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.

I thank Miss C. L. Watts for her help, especially with the growth and collection of the bacteria. I am also grateful to Mr S. Elsworth, Microbiological Research Establishment, Porton, Wilts., who prepared a quantity of bacteria for me.

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

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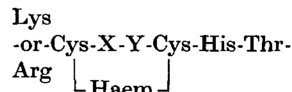
Pseudomonas cytochrome *c*-551 (*P*-cytochrome-551) was prepared in crystalline form by Horio *et al.* (1960), who also investigated the physical properties of the protein and showed it to have a molecular weight of about 8000.

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_2 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_2; besides *P*-cytochrome-551, several other *c*-type cytochromes with acid iso-

electric points are known, *Azotobacter vinelandii* cytochromes c_4 and c_5 (Neuman & Burris, 1959), *Pseudomonas* cytochrome *c*-554 (Horio, 1958*a*, *b*) and cytochrome *f* from higher plants (Davenport & Hill, 1951). It was considered desirable to study the haem sequence in *P*-cytochrome-551 as an example of this class of cytochromes, and to find out if the amino acid sequence was similar to that found in the cytochromes with alkaline isoelectric point.

At the time of writing, the complete sequences of only mammalian cytochrome *c* have been published [horse heart (Margoliash, Smith, Kreil & Tuppy, 1961; Margoliash, 1962); human heart (Matsubara & Smith, 1962)]. In the present investigation the complete amino acid sequence of *Pseudomonas* cytochrome *c*-551 has been determined, so that it has been possible to compare the complete sequence of two cytochromes from widely differing sources. This is of especial interest, as studies of the oxidation of various *c*-type cytochromes by mammalian cytochrome oxidase and by *Pseudomonas* cytochrome oxidase have been made (Yamanaka & Okunuki, 1963).

A preliminary account of part of the work reported below has been published (Ambler, 1962).

GENERAL EXPERIMENTAL APPROACH

When investigating the amino acid sequence around the haem group of a *c* cytochrome, at least three approaches are possible. Paleus & Tuppy (1959), after digesting cytochrome c_2 with trypsin, purified the resultant mixture of haem peptides by partition chromatography, and then removed the haem, oxidized the peptides and investigated the resultant cysteic acid peptides without further purification. Margoliash, Frohwirt & Wiener (1959) digested horse-heart cytochrome *c* with pepsin, separated the mixture of haem peptides from the remainder of the digestion mixture, oxidized the haem fraction and then began sequence studies on the crude mixture of cysteic acid peptides. In the present investigation it was considered more satisfactory to purify the peptides from the haem region as cysteic acid peptides after removal of the haem and oxidation, rather than to follow the Paleus & Tuppy (1959) procedure of purifying the haem peptides beforehand, because of the likelihood of producing by-products other than cysteic acid peptides during the haem-removal and oxidation steps.

In the investigation of the amino acid sequence of *P*-cytochrome-551, the experimental methods that could be used were limited by the amount of the protein available. Another limiting factor was quantitative amino acid analysis: the automatic amino acid analyser (Spackman, Stein & Moore, 1958) could measure seven samples each week, but the number of samples requiring analysis varied greatly at different stages in the investigation. This difficulty made it impossible for the experimental approach to be completely systematic.

The experimental approach used in the elucidation of the sequence depended on knowing the amino acid composition of the whole protein (Ambler, 1963). It was assumed, as there was no evidence to the contrary, that the protein consisted of a single peptide chain. This was confirmed when the sequence of all peptides obtained was shown to be consistent with such a structure. The peptides produced by tryptic digestion of the protein were investigated, by separation by gel-filtration and high-voltage paper electrophoresis followed by quantitative amino acid analysis. It was shown (Table 6) that the sum of the composition of the tryptic peptides was equal to the amino acid composition of the whole protein. The yields of the peptides were comparable, the cases of low yields being explicable (see the Discussion section). The tryptic peptides were then further degraded by other proteolytic enzymes, the particular enzyme being chosen so that about two to four peptide fragments were produced. These new peptides were analysed quantitatively if there was a possibility that they contained more than one residue of any amino acid. The complete amino acid sequence of the tryptic peptides was then worked out. In one or two cases the sequence of a small region of the protein was determined from a chymotryptic peptide, when this could be done more easily than from the tryptic peptide. The protein was also digested with chymotrypsin and with pepsin, and in each case all the peptides that were produced in good yield were purified and their quantitative amino acid compositions determined. This was followed by partial or complete amino acid-sequence determination. The tryptic peptides were then arranged in order, by using the positions of amino acids present in small amounts in the whole protein and the sequences of the chymotryptic and peptic peptides to determine the correct juxtaposition, and a unique amino acid sequence of the protein was obtained (Fig. 14).

In the sequence determination, the enzymes used were trypsin, chymotrypsin, pepsin, subtilisin B, leucine aminopeptidase and carboxypeptidase, and, to a limited extent, elastase and carboxypeptidase B. Chemical methods of end-group analysis, such as the fluorodinitrobenzene method and the phenyl isothiocyanate method, have also been used. The fluorodinitrobenzene method has not been quantitatively satisfactory, and so has been used only as a method for identifying the *N*-terminal residues of peptides. The phenyl isothiocyanate degradation has been used subtractively for small peptides, the residual peptide being purified and its amino acid composition determined by hydrolysis and paper electrophoresis. In carboxypeptidase investigations of peptides, both the liberated amino acids and the residual peptides were studied. Amide groups on aspartic acid and glutamic acid residues were detected by release of asparagine or glutamine after enzymic digestion of peptides and by consideration of the electrophoretic mobilities of peptides. Partial acid hydrolysis and cyanogen bromide splitting (Gross & Witkop, 1961) have been used to elucidate the sequence of some peptides.

MATERIALS

Trypsin. This was a 2 × crystallized salt-free preparation from Worthington Biochemical Corp., Freehold, N.J., U.S.A. (batch TRSF 813-14): this batch had the least chymotryptic activity of several assayed by Dr B. S. Hartley in this Laboratory.

Carboxypeptidase. This was a 3 × crystallized preparation, suspended in water, from Worthington Biochemical Corp.

