0.03\%$ (3) of nitrogen. Figures in parentheses indicate the number of estimations. On the basis of its iron content, the minimum molecular weight of *P*-cytochrome-551 is 8100.

Absorption spectra. *P*-cytochrome-551, recrystallized three times, was dissolved in 0.1M-sodium phosphate buffer, pH 7.0, and completely oxidized by the addition of a slight excess of potassium ferricyanide. The oxidized solution was dialysed against

Crystals of *Pseudomonas* cytochrome-551 (reduced form) from a solution in ammonium sulphate ($\times 800$).

the same buffer for 3 days in a cold room with several changes of buffer. This solution was used to obtain the oxidized absorption spectrum in Fig. 2, and the same solution, after treatment with sodium borohydride, gave the reduced spectrum. On the

Fig. 2. Absorption spectra of crystalline *Pseudomonas* cytochrome-551. The sample was recrystallized three times and its absorption spectrum was measured in 0.1 M-sodium phosphate buffer, pH 7.00, at 23°. The cytochrome was reduced by addition of a small amount of sodium borohydride. —, Oxidized form; - - - -, reduced form.

basis of iron content the molar extinction coefficient of the reduced pigment at 551 mμ is 28.3×10^6 mole⁻¹ cm.². The absorption maxima of *P*-cytochrome-551 are located at wavelengths significantly different from those of crystalline cytochrome *c* from ox-heart muscle. The ratios of extinctions at the absorption maxima of the two cytochromes also show small but definite differences (Table 2).

Ultracentrifugal and diffusion analyses. *P*-cytochrome-551, recrystallized three times, was dialysed against 0.02 M-sodium phosphate buffer, pH 7.0 (*I* 0.10, adjusted with NaCl), at 4–5° for 72 hr. Ultracentrifugal analyses of solution containing 0.4–1.1 % (w/v) of protein showed that the sample consisted of a homogeneous protein. $S_{20,w}$ was calculated to be 1.34 ± 0.07 s (4) and was nearly independent of concentration. Diffusion analyses were carried out with the same solutions of *P*-cytochrome-551. The value of $D_{20,w}$ $14.8 \pm 0.9 \times 10^{-7}$ cm.² sec.⁻¹ (4), was also nearly independent of concentration.

Partial specific volume. A sample recrystallized three times gave the value $\bar{v}$ 0.710 ml. g.⁻¹.

Molecular weight. The molecular weight of *P*-cytochrome-551 was calculated to be 7600 from the equation,

$$M = \frac{RTS}{(1 - \bar{v}\rho)D} = 2.44 \times 10^{10} \times \frac{S_{20,w}}{(1 - 0.9982 \bar{v})D_{20,w}}$$

(Carter, 1941; Lauffer & Stanley, 1940). This value is close to the minimum molecular weight based upon iron content (8100), which indicates that this cytochrome possesses one iron atom per molecule. Unlike the haem of haemoglobin, the haem of *P*-cytochrome-551 is not split from the protein moiety by treatment with acetone-HCl. The haem-protein linkage is thus of the *c*-type.

Table 2. Absorption spectra of crystalline *Pseudomonas* cytochrome-551 and ox-heart-muscle cytochrome *c*

<i>P</i> -cytochrome-551		Ox-heart-muscle cytochrome <i>c</i>	
Absorption max. (mμ)		Absorption max. (mμ)	
Reduced	$E/E_{551}^{red.}$	Reduced	$E/E_{550}^{red.}$
551	(1.0)	550*	1.0
521	0.58	520	0.60
416	5.3	415	4.7
313	1.1	313	1.3
290	—	290	—
280	—	280	—
Oxidized		Oxidized	
530	0.38	530	0.40
409	3.6	408	3.7
360	0.92	358	1.0
290	0.59	290	0.65
280	0.74	280	0.82

* Molar extinction at 550 mμ of crystalline ox-heart-muscle cytochrome *c* was 28.2×10^6 mole⁻¹ cm.

Electrophoretic analysis. A sample of *P*-cytochrome-551, recrystallized three times, was dialysed at 4–5° against 0.03 M-sodium phosphate buffer or sodium acetate buffer (*I* 0.10, adjusted with NaCl). Tests for homogeneity were carried out at 0° and at two pH values, 5.75 and 8.13. In all experiments the current was 10 mA. Both the descending and ascending schlieren patterns revealed the presence of a single protein. For calculation of electrophoretic mobility, runs were carried out at 20° with the results shown in Fig. 3. The current was 5.0 mA. In sodium phosphate buffer, pH 7.0 (*I* 0.10), the cytochrome migrated with a uniform descending boundary and had a mobility of 4.35×10^{-5} cm.² v⁻¹ sec.⁻¹. The isoelectric point of *P*-cytochrome-551 was found to be 4.70.

Normal oxidation-reduction potential. E'_0 of *P*-cytochrome-551 was found to be $+0.286 \pm 0.004$ v (3) at pH 6.50 and 20°.

