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## The Purification and Amino Acid Composition of *Pseudomonas* Cytochrome *c*-551

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Mammalian cytochrome *c* is a low-molecular-weight protein with a characteristic absorption spectrum and a very basic isoelectric point. Proteins with very similar properties have been isolated from the tissues of birds, fish, insects and higher plants, and from yeast. Many bacteria contain substances with an absorption spectrum like that of cytochrome *c* (Keilin, 1925; Fujita & Kodama, 1934), but when these proteins are purified it is found that their properties vary considerably between different bacterial species. The bacterial proteins differ in such properties as oxidation-reduction potential and isoelectric point, though all appear to have a low molecular weight.

The chemical structures of the *c* cytochromes from yeast and higher animals have been extensively studied (Tuppy, 1959), and the complete amino acid sequence of horse-heart cytochrome *c* has been determined (Margoliash, Smith, Kreil & Tuppy, 1961). Amino acid analyses of bacterial *c*-type cytochromes have been published (Takahashi, Titani & Minakami, 1959; Coval, Horio & Kamen, 1961), but the only investigation of the

amino acid sequence of a bacterial cytochrome *c* that has been published is the sequence near the haem group in *Rhodospirillum rubrum* cytochrome *c*<sub>2</sub> (Paleus & Tuppy, 1959).

*Pseudomonas* cytochrome *c*-551 (*P*-cytochrome-551) was prepared in crystalline form by Horio *et al.* (1960), and various physical properties of the protein were determined. The physiological role of the protein has been investigated by Yamanaka (1959) and Yamanaka, Ota & Okunuki (1961).

The work described in the present paper is the first part of an investigation of the chemical structure of the protein.

*P*-cytochrome-551 was chosen as a protein with which to try to investigate the correlation of genetic fine structure with amino acid sequence. The method of Horio *et al.* (1960) was not suitable for preparing small amounts of protein from many different strains of organism, but a satisfactory method of preparation has now been worked out and some of the chemical properties of the protein have been determined. As the amino acid sequence of the haem region of the protein (Ambler, 1962)

proved to be much less similar to mammalian cytochrome *c* than any of the other cytochromes that had been studied (Tuppy, 1959), determination of the whole amino acid sequence of the protein was undertaken, for comparison with mammalian cytochrome *c*.

### MATERIALS

The organism used was *Pseudomonas fluorescens* P6009/1, obtained from Dr N. O. Kaplan. Carboxymethylcellulose was made by the method of Ellis & Simpson (1956). Sephadex G-25 (Pharmacia, Uppsala, Sweden) was fractionated by dry-screening, and the portion that passed through a B.S. 60 sieve but was held by B.S. 100 used. Rivanol (2,5-diamino-7-ethoxyacridinium lactate monohydrate) was bought from L. Light and Co. Ltd., Colnbrook, Bucks. The enzymes used were deoxyribonuclease (Worthington Biochemical Co., St Louis, Mo., U.S.A.), subtilisin B (Bacterial Trypsin, from Novo, Copenhagen, Denmark) and Pronase (*Streptomyces griseus* protease, from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.).

### METHODS

**Medium.** The nitrate medium described by Lenhoff & Kaplan (1956) was used. To prepare 40 l. quantities, 4 l. of sterile tenfold concentrated medium was added to 36 l. of boiled tap water. The organism was maintained on nutrient agar (Difco) plates.

**Growth of organism.** The 40 l. batches were grown in deep unstirred culture in a modified 10-gallon tinned-steel milk-churn. This churn was fitted near the bottom with two 1.5 kw heating elements, which enabled the contents to be boiled. A portion (50 ml.) of nitrate medium was inoculated, and incubated at 30° without aeration for 24 hr. This culture was then added to 1 l. of the same medium, followed by incubation at 30° for 12 hr., and then by addition to 40 l. of warm (30°) medium contained in the milk churn. The churn was maintained at 30° for 12 hr. At the end of this period there was much gas formation; growth was allowed to continue until the foam began to subside, and then the culture was allowed to cool to 4° before the cells were harvested.

**Preparation of acetone-dried powder of cells.** The cells were collected by centrifuging (Sharples Super Centrifuge), and stored frozen until a sufficient quantity had been collected. Then 800 g. (wet wt.) quantities of cells were thawed, and added, in small quantities, to 10 l. portions of acetone that had been cooled to 0° with solid CO<sub>2</sub>. The mixture was kept stirred at 0° until all lumps had broken down, and then was filtered on a Buchner funnel. The pad was washed with 1 l. of cold acetone, 500 ml. of cold diethyl ether, and then dried to constant weight in a continuously evacuated desiccator at room temperature. The yield was 0.9–1.2 g./l. of culture.

**Chromatography on carboxymethylcellulose.** The buffers used for all experiments with carboxymethylcellulose were prepared by adjusting 0.05N-acetic acid to the required pH with 2N-ammonia. The carboxymethylcellulose (free acid form) was equilibrated with pH 3.9 buffer before the columns were poured. The adsorption of the cytochromes to the carboxymethylcellulose was not greatly affected by high flow rates, so that rates of 1–2 l./min. could be used on 8 cm. diam. columns. For elution of the material, lower

flow rates (50–100 ml./min. for an 8 cm. diam. column) were necessary to prevent tailing.