Carboxypeptidase B. This was from Worthington Biochemical Corp. (batch 6019).

Chymotrypsin. This was a 3× crystallized preparation from Worthington Biochemical Corp.

Subtilisin B. This was a preparation of 'bacterial trypsin' (Hunt & Ottesen, 1956) from Novo Terapeutisk Laboratorium, Copenhagen, Denmark.

Pepsin. This was a 2× crystallized (from ethanol) preparation from Worthington Biochemical Corp.

Leucine aminopeptidase. This was a preparation from Worthington Biochemical Corp. (batch 5913B).

Elastase. This was a 3× crystallized preparation, suspended in water, from Worthington Biochemical Corp.

Trypsin inhibitor. This was a crystalline preparation, from soya bean, from Worthington Biochemical Corp.

Pronase. This was Pronase-P (*Streptomyces griseus* protease), B-grade, from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.

Carboxymethylcellulose. This was prepared by the method of Ellis & Simpson (1956).

Sephadex G-25. Sephadex G-25 for gel-filtration was obtained from Pharmacia, Uppsala, Sweden. The material was dry-screened and the fraction that passed through sieve B.S. 200 was used.

Pseudomonas *cytochrome* c-551. The *P*-cytochrome-551 used was prepared by the method described by Ambler (1963). Several different batches, prepared at different times from independently grown lots of bacterial acetone-dried powder, were used during the investigation.

METHODS

Amino acid composition. Quantitative determinations of the amino acid composition of peptides were made with an automatic amino acid analyser (Spackman *et al.* 1958). The sample of peptide was hydrolysed in 0·5 ml. of 6N-hydrochloric acid at 105° for 24 hr. in evacuated sealed tubes, and the acid then removed under vacuum at 20°. Except where specifically mentioned in the Tables, the amount of peptide hydrolysate used for each column analysis was 0·1–0·3 μ-mole. To compensate for destruction during hydrolysis, 6% was added to recoveries of serine and 3% to those of threonine (these corrections are average values for protein samples hydrolysed in this Laboratory).

The amino acid compositions of very simple peptides and of peptides obtained only in very small amounts were determined by paper electrophoresis. The amino acids released by total acid hydrolysis were separated by electrophoresis at pH 2·0 on Whatman no. 1 paper for 40 min. at 100 v/cm.

Tryptophan-containing peptides. In some peptides tryptophan was determined by measuring the ultraviolet-absorption spectra of the pure peptide at pH 1 and pH 13 and comparing these with the spectrum of the free amino acid. In peptides where this method could not be used, the presence of tryptophan was detected by the colour reaction and fluorescence of the peptide on paper.

After acid hydrolysis, all peptides known to contain tryptophan yielded several fluorescent components that did not react with ninhydrin. The strongest of these had the same mobility on electrophoresis at pH 2·0 as glutamic acid.

Digestion of protein and peptides with proteolytic enzymes. (*a*) Trypsin, chymotrypsin, subtilisin B and elastase. The protein (5 μmoles; 45 mg.) was dissolved in 5 ml. of ammonium acetate solution (0·1N with respect to acetic acid), pH 8·5, and the pH was adjusted to 8·5 (glass electrode) with N-acetic acid or 2N-ammonia solution; 0·1 ml. of a freshly prepared aqueous solution of the enzyme (10 mg./ml.) was then added, and the mixture incubated at 37° for 7 hr. For peptides, 0·01–0·05 ml. of a fresh aqueous enzyme solution (1 mg./ml.) was added to a solution of the peptide (0·2 μmole) in 0·2 ml. of ammonium acetate solution (0·2N with respect to acetic acid), pH 8·5, and the mixture incubated at 37° for 7 hr. After hydrolysis the peptides were evaporated to dryness in a vacuum desiccator. The larger amount of enzyme was used when digesting peptides that experience had shown were only digested slowly by the protease. For digestion of the whole protein, the chymotrypsin was preincubated with soya-bean trypsin inhibitor (1:10, by wt.) at pH 8·5 and 37° for 1 hr. The elastase preparation used was insoluble in water, and so a solution (1 mg./ml.) was made up by the method described by Fraenkel-Conrat, Harris & Levy (1955) for preparing solutions of carboxypeptidase.

(*b*) Pepsin. Digestions with pepsin were carried out by the same method, except that the protein or peptide was dissolved either in 0·02N-hydrochloric acid and the pH adjusted to 2·0, or in formic acid–acetic acid–water (1:4:45, by vol.). An aqueous solution of pepsin was used.

(*c*) Pronase. Digestion of peptides with Pronase was achieved by the same method, but the peptides were dissolved in ammonium acetate (0·2N with respect to acetic acid), pH 6·5. The enzyme used was a solution (1 mg./ml.) in the same buffer.

Partial acid hydrolysis. The peptide (0·1–0·5 μmole) was evaporated to dryness, dissolved in 0·3 ml. of 6N-hydrochloric acid and heated at 100° for 30 min. The peptide mixture, after removal of the acid, was separated by paper electrophoresis.

Cyanogen bromide cleavage of peptides. The conditions described by Gross & Witkop (1961) were used.

Separation of haem peptides from remainder of digest. After digestion the protein reaction mixture (5 μmoles) was adjusted to pH 4·0 (glass electrode) with N-acetic acid or 2N-ammonia solution and diluted to 100 ml. The solution was then passed through a column (5 cm. × 1 cm. diam.) of carboxymethylcellulose. The haem peptides were retained as a dark-brown band at the very top of the column. The column was washed through with 50 ml. of ammonium acetate solution (0·05N with respect to acetate), pH 5·0, and then the haem peptide was eluted with 0·01N-ammonia solution. All the solution that came through the column before the coloured material was combined, freeze-dried and used for investigation of the non-haem peptides of the cytochrome. All the coloured material was eluted in about 4 ml. of solution, and was then freeze-dried. Results indicated that only very small amounts of non-haem peptides remained with the haem fraction.