DISCUSSION

The first successful water-solubilization and partial purification of bacterial *c*-type cytochrome was obtained with *Rhodospirillum rubrum* (Vernon, 1953). Vernon & Kamen (1954) reported that this cytochrome has the following physicochemical properties: absorption maxima, 550 mμ, 521 mμ, and 415 mμ in its reduced form; 530–535 mμ and 408 mμ in its oxidized form; mol.wt., 16 000; E'_0 , 0.338 v at pH 7.0. Kamen & Takeda (1956) purified a bacterial cytochrome *c* from *P. aeruginosa*, which showed absorption maxima in its reduced form at 552, 520 and 416 mμ, and in its oxidized form at 530 and 408–410 mμ. A *c*-type cytochrome obtained in a pure form from *P. aeruginosa* by Horio (1958*a*) showed in its reduced state an absorption maximum at 550.5 mμ, which differs slightly but de-

finitely from the wavelength given by Kamen & Takeda (1956). Besides this cytochrome, Horio (1958*a, b*) found another cytochrome easily extractable from the same micro-organism, and, after considerable purification, the latter, in its reduced form, showed an absorption maximum at 554 mμ. These two bacterial cytochromes were tentatively called *P*-cytochrome-551 and *P*-cytochrome-554, according to the nomenclature of Scarisbrick (1947). At present it seems likely that the cytochrome *c* purified by Kamen & Takeda (1956) could be a mixture of *P*-cytochrome-551 and *P*-cytochrome-554.

Unlike *P*-cytochrome-551, typical cytochrome *c* compounds can be easily purified by column chromatography on a weak cationic resin such as Amberlite IRC-50. For example, the oxidized form of cytochrome *c* extracted from baker's yeast can be separated into four main fractions. All of them show the same absorption spectrum in their reduced as well as in their oxidized forms, and it could be inferred that this yeast contains at least four different types of cytochrome *c*. Comparative studies of types of cytochrome *c* extractable from baker's yeast, heart muscle of ox and horse and breast muscle of pigeon have shown that it is a common occurrence for several *c*-type pigments to appear during chromatographic purification. However, it can now be accepted that there is only one native cytochrome *c* in each living organism and that all additional chromatographic species or 'modified cytochrome *c*' result from certain changes in the coiling of the protein structure (Nozaki *et al.* 1957; Nozaki, Mizushima, Horio & Okunuki, 1958; Mizushima, Nozaki, Horio & Okunuki, 1958; Yamanaka, Mizushima, Nozaki, Horio & Okunuki, 1959). Nozaki (1960) has found that ox-heart cytochrome *c*, when modified by acids, exists in a dimeric form within a neutral pH range. Cytochrome *c* compounds which have been partially digested by proteolytic enzymes fail to be adsorbed on weakly acid resins, and they show absorption spectra notably different from those of the native and modified cytochrome *c* (Nozaki, Mizushima, Yamanaka, Horio & Okunuki, 1959). Since crystallizability is a common property of native cytochrome *c*, we believe that crystalline *P*-cytochrome-551 is the native form of the pigment.

Egami *et al.* (1957) proposed a system of nomenclature for cytochromes based upon the nature of the haem group, on the species of organism and upon the oxidation-reduction potential. According to their system, *P*-cytochrome-551 would be called 'cytochrome *c* (*Pseudomonas aeruginosa*, E'_0 0.286 v)'. We propose this as the formal name, and '*Pseudomonas* cytochrome *c*-551' as the common name, for the pigment described in this paper.

Fig. 3. Ionic mobility of crystalline *Pseudomonas* cytochrome-551. ○, Acetate buffer; ●, phosphate buffer; *I* 0.10; temperature 20°.

SUMMARY

1. *Pseudomonas* cytochrome c-551 has been isolated and crystallized from *P. aeruginosa*, which was grown in submerged culture with nitrate as a terminal electron acceptor. The purification involves collection by H-form Duolite CS-101, chromatography on activated aluminium oxide, zone electrophoresis on a starch column and precipitation followed by crystallization from ammonium sulphate solution.

2. After three recrystallizations, *Pseudomonas* cytochrome c-551 was a homogeneous protein according to electrophoretic and ultra-centrifugal criteria.

3. The cytochrome shows absorption maxima at 551, 521, 416, 316, 290 and 280 m μ in the reduced form, and at 530, 409, 360, 290 and 280 m μ in the oxidized form. The molar extinction coefficient at 551 m μ (α -absorption maximum) is 28.3×10^6 mole⁻¹ cm.² at pH 7.00.

4. The cytochrome contains 16.2% of nitrogen and 0.69% of iron. The latter figure indicates a minimum molecular weight of 8100.

5. Values of sedimentation constant, diffusion constant and partial specific volume at pH 7.0 (*I* 0.10) are $S_{20,w}$, 1.34×10^{-13} cm. sec.⁻¹; $D_{20,w}$, 14.8×10^{-7} cm.² sec.⁻¹; $\bar{v}$, 0.710. The molecular weight calculated from these values is 7600 and the cytochrome thus contains one iron atom per molecule.

6. The isoelectric point of the cytochrome is 4.70, and E'_0 (at pH 6.50 and at 20°) is +0.286 v.

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