**Spectra.** Absorption spectra were measured by using Unicam model SP. 500 and Beckman Spinco model DK-2 spectrophotometers, and quartz cuvettes of 1 cm. light-path and 1.5 ml. capacity. The positions of the  $\alpha$ - and  $\beta$ -bands of the reduced cytochrome were determined by using a Hartridge reversion spectroscope. The amount of cytochrome *c* in preparations was determined by diluting the sample to a suitable concentration with 0.1M-sodium phosphate, pH 7.4, and measuring the difference in extinction of the oxidized and the reduced sample at the wavelength of the reduced  $\alpha$ -band. The sample was first oxidized by adding 1 mM-K<sub>3</sub>Fe(CN)<sub>6</sub> (0.05 ml./1.5 ml.), and after measurement reduced with an excess of sodium dithionite. In all calculations it was assumed that the difference in molar extinction coefficients at this wavelength was  $20 \times 10^6$  mole<sup>-1</sup> cm.<sup>2</sup>. The corresponding value for horse-heart cytochrome *c* (Margoliash & Frohwirt, 1959) is  $18.7 \times 10^6$  mole<sup>-1</sup> cm.<sup>2</sup>.

**Ultracentrifugal analysis.** A sedimentation measurement was made by using a Beckman Spinco model E ultracentrifuge. The run was at 17° and 260 000 g. The value of *S*<sub>20</sub> was calculated by the method of Markham (1960).

**Electrophoresis of protein.** For moving-boundary electrophoresis, the protein was equilibrated with the buffer by gel-filtration on a column (60 cm. × 1 cm. diam.) of Sephadex G-25, and electrophoresis was carried out in a Tiselius apparatus. The method of Durrum (1950) was used for electrophoresis of the protein on paper. The buffers used were: 0.05N-acetic acid, adjusted to pH 4.7 with ammonia; 0.05M-sodium cacodylate, adjusted to pH 6.0 with hydrochloric acid; 0.05M-sodium veronal, adjusted to pH 8.5 with hydrochloric acid. After electrophoresis, the paper was stained with bromophenol blue.

**Amino acid analysis.** The method of Spackman, Stein & Moore (1958) was used. Protein samples (about 2 mg.) were hydrolysed in 0.5 ml. of 6N-hydrochloric acid in evacuated sealed tubes at 105° for 12–72 hr. The hydrochloric acid was then removed in a vacuum desiccator at room temperature (20°). Tryptophan was determined by the method of Spies & Chambers (1948).

**N-Terminal-group identification.** Phenyl isothiocyanate degradation of the protein was carried out by the paper-strip method (Fraenkel-Conrat, Harris & Levy, 1955; section II.2.B).

Two versions of the 1-fluoro-2,4-dinitrobenzene method were used to study the protein. In the first instance the method of Fraenkel-Conrat *et al.* [1955; sections I.3.A (3) and I.3.C] was followed, except that the protein was dissolved in 8M-urea before reaction with 1-fluoro-2,4-dinitrobenzene, and that for the paper chromatography of ether-soluble DNP-amino acids the first solvent system used was 2-methylbutan-2-ol-2N-ammonia instead of the Biserte & Osteux (1951) 'toluene' solvent. In the second method for the preparation of the DNP-protein, the cytochrome (1  $\mu$ mole) was dissolved in 2.5 ml. of buffer (0.2M-N-ethylmorpholine, adjusted to pH 8.4 with acetic acid), and 0.025 ml. of 1-fluoro-2,4-dinitrobenzene dissolved in 5 ml. of ethanol was added. The single-phase mixture was allowed to react at 37° for 3 hr., and then the excess of fluorodinitrobenzene removed by extraction into ether. The aqueous phase was fractionated by gel-filtration on a column (60 cm. × 1 cm. diam.) of Sephadex G-25 that had been

equilibrated with 0.1N-ammonia. The DNP-protein remained soluble throughout the preparation. The pH of the protein solution was then adjusted with acetic acid to pH 8.5 (for digestion with subtilisin B) or pH 6.5 (for digestion with Pronase), and 0.2 mg. of enzyme added. Digestion was allowed to continue for 16 hr. at 37°, and then the peptide mixtures were evaporated to dryness in a vacuum desiccator ready for fractionation by paper electrophoresis. The acidic yellow peptides were separated by high-voltage paper electrophoresis with pH 6.5 buffer (pyridine-acetic acid-water; 25:1:225, by vol.) and the Michl (1951) apparatus. These peptides were then hydrolysed, or further purified by paper chromatography [butan-1-ol-acetic acid-water (3:1:1, by vol.)] before hydrolysis. After hydrolysis, the DNP-amino acids present were extracted into ether and identified by paper chromatography, and the amino acids identified by high-voltage paper electrophoresis at pH 2.0 [formic acid-acetic acid-water (1:4:45, by vol.)], or determined by the method of Spackman *et al.* (1958). The identity of DNP-glutamic acid was confirmed by electrophoresis at pH 9 in 1% (w/v) ammonium carbonate buffer.