Removal of haem from cytochrome or haem peptide. The basis of the method is due to M. Morrison (personal communication). The peptide (5 μmoles) was dissolved in 5 ml. of 5% (v/v) formic acid, and 50 mg. of finely ground mercuric chloride was added. The mixture was incubated, with occasional shaking, for 6 hr. at 37°. Acetone (5 ml.) was then added, and the turbid brown mixture was extracted five times with 15 ml. portions of peroxide-free ether. The bulk of the haem was extracted into the ether,

together with most of the mercuric chloride. The aqueous phase, which was still brown and turbid, was evaporated to dryness in a rotary vacuum evaporator at 37°, and then dissolved, at 0°, in 0·5 ml. of 98–100% (v/v) formic acid. Then 1 ml. of ice-cold preformed performic acid (Hirs, 1956) was added, and oxidation allowed to continue at 0° for 1 hr. The mixture was freeze-dried without dilution, and then dissolved in about 0·5 ml. of water and again freeze-dried. The residue was then extracted with 0·3 ml. of 0·1 N-ammonia, and the extract, together with some of the black insoluble material, applied to paper for high-voltage electrophoresis.

High-voltage paper electrophoresis. The apparatus used was similar to that described by Michl (1951). The buffer systems were pyridine–acetic acid–water (25:1:225, by vol.), pH 6·5, pyridine–acetic acid–water (1:10:89, by vol.), pH 3·5, formic acid–acetic acid–water (1:4:45, by vol.), pH 2·0, and ammonium carbonate (1%, w/v), pH 9·0. Toluene was the coolant with the pH 6·5 buffer. With the other buffers light petroleum ('white spirit 100'; Esso) was used. For the separations at pH 2·0, 100 v/cm. was used, and for separations in the other systems 60 v/cm. The times of separation were 0·5–3 hr.

For separating small quantities of peptide, Whatman no. 1 paper was used, and 0·02–0·1 μmole of peptide/cm. separated. For larger amounts of material, Whatman no. 3 MM paper was used, with loads of 0·1 μmole/cm. (for a digest of the whole protein) to 0·5 μmole/cm.

For larger-scale peptide preparation, a piece of paper 20 cm. wide was used, with a 15 cm. band of peptide in the middle with 1 cm. bands of peptide on each side. After electrophoresis the paper was allowed to dry at room temperature. The 1 cm. bands were cut off and stained to locate the peptide, and the 15 cm. band was then cut out and the peptide eluted with 0·01 N-ammonia solution.

Paper chromatography. Peptides were separated by descending chromatography with the solvent mixture butan-1-ol–acetic acid–water (3:1:1, by vol.) for 12 hr. at 20°. The paper used and loads applied were the same as for separations by high-voltage paper electrophoresis. It was necessary to purify the peptides after chromatography by re-electrophoresis at pH 6·5 or pH 3·5, as otherwise very bad yields of some amino acids were recovered in quantitative analyses (see the Discussion section).

Identification and location of peptides after paper electrophoresis. Most peptides were located by dipping the paper in 0·25% (w/v) ninhydrin solution in acetone, and heating at 80–100° for 5 min. As some peptides gave a very weak colour with ninhydrin, the chlorination method of Reindel & Hoppé (1954) was used after reaction with ninhydrin. The specific colour tests for peptides containing histidine (Dent, 1947), tyrosine (Jepson & Smith, 1953), tryptophan (Harley-Mason & Archer, 1958) and arginine (Jepson & Smith, 1953) were also used. Proline and peptides containing *N*-terminal proline were detected with isatin (Acher, Fromageot & Jutisz, 1950). The methods for arginine, proline and tryptophan could not be used satisfactorily after the ninhydrin reaction.

Before treating with ninhydrin, the paper was examined by ultraviolet light, as the tryptophan peptides were always found to be associated with a fluorescent band.

Peptide maps. An amount of about 0·05–0·1 μmole of peptide mixture was separated by electrophoresis as a 2 cm. band on Whatman no. 1 paper at pH 6·5 for 1·5 hr. at 60 v/cm. The strip of paper (about 7 cm. wide) containing the separated peptides was then sewn (with 0·2 cm. straight machine stitches, within 0·1–0·2 cm. of the edge of the strip) on to a sheet of Whatman no. 1 paper, and the underlying part of this sheet cut off as close (0·1–0·2 cm.) to the stitching as possible. The peptides were then separated in the second dimension, either by paper electrophoresis at pH 3·5 for 1·5 hr. at 60 v/cm., or by descending paper chromatography with butan-1-ol–acetic acid–water (3:1:1, by vol.) for 12 hr. at 20°.

Separation of peptides by gel-filtration. Sephadex G-25 ('through B.S. 200' fraction) was allowed to swell in 0·01 N-ammonia solution, and the finest particles were removed by several decantations in this solution. A column (120 cm. × 1 cm. diam.), fitted with a porosity-1 glass sinter, was then packed, by the method recommended by Flodin (1961). The peptide sample, up to 10 μmoles (about 80 mg.) of mixed peptides, was put on the column dissolved in about 1 ml. of 0·1 N-ammonia solution, and eluted with 0·01 N-ammonia solution at a flow rate of about 6 ml./hr. The eluate was collected in 3·5 ml. fractions and peptides were located by the measurement of the ultraviolet absorption of the fraction at 280 mμ and by electrophoretic separation (on paper, at pH 6·5) of portions of the fractions.

N- *and* C-*Terminal groups of peptides.* The fluorodinitrobenzene method was used to identify the *N*-terminal amino acid of peptides. The peptide (0·1–0·2 μmole) was dissolved in 0·2 ml. of 0·2 M-*N*-ethylmorpholine adjusted to pH 8·4 with acetic acid (a volatile buffer that did not react with fluorodinitrobenzene), and 0·4 ml. of ethanol containing 0·005 ml. of 1-fluoro-2,4-dinitrobenzene was added. The single-phase mixture was incubated at 37° for 4 hr., and then the excess of fluorodinitrobenzene was extracted with ether. The aqueous phase was then evaporated to dryness, and most of the dinitrophenol was removed by sublimation under high vacuum. The DNP-peptide was then hydrolysed with 0·5 ml. of 6 N-hydrochloric acid for 6–24 hr. at 105° in an evacuated sealed tube. The liberated DNP-amino acid was then extracted from the acid into ether, dried under vacuum, dissolved in ethyl acetate, and applied to paper for chromatography or electrophoresis. The ether-soluble DNP-amino acids were separated and identified by ascending two-dimensional chromatography, with 2-methylbutan-2-ol–2 N-ammonia solution in the first dimension (Fraenkel-Conrat *et al.* 1955), and 1·5 M-sodium phosphate, pH 6·0, in the second (Levy, 1954). Several samples and markers were separated at once, the papers being held in frames (Datta, Dent & Harris, 1950). DNP-cysteic acid, which was not extracted from the acid into ether, was identified by electrophoresis at pH 2·0. The differentiation between DNP-aspartic acid and DNP-glutamic acid was made by electrophoresis at pH 9·0. As the *N*-ethylmorpholine salts of very small DNP-peptides with non-polar side chains tended to be extracted into ether, 1% (w/v) sodium hydrogen carbonate was used as the buffer for the dinitrophenylation of such peptides.

The phenyl isothiocyanate method was used subtractively to determine the sequence of small peptides. The peptide (0·05–0·2 μmole) was dissolved in 0·2 ml. of water, and 0·4 ml. of 10% (v/v) phenyl isothiocyanate in pyridine added. The mixture was kept at room temperature (20°) for 8–12 hr. and then freeze-dried. This did not remove all the phenyl isothiocyanate. The dry residue was then treated with 0·3 ml. of acetic acid saturated with anhydrous

hydrogen chloride, at room temperature (20°) for 0·5 hr. At the end of this time, 0·5 ml. of water was added, and the mixture then quickly extracted three times with 2 ml. of diethyl ether. The ether washings were discarded. The aqueous phase, containing the residual peptide, was freeze-dried and purified by paper electrophoresis, and a sample (0·02–0·05 μmole) was hydrolysed and the amino acid content determined by electrophoresis at pH 2·0. The remainder of the residual peptide was then again treated with phenyl isothiocyanate to remove the next amino acid.

For reaction with carboxypeptidase, the peptide (0·05–0·2 μmole) was dissolved in 0·2 ml. of 0·2M-*N*-ethylmorpholine adjusted to pH 8·4 with acetic acid. Then 0·01–0·05 ml. (0·01 mg.) of carboxypeptidase solution was added, and the mixture was incubated at 37°, normally for 4 hr., and then dried under vacuum in readiness for paper electrophoresis. The enzyme solution was prepared according to the method of Fraenkel-Conrat *et al.* (1955: p. 406, method 1, but without the use of di-isopropyl phosphorofluoridate) and diluted to 1 mg./ml. with the *N*-ethylmorpholine buffer. The residual peptide was separated from the released amino acids by paper electrophoresis: at pH 6·5 if the original peptide carried a net charge at this pH, and at pH 3·5 if the original peptide was electrically neutral at pH 6·5. After electrophoresis, the region of paper containing the free amino acids was not stained, but sewn (with 0·2 cm. straight machine stitches) on to another piece of paper, and the amino acids were separated and identified by electrophoresis at pH 2·0. The region of the original paper containing the residual peptide (or peptides) was stained, and the mobility recorded. In cases where there was sufficient material, only a portion of the residual peptide(s) was stained, the rest being eluted, hydrolysed and the constituent amino acids identified by paper electrophoresis. In a few cases the ratios of amounts of liberated amino acids were measured quantitatively. Leucine aminopeptidase was used in exactly the same way as carboxypeptidase. The freeze-dried enzyme was dissolved in 1 ml. of 2 mM-magnesium chloride, pH 8, and stored frozen. Portions (0·1 ml.) were diluted with 0·2M-*N*-ethylmorpholine (adjusted to pH 8·4 with acetic acid) to give a final concentration of 1 mg./ml. Portions (0·01–0·05 ml.) of this solution were then added to each peptide, in solution in the same buffer.

RESULTS

Peptide nomenclature

Peptides are designated by a letter (indicating the enzyme used for the original hydrolysis of the protein) followed by an arabic numeral (indicating the relative mobility of the peptide on electrophoresis at pH 6·5, the most basic peptide of the digest having the lowest number). In some cases the series of numbers is incomplete. Suffixes 'a', 'b' etc. are used when more than one peptide in the mixture has the same electrophoretic mobility at pH 6·5. If the peptide from the initial enzymic digest is then degraded, by enzymic or chemical means, the products of this treatment are similarly designated, by a letter to indicate the type of degradation, and a number for each of the products.

The letters used are as follows. For enzymic digestion: T, trypsin; C, chymotrypsin; P, pepsin; S, subtilisin B; E, elastase; Q, Pronase; λ, leucine aminopeptidase; κ, carboxypeptidase. For chemical treatment: X, cleavage with cyanogen bromide; π, partial acid hydrolysis and ψ (with a number indicating the number of steps) the residual peptide after phenyl isothiocyanate degradation. Peptides that were isolated with haem attached are designated by 'h' after the letter for the enzyme used in the initial degradation.

An example illustrates the system: C8 is a peptide from the chymotryptic digest of *P*-cytochrome-551 that separates from all the other peptides in the digest when subjected to electrophoresis at pH 6·5. C8P1a is one of the most basic peptides produced when C8 is digested with pepsin. C8P1aX2a is one of the less basic peptides formed when C8P1a is cleaved with cyanogen bromide. C8P1aX2aψ1 is the residual peptide after the *N*-terminal amino acid of C8P1aX2a had been removed by phenyl isothiocyanate degradation.

Abbreviations used in the Tables

As the same general method was used for the elucidation of the sequence of each peptide, the results of these investigations are most conveniently given in Tables.

In the Tables the 'relative yield' of the peptide is an approximate means of showing how much of each peptide in the mixture was produced by the digestion treatment. This was estimated by total acid hydrolysis of an equal portion of each purified peptide (0·005–0·1 μmole) and visual comparison of the amounts of free amino acids after separation by electrophoresis at pH 2·0 and staining with ninhydrin.