## RESULTS

### *Purification of Pseudomonas cytochrome c-551*

*Step 1. Extraction of acetone-dried powder of cells.* The acetone-dried cells (200 g.) were ground in a mortar with 15 g. of alumina powder (polishing alumina 5/20; Griffin and Tatlock Ltd.). Portions (70 g.) of this mixture were then blended at 45° with 600 ml. of 0.1M-ammonium acetate, pH 6.5, for 1 min. The mixture was maintained at 45° for 10 min. and then again blended for 1 min. The mixture at this stage was very viscous. It was cooled to 20°, and 1 mg. of deoxyribonuclease/600 ml. was added. The viscosity decreased very rapidly. The mixture was then cooled to 0°, and centrifuged for 15 min. at 15 000 g. The supernatant (1400 ml.) was clear and dark red. The precipitate was re-extracted, and yielded about one-eighth as much cytochrome as the first extraction. The first and second extracts (1800 ml.) were combined.

*Step 2. Treatment of extract with rivanol.* Aqueous 4% rivanol (120 ml.) was added to the 1800 ml. of ice-cold extract. The bulky precipitate was removed by centrifuging for 10 min. at 5000 g. The golden red supernatant had a volume of about 1800 ml.

*Step 3. Acidification of extract before chromatography.* The pH of the solution was now about 7.0. The solution was cooled to 4°, and 50% (v/v) acetic acid added, with thorough mixing, until the pH was 3.9. A precipitate had begun to form when the pH had reached about 5.5. The solution was centrifuged for 10 min. at 10 000 g. The clear supernatant was orange, most of the rivanol being in the precipitate. The supernatant was freed from the remaining rivanol by filtration through a 2 cm. layer of

carboxymethylcellulose on a 5 cm. diam. Buchner funnel. No cytochrome was adsorbed from the undiluted extract. The carboxymethylcellulose was washed with pH 3.9 buffer, and the washings were added to the filtrate.

*Step 4. Adsorption of respiratory components on to carboxymethylcellulose.* The pH 3.9 solution (2 l.) was diluted to 50 l. with cold (4°) distilled water. The clear dilute solution was then divided into three equal parts, and each portion passed under suction through a 1.5 cm. thick pad of carboxymethylcellulose on a 5 cm. diam. Buchner funnel. The carboxymethylcellulose became charged with coloured material. The combined 50 l. of filtrate was then passed again under suction through three successive pads (2 cm. × 8 cm. diam.) of carboxymethylcellulose. Little coloured material was adsorbed on to the third pad. No cytochrome bands could be seen with a hand spectroscope in a 20 cm. layer of dithionite-reduced final filtrate. The coloured material was eluted from the carboxymethylcellulose pads with pH 6.5 buffer, and all the eluates were combined to form about 1.5 l. of dark-red solution.

*Step 5. Chromatography on carboxymethylcellulose.* The dark-red solution was adjusted to pH 3.9 with 0.05N-acetic acid, and then passed through a column (8 cm. × 3 cm. diam.) of carboxymethylcellulose. The eluate was colourless, and three coloured bands, red, blue and a second lower red band, developed at the top of the column. When the whole of the solution had passed through, the fastest-moving red band had extended about 6 cm. down the column, whereas the blue band and the top red band were still in the top 2 cm. The column was washed with 500 ml. of pH 3.9 buffer, which did not alter the position of any of the bands, and then pH 4.45 buffer passed through. The lower red band was eluted (in about 500 ml.), and the blue band moved about half-way down the column. The blue material was eluted with pH 4.65 buffer, and the remainder of the coloured material with pH 5.1 buffer. The red eluates were examined in the Hartridge reversion spectroscope, and, after reduction with dithionite, the pH 4.45 fraction was seen to contain *P*-cytochrome-551, and the pH 5.1 fraction *Pseudomonas cytochrome c-554* (*P*-cytochrome-554). In some preparations the pH of pH 4.45 buffer was not adjusted with sufficient accuracy, and it was necessary to again chromatograph the *P*-cytochrome-551 on carboxymethylcellulose to separate it from some of the blue material [*Pseudomonas* blue protein (*P*-blue protein)].

*Step 6. Concentration and ammonium sulphate precipitation of Pseudomonas cytochrome c-551.* The pH of the *P*-cytochrome-551 solution was adjusted to 3.9 with 0.05N-acetic acid. The cytochrome was then adsorbed on to a column (5 cm. × 2 cm. diam.)

of carboxymethylcellulose, and eluted with pH 5.1 buffer to form 30 ml. of very strongly coloured solution. The pH of this solution was adjusted to 7.0 with ammonia, and the volume made up to 50 ml. Solid ammonium sulphate (22 g.) was then added to give 65% saturation, and the mixture stirred at room temperature (17°) for 30 min. The solution was then centrifuged at 10 000 *g* for 10 min., and a small amount of dirty-brown precipitate collected. The supernatant was then made up to 80% saturation by adding 7 g. more of ammonium sulphate, and a heavy precipitate formed. After stirring for 30 min. at 17°, the precipitate was collected by centrifuging at 10 000 *g* for 10 min. The pale-orange supernatant was discarded. The precipitate was dissolved in 5 ml. of water, and prepared for diethylaminoethylcellulose chromatography or for proteolytic enzyme digestion by gel-filtration through a column (60 cm. × 1 cm. diam.) of Sephadex G-25 with the appropriate buffer.