The 'electrophoretic mobility', *m*, was measured at pH 6·5. As different peptide mixtures were subjected to electrophoresis for different lengths of time, and as in the system used the endosmotic movement of uncharged substances was appreciable, the measurement is of relative mobility. In all separations a parallel separation of an amino acid mixture was made on the same sheet of paper and the position of the 'neutral' (monoaminomonocarboxylic) amino acids used to locate the true origin. The mobility of basic peptides was then measured relative to that of lysine, indicated by a positive sign, and acidic peptides relative to that of aspartic acid, indicated by a negative sign. The mobility of the same peptide varied by about 0·02–0·05 as measured on different occasions, but the mobility relative to adjacent peptides in the same mixture did not alter.

The 'amino acid composition' was either measured on the automatic amino acid analyser or estimated visually after separation of the hydro-

Table 1. *Properties of peptides from haem region of* Pseudomonas *cytochrome* c-551

The 'tryptic haem fraction' was purified only by adsorption on to carboxymethylcellulose and elution; the haem peptides were further purified by electrophoresis at pH 6·5 and 3·5. Experimental details and definition of the symbols are given in the text. The impurities of peptides Th8 and Th10 are shown in Table 6, and those of peptides Cha and Chb in Table 18.

Peptide	Electrophoretic mobility (*m*)	Amount analysed (µmole)	Amino acid composition											Other details
			Gly	Ala	Val	Ileu	Thr	Asp	Phe	$CySO_3H$	$MetSO_2$	Lys	His	
Tryptic haem fraction	.	.	0·99	1·88	1·00	0·93	0·87	1·48	.	1·03	.	1·67	0·89*	.
Th8	−0·09	.	1·09	2·11	1·04	1·02	1·01	1·97	.	1·72	.	2·08	1·03	DNP: Asp LAP: Asp(NH_2) released
Th10	−0·29	.	1·03	2·15	0·96	1·01	1·02	1·01	.	1·71	.	0·97	0·92	DNP: Gly CP: Lys released LAP: no action
Cha	+0·16	0·04	1·25	1·96	1·02	1·10	1·00	2·11	.	2·01	.	2·74	0·87	DNP: Lys† CP: Lys released
Chb	+0·16	0·05	1·12	2·02	1·06	1·01	1·00	2·02	.	1·96	0·76	2·74	0·86	DNP: Lys† CP: Lys and $MetSO_2$ released
Pha + Phb	−0·02‡	.	1·02	2·06	1·05	0·99	0·34	2·00	0·92	1·88	.	2·08	0·99	DNP: Phe CP: Pha: small amounts of Ala, Ileu and Asp released. Phb: no action
Sh	−0·41	.	0·98	2·00	1·06	.	.	.	.	2·00	.	.	1·04	DNP: Gly CP: Ala released

* Other amino acids present (mole/mole of peptide): Leu (0·19), Ser (0·20), Glu (0·21).
† DNP-end-group analysis was performed on a mixture of peptides Cha and Chb.
‡ Mobilities of peptides Pha and Phb not determined separately.

lysate by paper electrophoresis at pH 2·0 and staining with ninhydrin. For quantitative determinations, the analysis values are shown to two decimal places, the calculation assuming that the average of the amounts of the different amino acids present was integral. The visually estimated compositions were normally used when the peptide was very simple, the number of '+' signs indicating the relative intensity of the ninhydrin spot.

Other properties of the peptides are given in unheaded columns in some of the Tables. 'CP' indicates treatment with carboxypeptidase, 'LAP' treatment with leucine aminopeptidase, 'PTC' phenyl isothiocyanate degradation and 'DNP' the use of the fluorodinitrobenzene method for determination of the *N*-terminal residue of the peptide. Other properties also mentioned are the colours given by the peptide when stained on paper with ninhydrin, and the results of colour tests on paper, especially the isatin test for *N*-terminal proline residues.

In a few Tables the methods used for the purification of the peptides are given: the abbreviations used are: 2, paper electrophoresis at pH 2·0; 3, paper electrophoresis at pH 3·5; 6, paper electrophoresis at pH 6·5; B, paper chromatography with butan-1-ol–acetic acid–water (3:1:1, by vol.); Cm, chromatography on carboxymethylcellulose; G, gel-filtration on Sephadex G-25 column. The symbols are in the same order as the purification methods that were used in preparing the peptide.

Haem-containing peptides

The haem-containing fractions produced by tryptic, chymotryptic, peptic and subtilisin B digestion of native *P*-cytochrome-551 were separated from the remainder of the digests by the carboxymethylcellulose method. The amino acid composition of the whole, unpurified, tryptic haem fraction is shown in the top line of Table 1. The haem was removed from the peptides in the haem fractions, the mixture was oxidized, and the cysteic acid peptides were purified by paper electrophoresis at pH 6·5 followed by electrophoresis at pH 3·5. In each case the predominant ninhydrin-positive component(s) contained histidine, as recognized by the colour reaction on the paper. Table 1 shows the electrophoretic mobility, amino acid composition and end groups of these peptides.

The peptides Pha and Phb were completely separated by electrophoresis at pH 6·5 for 3 hr. at 60 v/cm., but not in sufficient amounts for quantitative amino acid analysis. Paper electrophoresis of acid hydrolysates of these peptides showed that peptide Pha contained threonine in amount about equal to that of isoleucine and valine, whereas peptide Phb contained no detectable threonine.

Table 2. *Peptides formed by subtilisin B digestion of peptide* Sh

The peptides were purified by electrophoresis at pH 6·5 and 3·5. Experimental details and definition of the symbols are given in the text.

Peptide	Electrophoretic mobility (m)	Relative yield	Amino acid composition	Other details
ShS1	−0·30	8	Ala (+), $CySO_3H$ (+), His (+)	DNP: $CySO_3H$ CP: Ala released; residual peptide and/or unchanged peptide (m = −0·30) formed
ShS2	−0·54	8	Gly (+), Ala (+), Val (+), $CySO_3H$ (+)	DNP: Gly CP: Ala and Val released; residual peptide (m = −0·86) formed

Table 3. *Peptides formed by elastase digestion of peptide* Th10

The peptides were purified by electrophoresis at pH 6·5 and 3·5. The free amino acids Lys (relative yield 8) and Ala (10) were also found in the digest. Experimental details and definition of the symbols are given in the text.

Peptide	Electrophoretic mobility (m)	Relative yield	Amino acid composition	Other details
Th10E2	−0·30	1	Ala (+), $CySO_3H$ (+), His (+)	.
Th10E3	−0·46	2	Ala (+), Ileu (+), Thr (+), Asp (+)	.
Th10E4	−0·56	6	Ala (+), Ileu (+), Thr (+), Asp (+), $CySO_3H$ (+), His (+)	CP: no action
Th10E5	−0·64	8	Gly (+), Val (+), $CySO_3H$ (+)	CP: Val released; residual peptide (Gly,$CySO_3H$) (m = −0·86), formed

Subtilisin haem peptide

The peptide Sh obtained after subtilisin B digestion of the protein was the smallest peptide isolated with haem attached. After removal of the haem, and oxidation of the cysteine to cysteic acid, subtilisin B was able to split the peptide into two parts. The mobilities, amino acid composition and end groups of these peptides are shown in Table 2. The presence of glycine as the N-terminal residue of peptide Sh was confirmed by removing this residue by the phenyl isothiocyanate method. The residual peptide, Shψ1, had an electrophoretic mobility, m, of −0·50, and on acid hydrolysis was found to have the amino acid composition Ala (++), Val (+), $CySO_3H$ (++), His (+), containing no glycine.

From this evidence the sequence deduced for peptide Sh was:

Gly-$CySO_3H$(Ala,Val)$CySO_3H$-His-Ala

Tryptic haem peptides

From tryptic digests, two peptides, Th8 and Th10, were isolated in approximately equal amounts. Their amino acid compositions (Table 1) were the same, except that Th8 contained one additional residue each of lysine and aspartic acid. On treatment with trypsin, peptide Th8 gave a mixture of unaltered peptide, a peptide with the same electrophoretic mobility at pH 6·5 as that of a peptide known to be asparaginyl-lysine (peptide T2; see Table 6), and a histidine-containing peptide with the same electrophoretic mobility as that of peptide Th10. From this evidence, and the end-group results of Table 1, it was concluded that peptide Th8 differed from peptide Th10 only in having asparaginyl-lysine attached at the N-terminal end.

The sequence of peptide Th10 was studied by digesting the peptide with elastase: the properties of the peptides produced are shown in Table 3. Peptide Th8 was digested with Pronase, and the peptides shown in Table 4 were isolated.

A mixture of peptides Th8 and Th10 was partially hydrolysed with acid: electrophoresis at pH 6·5 separated the hydrolysate into at least 16 bands. The major compounds were: peptide Thπ2, with m = +0·57 and composition (Thr,Lys); peptide Thπ7, with m = −0·27 and composition ($CySO_3H$,-His); peptide Thπ11, with m = −0·69 and composition (Ala,Val,$CySO_3H$). When peptide Thπ11 was treated with carboxypeptidase, alanine was released, and a residual peptide with m = −0·79 and composition (Val,$CySO_3H$) was formed.

In Fig. 1 is shown the interpretation of these results.

Other haem peptides

As stated above, two peptides, Pha and Phb, with very similar electrophoretic properties, were isolated after peptic digestion of the protein. It

was anticipated that peptide Pha would release threonine on treatment with carboxypeptidase, but no amino acid at all was released. Peptide Phb released small amounts of alanine, isoleucine and aspartic acid. This is the only case encountered during the investigation of the sequence of *P*-cytochrome-551 in which carboxypeptidase (used under the conditions described above) released an acidic amino acid.

A mixture of peptides Pha and Phb was digested with subtilisin B. The peptides formed were purified by paper electrophoresis, and their properties found to be as shown in Table 5. None of these peptides contained threonine, and threonine was not detected as a free amino acid. As expected, peptides corresponding to ShS1 and ShS2 (Table 2) were found (peptides PhS5a and PhS7), showing that the same bonds were susceptible to subtilisin B

Table 4. *Peptides formed by Pronase digestion of peptide* Th8

The peptides were purified by electrophoresis at pH 6·5 and 3·5. The free amino acids Lys (relative yield 4), His (1), Ala (4), Val (4) and Asp(NH_2) were also found in the digest. Experimental details and definition of the symbols are given in the text.

Peptide	Electrophoretic mobility (*m*)	Relative yield	Amino acid composition	Other details
Th8Q2	+0·60	1	Asp (+), Lys (+)	Same mobility and composition as T2 (see Table 6)
Th8Q4d	0	1	Gly (+), Asp (+), $CySO_3H$ (+), Lys (+)	.
Th8Q5a	−0·28	1	Ala (+), $CySO_3H$ (+), His (+)	CP: Ala released LAP: no action
Th8Q5b	−0·28	1	$CySO_3H$ (+), His (+)	.
Th8Q6	−0·53	4	Ileu (+), Thr (+), Asp (+)	CP: no action LAP: Ileu released; residual peptide (*m* = −0·70) (the same as Th8Q7) formed
Th8Q7	−0·70	1	Thr (+), Asp (+)	.
Th8Q8	−0·78	1	Ala (+), $CySO_3H$ (+)	.
Th8Q9	−0·85	3	Gly (+), $CySO_3H$ (+)	.

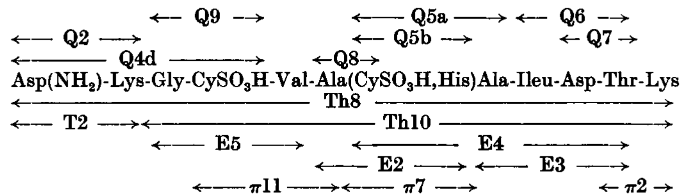


Fig. 1. Amino acid sequence of peptides Th8 and Th10 (see Tables 3 and 4). Definition of symbols is given in the text.

Table 5. *Peptides formed by subtilisin B digestion of peptide* Ph

The peptides were purified by electrophoresis at pH 6·5 and 3·5. Experimental details and definition of the symbols are given in the text.