*Step 7. Chromatography of Pseudomonas cytochrome c-551 on diethylaminoethylcellulose.* *P*-cytochrome-551 (4 μmoles) from step 6 was equilibrated with 0.1 M-ammonium acetate solution, pH 9.1, by gel-filtration, and the cytochrome solution then diluted from 12 ml. to 50 ml. The solution was passed through a column (8 cm. × 1 cm. diam.) of diethylaminoethylcellulose, which had been equilibrated with 0.025 M-ammonium acetate, pH 9.1. The protein was adsorbed in the top 1 cm. of the column, and then removed by gradient elution which involved increasing the acetate ion concentration in the eluting buffer, by adding 0.1 M-ammonium acetate, pH 8.5, to the 0.025 M-ammonium acetate, pH 9.1, in the 150 ml. buffer reservoir. The bulk of the protein was eluted in a single symmetrical peak of 40 ml., beginning 30 ml. after the gradient was started. Some other red-brown material was firmly bound to the column material, but was eluted with 0.05 M-

ammonium acetate, pH 3.9. The main coloured fraction was freeze-dried.

The yields and relative purity of the *P*-cytochrome-551 at each step are shown in Table 1.

#### *Purification of Pseudomonas blue protein*

The blue fraction from step 5 had a spectral purity of about 0.3 ( $E_{625}^{ox}/E_{280}$  ratio, where  $E_{625}^{ox}$  represents the extinction at 625 mμ of the ferricyanide-oxidized sample and  $E_{280}$  the extinction at 280 mμ of the sample to which neither oxidant nor reductant had been added). Repeating the chromatography on carboxymethylcellulose produced material with purity up to 0.45, and precipitation with ammonium sulphate (95% saturation at pH 7 and at 17°) produced material of spectral purity 0.5. Chromatography on diethylaminoethylcellulose, with the same conditions as step 7 of the purification of *P*-cytochrome-551, produced material of spectral purity 0.59.

#### *Pseudomonas cytochrome c-554*

The *P*-cytochrome-554 fraction was concentrated by adsorption on to and elution from carboxymethylcellulose, but has not yet been obtained in a pure state.

#### *Properties of Pseudomonas cytochrome c-551*

*Spectrum.* The shapes of the spectra of the oxidized and reduced protein were very similar to those reported by Horio *et al.* (1960). The positions of the maxima are shown in Table 2. The Japanese workers used the  $(E_{551}^{red} - E_{570}^{red})/E_{280}$  ratio (where  $E_{551}^{red}$  and  $E_{570}^{red}$  were the extinctions of the dithionite-reduced sample at 551 mμ and 570 mμ respectively, and  $E_{280}$  the extinction at 280 mμ of the sample not treated with either oxidizing or reducing agent) as a measure of the purity of the protein. The values of this ratio at various stages in the purification are shown in Table 1. The molar extinction coefficient of the pure reduced protein at 551 mμ was esti-

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

Full experimental details are given in the text.

Main step	Purity [( $E_{551}^{red} - E_{570}^{red}$ )/ $E_{280}$ ]	Cytochrome present (μmoles)	Total material present	
			Dry wt. (g.)	Vol. (ml.)
Acetone-dried powder of cells	—	—	200	—
pH 6.5 extract of acetone-dried powder of cells (Step 1)	*	26	27	1800
pH 3.9 supernatant (Steps 2 and 3)	*	14†	4	2000
Eluate after chromatography and concentration on carboxymethylcellulose (Steps 4 and 5)	1.00	11	0.11	30
Fractionation by ammonium sulphate precipitation (65–85% saturation) (Step 6)	1.05	10	0.10	10
Eluate after chromatography on diethylaminoethylcellulose (Step 7)	1.13–1.17	6	0.06	40

\* Purity not determined, as  $E_{280}$  very large.

† Amount determined in preparation in which rivanol treatment was omitted. If rivanol is present,  $E_{280}$  is very large.

mated to be  $30 \times 10^6$  mole<sup>-1</sup> cm.<sup>2</sup>, the amount of protein being determined by amino acid analysis, and the assumption made that there was one residue of each of arginine, histidine and tyrosine/molecule.

Table 2. *Spectral properties of Pseudomonas cytochrome c-551 preparations*

Full experimental details are given in the text.

	Present investigation	Horio <i>et al.</i> (1960)
Position of maxima (m $\mu$ )		
Reduced $\alpha$ -band	550-551	551
Reduced $\beta$ -band	520	521
Reduced $\gamma$ -band	416	416
Reduced $\delta$ -band	316	313
Oxidized $\gamma$ -band	408	409
Extinction ratios ( $E/E_{\alpha}^{\text{red}}$ )		
Reduced $\alpha$ -band	(1.0)	(1.0)
Reduced $\beta$ -band	0.59-0.62	0.58
Reduced $\gamma$ -band	4.9-5.1	5.3
Oxidized $\gamma$ -band	3.6-3.9	3.6
280 m $\mu$	0.8	0.74
Molar extinction coefficient of reduced protein at 551 m $\mu$ (mole <sup>-1</sup> cm. <sup>2</sup> )	$30 \times 10^{6**}$	$28.3 \times 10^{6\dagger}$

\* On the basis of tyrosine, histidine and arginine content.

† On the basis of iron content.

*Electrophoretic and ultracentrifugal analysis.* The protein migrated as a single component when subjected to paper electrophoresis at pH 4.7, pH 6.0 and pH 8.5. At pH 4.7 the protein moved very little, but at pH 6.0 and pH 8.5 the protein migrated towards the anode. At each pH the haem-coloured material coincided exactly with the material that stained blue with bromophenol blue. In moving-boundary electrophoresis (1% solution in 0.05M-sodium cacodylate, pH 6.0), a single symmetrical peak was observed, diffusing rapidly and moving towards the anode. In the ultracentrifuge, a 1% solution in 0.05M-sodium cacodylate, pH 6.0, sedimented as a homogeneous rapidly-diffusing component, with a sedimentation constant ( $S_{20}$ ) of 1.3s. In both the ultracentrifuge and moving-boundary electrophoresis, the colour boundary coincided with the centre of the schlieren peak.

*Amino acid composition.* Equal samples of the protein were hydrolysed for 12, 24, 48 and 72 hr. A fifth sample was treated, before hydrolysis, to remove the haem by the mercuric chloride method (M. Morrison, personal communication; Ambler, 1963) and to oxidize the sulphur-containing amino acids by preformed performic acid (Hirs, 1956). The results are shown in Table 3. The haem present

Table 3. *Amino acid composition of Pseudomonas cytochrome c-551*

Full experimental details are given in the text.

	Amount of amino acid recovered ( $\mu$ moles)				Residues/molecule				
	Hydrolysis time (hr.)				Ox.*	Best value	Best value/0.088†	Nearest integer	Covalent <i>et al.</i> (1961)
	12	24	48	72					
Glycine	0.604	0.594	0.618	0.621	0.622	0.609	6.9	7	9
Alanine	1.121	1.107	1.143	1.151	1.160	1.140	12.9	13	7
Valine	0.458	0.554	0.607	0.618	0.584	0.623‡	7.1	7	4
Leucine	0.302	0.335	0.356	0.365	0.347	0.370‡	4.2	4	4
Isoleucine	0.213	0.240	0.254	0.260	0.249	0.263‡	3.0	3	3
Serine	0.246	0.235	0.230	0.223	0.238	0.245§	2.8	3	4
Threonine	0.165	0.167	0.167	0.165	0.170	0.166	1.9	2	4
Aspartic acid	0.713	0.692	0.705	0.708	0.717	0.704	8.0	8	7
Glutamic acid	0.902	0.898	0.901	0.923	0.921	0.906	10.3	10	7
Phenylalanine	0.168	0.171	0.178	0.174	0.165	0.173	2.0	2	2
Tyrosine	0.084	0.086	0.083	0.080	0	0.084	1.0	1	2
Tryptophan								2	
Methionine	0.164	0.161	0.168	0.176	0.069¶	0.167	1.9	2	1
Cysteic acid					0.129	0.129	1.5	2	1
Proline	0.524	0.524	0.542	0.557	0.512	0.537	6.1	6	5
Lysine	0.647	0.682	0.705	0.725	0.697	0.735‡	8.3	8	3
Histidine	0.083	0.086	0.086	0.091	0.090	0.086	1.0	1	1
Arginine	0.080	0.083	0.086	0.086	0.082	0.086‡	1.0	1	1
Total	—	—	—	—	—	—	—	82	65

\* Ox.: sample treated with mercuric chloride to remove the haem, oxidized with performic acid and then hydrolysed for 24 hr. The recoveries were not used for calculation of 'best values' other than cysteic acid.

†  $0.088 = [\Sigma (\text{best value})]/78$ , where 78 is the total number of residues in the protein, excluding tryptophan and cysteine. The number 78 was calculated in the first place by assuming that there was 1 residue each of tyrosine, histidine and arginine in the molecule.

‡ Recovery extrapolated to infinite time of hydrolysis.

§ Recovery extrapolated to zero time of hydrolysis.

|| Estimated colorimetrically (see the text).

¶ As methionine sulphone.

in cytochrome interfered with the colorimetric determination of tryptophan by the Spies & Chambers (1948) method, a sample of horse-heart cytochrome *c* giving a result 15% higher than the known content of 1 residue/molecule (Margoliash, Kimmel, Hill & Schmidt, 1962). With *P*-cytochrome-551, a content of 2.37 residues/molecule

was recorded, consistent with a true content of 2 residues/molecule.