Peptide	Electrophoretic mobility (*m*)	Relative yield	Amino acid composition	Other details
PhS2	+0·83	4	Asp (1·10), Phe (0·87), Lys (2·0)*	.
PhS3a	+0·65	2	Asp (+), Lys (+)	Same mobility (at pH 6·5 and pH 3·5) as T2 (see Table 6)
PhS3b	+0·65	1	Phe (+), Lys (+)	Same mobility (at pH 6·5 and pH 3·5) as T11π1 (see Table 15)
PhS5a	−0·29	6	Ala (+), $CySO_3H$ (+), His (+)	DNP: $CySO_3H$ CP: Ala
PhS5b	−0·29	2	$CySO_3H$ (+), His (+)	.
PhS6	−0·53	2	Ala (+), Ileu (+), Asp (+)	.
PhS7	−0·57	8	Gly (+), Ala (+), Val (+), $CySO_3H$ (+)	DNP: Gly
PhS9	−0·65	4	Ileu (+), Asp (+)	Ninhydrin on paper: no colour

* 0·03 μmole of hydrolysate was used on each column for analysis.

in a cysteic acid peptide derived from the haem region as those in the intact protein. Peptide PhS9 (Table 5) was of interest; it contained only isoleucine and aspartic acid, but could not be detected on paper by the ninhydrin reaction, being located by the Reindel–Hoppé reagent.

Both of the peptides obtained (in approximately equal amounts) after chymotryptic digestion of *P*-cytochrome-551 had the same amino acid composition (peptides Cha and Chb; see Table 1), except that peptide Chb contained in addition a residue of methionine sulphone. This residue was located at the *C*-terminus of the peptide, as carboxypeptidase released methionine sulphone and lysine from peptide Chb, whereas from peptide Cha only lysine was released. The only *N*-terminal amino acid in a mixture of peptides Cha and Chb was lysine (Table 1).

By combining the evidence for the sequence of peptides Sh, Th8 and Th10 (Fig. 1) the complete sequence of peptide Th8 was deduced. This sequence was in accordance with the evidence for the sequence of peptides Pha and Phb (Table 5) and Cha and Chb, and the complete sequence shown in Fig. 2 was deduced.

Peptides from tryptic digestion of Pseudomonas-*cytochrome* c-551

The number of peptides present in the non-haem portion of the tryptic digests was determined from peptide maps (Figs. 3, 4). The peptide mixture was separated by gel-filtration (Fig. 5) and the peptides were purified by paper electrophoresis, paper chromatography or by a second gel-filtration. The combination and sequence of methods used is shown in Table 6. The overall yield and amino acid composition of each peptide was determined by quantitative amino acid analysis (Table 6). If peptide Th8 (shown in the preceding section to yield peptides T2 and Th10 on further digestion) and peptide T10b (of composition identical with that of peptide T9b) are excluded, the sum of the amino acid composition is the same as that of the whole protein (Ambler, 1963).

In the succeeding section the peptides are considered individually, and the evidence for amino acid sequence is presented.

Peptide T1. The composition was (Ileu,Lys). Dinitrophenylation and hydrolysis gave DNP-Ileu and very stable DNP-Ileu-ε-DNP-Lys, which on further acid hydrolysis (for 24 hr. at 105°) formed DNP-Ileu, ε-DNP-Lys and unchanged bis(DNP)-peptide. With leucine aminopeptidase, Ileu and Lys were released, and a trace of unchanged peptide remained. With carboxypeptidase B, no action was obtained.

The sequence of the peptide is therefore:

Ileu-Lys

Peptide T2. The composition was (Asp,Lys). Dinitrophenylation and hydrolysis gave DNP-Asp. With leucine aminopeptidase, $Asp(NH_2)$ and Lys were released; unchanged peptide was still present. With carboxypeptidase B, no action was obtained. The presence of the amide group is also shown by the electrophoretic mobility ($m = +0{\cdot}57 - +0{\cdot}60$).

The sequence of the peptide is therefore:

$Asp(NH_2)$-Lys

Peptide T4a. The composition was (Gly,Ala,Val,-Pro,Tyr,Met,Lys). Dinitrophenylation and hydrolysis gave what might have been either DNP-Val or DNP-Met; after performic acid oxidation of the peptide DNP-$MetSO_2$ was obtained. With leucine aminopeptidase, Met and Val were released, and a residual peptide ($m = +0{\cdot}42$) was formed; a trace of unchanged peptide remained. With carboxypeptidase, Lys and Tyr were released and a residual peptide ($m = 0$) was formed. With carboxypeptidase B, Lys was released, and a residual peptide ($m = 0$) was formed.

The peptide was then treated with a mixture of carboxypeptidase and leucine aminopeptidase. The principal product was peptide T4aλκ4, of composition (Gly,Ala,Pro), which initially gave a yellow–green colour with ninhydrin, and smaller amounts of peptide T4aλκ1, of composition (Gly,-Ala,Pro,Tyr,Lys). Peptide T4aλκ4 was subjected to phenyl isothiocyanate degradation, the residual peptide after one cycle having the composition (Ala,Pro), which showed faintly and permanently yellow with ninhydrin and blue with the isatin reagent on paper. The residue after two cycles of degradation was free alanine.

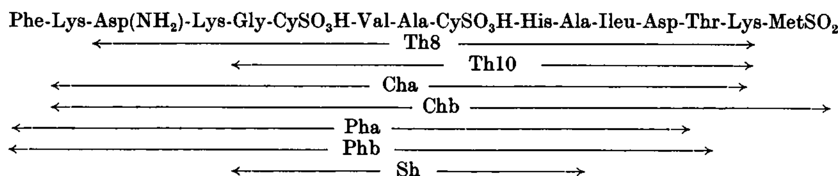


Fig. 2. Amino acid sequence of the haem region of *Pseudomonas* cytochrome *c*-551. Definition of symbols is given in the text.

Both pepsin and subtilisin B hydrolysed peptide T4a to peptides of composition (Gly,Ala,Val,Met,-Pro) and (Tyr,Lys).