*N-Terminal-group investigations.* With the phenyl isothiocyanate method, glutamic acid phenylthiohydantoin (0.8 mole/mole of protein) was released at the first step, and the phenylthiohydantoin of aspartic acid or asparagine or glutamine (0.7 mole/

Table 4. *Dinitrophenylpeptides isolated after enzymic digestion of dinitrophenylated Pseudomonas cytochrome c-551*

Full experimental details are given in the text. The purification symbols indicate the successive steps in the purification of the peptide: 6, paper electrophoresis in pyridine-acetic acid-water (25:1:225, by vol.), pH 6.5, on Whatman no. 1 paper, at 60 v/cm. for 1.5 hr.; B, paper chromatography with butan-1-ol-acetic acid-water (3:1:1, by vol.). Electrophoretic mobility at pH 6.5 is defined as the distance of substance from uncharged marker substance (glucose) at the end of the electrophoretic separation divided by the distance of the aspartic acid marker from the glucose marker. The  $R_F$  is that in butan-1-ol-acetic acid-water (3:1:1, by vol.). Where numerical values are given for the amino acid composition, these refer to moles/mole of peptide; where '+' and '++' are given, these refer to visual estimations of the relative intensities of ninhydrin spots.

Digestion	Purification	Electrophoretic mobility at		Amino acid composition
		pH 6.5	$R_F$	
Subtilisin B	6, 6, B	-1.0	0.9	DNP-Glu (+), Val (0.96), Leu (1.04), Asp (0.78), Glu (1.35), Pro (0.98)
Pronase	6	-0.42	—	$\epsilon$ -DNP-Lys and large amounts of many free amino acids
	6	-1.04	—	DNP-Glu (+), Val (+), Asp (+), Glu (++) , Pro (+)
	6	-1.14	—	DNP-Glu (+), Asp (+), Glu (++), Pro (+)

Table 5. *Amino acid composition of Pseudomonas blue protein*

The copper was removed from the protein before hydrolysis. Different amounts of protein were analysed at each different time (see the text), so for comparison the results were calculated such that at each time the recoveries of leucine and glutamic acid were 10 residues. In all cases cysteine was recovered as cysteic acid: recoveries of cysteic acid were not increased by performic acid oxidation of the protein before hydrolysis.

	Amino acid recovery (residues/molecule)						
	Hydrolysis time (hr.)				Best value	Nearest integer	Coval <i>et al.</i> (1961)
	12	24	36	60			
Glycine	11.26	10.70	11.26	10.84	11.0	11	14
Alanine	7.34	7.42	7.32	7.24	7.3	7	8
Valine	8.14	8.28	9.66	9.96	10.0*	10	10
Leucine	10.06	10.16	10.14	10.00	10.1	10	10
Isoleucine	3.30	3.52	3.80	3.88	3.9*	4	4
Serine	9.64	7.62	7.68	7.22	10†	10	11
Threonine	10.12	9.52	9.54	9.26	10†	10	11
Aspartic acid	18.72	19.80	17.88	18.04	18.6	19	19
Glutamic acid	9.94	9.82	9.86	10.00	9.9	10	11
Phenylalanine	5.86	5.90	5.78	5.68	5.8	6	6
Tyrosine	1.98	1.98	1.84	1.54	2.0†	2	2
Tryptophan					< 0.2	0	.
Methionine	5.70	5.64	5.40	5.16	5.8†	6	6
Cysteic acid	2.82	2.54	0.70	0.42	2.9†	3	2
Proline	4.42	4.34	3.86	3.96	4.1	4	5
Lysine	10.54	10.16	11.42	9.82	10.5	10-11	12
Histidine	4.24	3.00	4.50	4.16	4.0	4	4
Arginine	0.94	1.04	1.28	0.86	1.0	1	1
Total						127-128	136

\* Recovery extrapolated to infinite time of hydrolysis.

† Recovery extrapolated to zero time of hydrolysis.

mole of protein) at the second step. The phenylthiohydantoin released were chromatographically pure, and had adequate spectrophotometric purities ( $E_{260}/E_{267.5}$  ratios) of 0.92 at each step.

Acid hydrolysis of the DNP-protein prepared by the first method described resulted in the formation of DNP-glutamic acid. No other ether-soluble DNP-amino acid was detected, and no DNP-arginine was found. The best yield obtained was 1 mole of DNP-glutamic acid from 20 000 g. of DNP-protein; this did not include corrections for destruction of the DNP-amino acid during hydrolysis or losses during paper chromatography.

Enzymic hydrolysis of the DNP-protein prepared by the second method described was followed by the isolation of the acidic yellow peptides shown in Table 4. The overall recovery of the purified subtilisin B peptide was low (15%), but it had been necessary to prepare the peptide by three paper purification steps. After digestion with both subtilisin B and Pronase the bulk of the yellow material had zero electrophoretic mobility, and the greatest part of the ninhydrin-positive material was also concentrated in this band.

#### *Properties of Pseudomonas blue protein*

The spectrum of the oxidized protein was exactly the same as that reported by Horio, Sekuzu, Higashi & Okunuki (1961), and the same sharp subsidiary maximum at 292  $m\mu$  was observed. The absorption spectrum of the reduced protein was exactly the same as that of the oxidized protein, except for the absence of the peak at 630  $m\mu$ . Material of spectral purity ( $E_{625}^{25}/E_{260}$ ) of 0.5 had a copper content (determined by the method of Gahler, 1954) of 0.37%, corresponding to 1 atom of copper/molecule in a protein of 16 000 molecular weight. Material of spectral purity 0.59 (the best obtained) was freed of copper by precipitating the protein with 5% (w/v) trichloroacetic acid, which left the copper in the supernatant. The protein was washed with ethanol, acetone and ether, and hydrolysed for amino acid analysis. The results for 12, 24, 36 and 60 hr. hydrolyses are shown in Table 5. Owing to losses during the washing of the precipitated protein, slightly different amounts of protein were found in each hydrolysate. It was observed that the amount of leucine equalled the amount of glutamic acid in each sample irrespective of the time of hydrolysis. It was therefore assumed that complete recoveries of these two amino acids were obtained after each hydrolysis, and these amounts were used for the calculation of the recoveries of the other amino acids. It was found that the cysteine of the protein was all converted into cysteic acid during hydrolysis, performic acid oxidation before hydrolysis not increasing the yields of cysteic acid.

The tryptophan content of the protein was less than 0.3%, the carbohydrate content (Devor, 1950) less than 0.2%, and the nitrogen content (by the micro-Kjeldahl method) 15.8%.

#### DISCUSSION

The method of preparing *P*-cytochrome-551 developed in the present research is summarized in Table 1. This method was used in a considerably simplified form when small quantities of bacteria were to be extracted. The rivanol treatment (step 2) was sometimes omitted even in large-scale preparations, and was always left out in small preparations. It was included because the removal of some material at this stage rather than on acidification (step 3) seemed to give slightly better yields of *P*-cytochrome-551. For small-scale preparations, step 6 was left out, the protein solution being freeze-dried immediately after chromatography on carboxymethylcellulose, and step 7 was usually omitted when the protein prepared was to be used for sequence investigations, as the further purification obtained did not warrant the loss in yield that the step involved.

The electrophoretic and ultracentrifugal analyses, and the *N*-terminal group investigations, provided evidence that the preparation was homogeneous. The protein was shown to contain glutamic acid at the *N*-terminus of a peptide chain. The author has not found the fluorodinitrobenzene method satisfactory for quantitative determination of protein end groups, but, with the phenyl isothiocyanate method, the yield of *N*-terminal residue was 80% of the theoretical value for a protein of 9000 molecular weight. The results obtained by enzymic digestion of the DNP-protein were clear-cut because of the extremely acidic nature of the *N*-terminal sequence, and because of the unusual solubility of the DNP-protein. The enzymes used (Pronase and subtilisin B) both cause very extensive degradation of proteins. From degradation of a DNP-protein the peptide mixture produced would contain basic peptides containing arginine residues, neutral peptides containing  $\epsilon$ -DNP-lysine, 1(3)-DNP-histidine and neutral amino acids, and acidic peptides containing aspartic acid or glutamic acid. The *N*-terminal peptide would be acidic (unless an arginine residue was very close to the terminus), and would be expected to have a large mobility on electrophoresis if the *N*-terminal residue were glutamic acid or aspartic acid. Most of the yellow peptides would be neutral (containing  $\epsilon$ -DNP-lysine and neutral amino acids), though some acidic peptides, containing  $\epsilon$ -DNP-lysine joined to aspartic acid or glutamic acid, would be coloured. In the Pronase digest (Table 4) one such peptide was identified, but the other acidic yellow peptides,

and the only acidic yellow peptide obtained after digestion with subtilisin B, yielded DNP-glutamic acid on hydrolysis, together with the other amino acids shown in the Table. From their compositions, the *N*-terminal sequence of the protein was deduced to be



which was compatible with the evidence from the second step of phenyl isothiocyanate degradation. The complete structure results (Ambler, 1963) confirmed the *N*-terminal sequence.

The amino acid composition determined for *P*-cytochrome-551 is shown in Table 3. The best yield of cysteine recovered (as cysteic acid) corresponded to 1.5 residues/molecule. This was taken as evidence that the true cysteine content was 2 residues/molecule, the low yield being ascribed to incomplete release of cysteine when the thioether bonds to the haem were broken. The recoveries of some of the amino acids increased with time of hydrolysis. This was expected for isoleucine and valine, as it is well known that these amino acids are often released only slowly by acid hydrolysis (Harfenist, 1953). The other amino acids concerned (leucine, lysine and arginine) were found (Ambler, 1963) to occur in the sequence in positions adjacent to isoleucine or valine residues. The protein was found to contain only small amounts of tyrosine, arginine and histidine, and the amounts of these amino acids corresponded in each case to 1 residue/molecule in a protein of about 9000 molecular weight.

With the value of 9000 assumed to be the molecular weight, the molar extinction coefficient of the reduced protein at 551 m $\mu$  was calculated, and found to be  $30 \times 10^6$  mole<sup>-1</sup> cm.<sup>2</sup>. This is similar to the value of  $27.7 \times 10^6$  mole<sup>-1</sup> cm.<sup>2</sup> determined for

horse-heart cytochrome *c* (Margoliash & Frohwirt, 1959), based on iron (and hence haem) content. This value therefore is evidence that the present *P*-cytochrome-551 preparation contains 1 haem group/molecule for a molecular weight of 9000. The sedimentation constant ( $S_{20} = 1.3$  s) was similar to the value found by Horio *et al.* (1960), who also measured the diffusion constant, and from the two values deduced the molecular weight to be 7800. The results of the phenyl isothiocyanate degradation approached a yield of 1 *N*-terminal group/haem group. Thus all the available evidence is consistent with the conclusion that *P*-cytochrome-551 consists of a single peptide chain, with the *N*-terminal residue glutamic acid, and a molecular weight of about 9000.

It is not known whether the crystalline protein prepared by Horio *et al.* (1960) from *P. aeruginosa* is identical with the present material, but many of the properties are very similar. These properties are shown in Tables 2, 3 and 6. Coval *et al.* (1961) determined the amino acid composition of the Japanese preparation, and their results differ appreciably from those obtained in the present investigation, especially in the lysine content of the samples (Table 3). Coval *et al.* (1961) did note that their results for *P*-cytochrome-551 were based on only a single analysis, because of the extremely limited amount of material available to them. In both investigations, the cytochrome was prepared from a denitrifying culture, though the organisms used were different. Many diagnostic tests are given equally by *P. aeruginosa* and *P. fluorescens*, the characteristic difference being growth temperature, *P. aeruginosa* growing at up to 41°, whereas the optimum for *P. fluorescens* is 30° (Haynes, 1951; Gaby, 1955; Rhodes, 1959). The close similarity between the two organisms was demonstrated by the close similarities in the amino acid compositions of the two blue proteins [Coval *et al.* (1961) and Table 5].

Table 6. Comparison of properties of *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* cytochrome *c*-551

	<i>P. aeruginosa</i> (Horio <i>et al.</i> 1960; Coval <i>et al.</i> 1961)	<i>P. fluorescens</i> (present investigation)
Spectra	Very similar (see Table 2)	
Isoelectric point	4.7	About 4.7
Sedimentation constant	1.34 ± 0.07	1.3*
Molecular weight:		
Sedimentation and diffusion	7800	—
Iron content	8100	—
Histidine, arginine and tyrosine content	—	About 9000
Amino acid composition		
Total residues	65	82
Lysine (residues)	3	8
Alanine (residues)	7	13

(For other amino acids see Table 3)

\* Single determination, not corrected to solution in water.

## SUMMARY

1. *Pseudomonas* cytochrome *c*-551 has been prepared in a highly purified form from denitrifying cultures of *P. fluorescens*. The purification involved chromatography on carboxymethylcellulose and diethylaminoethylcellulose.

2. The final preparation was a homogeneous protein according to electrophoretic, ultracentrifugal and *N*-terminal-group criteria.

3. The physical properties of the preparation were similar to those of a thrice-crystallized preparation of *Pseudomonas* cytochrome *c*-551, prepared from *P. aeruginosa* by a different method.

4. The amino acid composition of *Pseudomonas* cytochrome *c*-551 was determined after acid hydrolysis.



5. The *N*-terminus of the protein was investigated by the phenyl isothiocyanate and fluorodinitrobenzene methods, and the *N*-terminal sequence of the protein deduced to be

Glu(Asp,Glu,Pro)Val-Leu...

6. All evidence obtained showed that the protein contained 1 haem group/molecule, and had a single peptide chain with 82 amino acid residues.

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## The Amino Acid Sequence of *Pseudomonas* Cytochrome *c*-551

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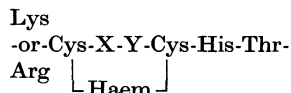
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*Pseudomonas* cytochrome *c*-551 (*P*-cytochrome-551) was prepared in crystalline form by Horio *et al.* (1960), who also investigated the physical properties of the protein and showed it to have a molecular weight of about 8000.

The molecular weight was considerably lower than that of mammalian cytochrome *c* (12000), and there was a very considerable difference in the isoelectric points of the cytochromes, that of *P*-cytochrome-551 being pH 4.7 (Horio *et al.* 1960) instead of the pH of about 11 of mammalian cytochrome *c*. This difference is reflected in the much lower basic amino acid content of the bacterial protein.

The amino acid sequence of cytochrome *c*, in the immediate vicinity of the haem group, has been determined for the protein from various vertebrate

sources, silkworm (*Bombyx mori*), yeast, and for one bacterial cytochrome (*c*<sub>2</sub> from *Rhodospirillum rubrum*) (Table 26). Although there were differences in the sequences in these proteins, in all there was the common pattern:



This pattern was also found in the vicinity of one of the haem residues in the 'RHP' from *Chromatium* (Dus, Bartsch & Kamen, 1962).

All the cytochromes *c* thus investigated had alkaline isoelectric points, with the possible exception of cytochrome *c*<sub>2</sub>; besides *P*-cytochrome-551, several other *c*-type cytochromes with acid iso-