The sequence of the peptide is therefore:

Met-Val-Gly-Pro-Ala-Tyr-Lys

Peptide T4b. The composition was (Val,Leu,Ser,-Glu,Try,Lys). The tryptophan content was determined from the absorption spectrum of the purified peptide. The peptide (0·3 μmole) was digested with leucine aminopeptidase, and the reaction products were separated by the automatic amino acid analyser. The products were (per unit amount of starting peptide): Val (1·06), Leu (0·98), Try (0·8), Lys (0·3). There were small amounts of Ser plus Glu(NH_2) (not resolved) and a peak eluted at the same volume as was peptide C3b (Ser-Glu(NH_2)-Lys). With carboxypeptidase B, Lys was released. With carboxypeptidase: Lys (++), Glu(NH_2) (+), and traces of Ser, Val and Leu were released. The peptide (0·3 μmole) was digested with subtilisin B. The properties of the peptides isolated are shown in Table 7. Peptides T4bS1 and T4bS2 were separated from peptide T4bS4 by gel-filtration and then purified by electrophoresis at pH 6·5. Peptide T4bS4 prepared by gel-filtration was homogeneous, as judged by electrophoresis at pH 3·5.

The sequence of the peptide is therefore:

(Try,Val)Leu-Ser-Glu(NH_2)-Lys

Peptide T7. The composition was (Ala_2,Val,-Asp,Lys). Dinitrophenylation and hydrolysis gave DNP-Asp; partial acid hydrolysis produced DNP-Asp(Val,Ala) and DNP-Asp. With leucine aminopeptidase, small amounts of Asp were released, but the bulk of the peptide was unaffected. With carboxypeptidase B, Lys was released, and an acidic residual peptide was formed. With carboxypeptidase, Lys and Ala were released, and a residual peptide ($m = -0{\cdot}71$) was formed. As the peptide had no net charge at pH 6·5 ($m = 0$), the peptide must contain aspartic acid rather than asparagine.

On partial acid hydrolysis, peptide T7 formed the peptides described in Table 8, as well as small quantities of free amino acids. The effect of enzymic digestion of peptide T7 by elastase, subtilisin B and Pronase was investigated, but none gave as satis-

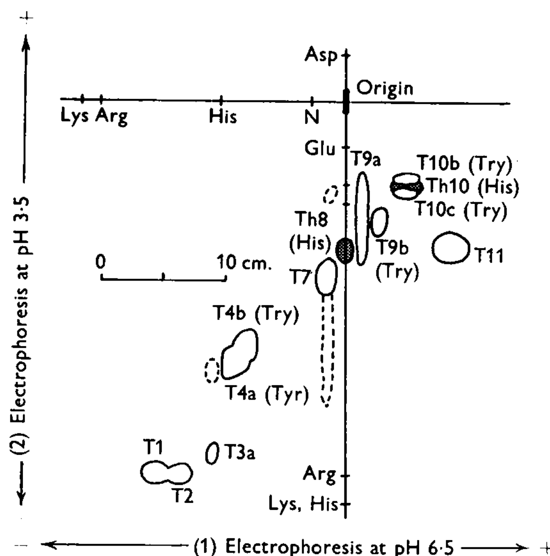


Fig. 3. Products of tryptic digestion of *Pseudomonas* cytochrome *c*-551. Separation in the first dimension was by electrophoresis at pH 6·5 [pyridine–acetic acid–water (25:1:225, by vol.)] at 60 v/cm. for 1·5 hr.; and in the second dimension by electrophoresis at pH 3·5 [pyridine–acetic acid–water (1:10:89, by vol.)] at 60 v/cm. for 1·5 hr. (His), (Tyr) and (Try) indicate peptides shown to contain these amino acids by staining reactions. Lys, His, Arg, N, Glu and Asp (along the axes) indicate respectively the positions reached by lysine, histidine, arginine, the neutral amino acids, glutamic acid and aspartic acid after separation in each dimension. The positions reached by peptides Th8 and Th10 are shown, though these peptides had normally been removed before the remainder of the peptides were separated (see the text). Definition of the symbols is given in the text.

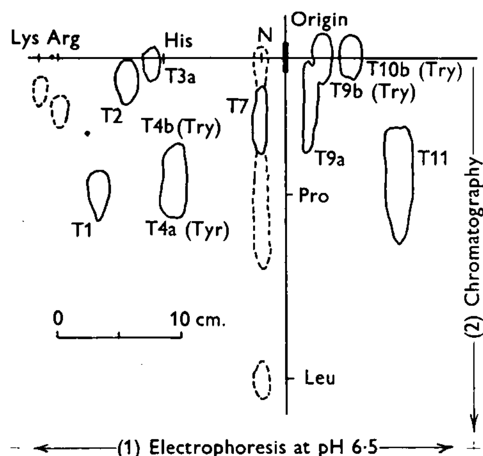


Fig. 4. Products of tryptic digestion of *Pseudomonas* cytochrome *c*-551. Separation in the first dimension was by electrophoresis at pH 6·5 [pyridine–acetic acid–water (25:1:225, by vol.)] at 60 v/cm. for 1·5 hr.; and in the second dimension by chromatography in butan-1-ol–acetic acid–water (3:1:1, by vol.). (Tyr) and (Try) indicate peptides shown to contain these amino acids by staining reactions. Lys, Arg, His, N, Pro and Leu (along axes) indicate respectively the positions reached by lysine, arginine, histidine and the neutral amino acids after electrophoresis and proline and leucine after chromatography. Definition of the symbols is given in the text.

factory a peptide pattern as did partial acid hydrolysis, a larger proportion of free amino acids being produced.

The sequence of the peptide is therefore:

Asp-Val-Ala-Ala-Lys

Peptide T9a. The composition was (Gly_2,Ala_5,Leu,Glu_4,Phe,Arg). Dinitrophenylation and hydrolysis gave DNP-Phe. With leucine aminopeptidase, Phe (++) and Ala (+) were released, and a residual peptide ($m = -0{\cdot}24$) was formed. With carboxypeptidase B, Arg was released. With carboxypeptidase, small amounts of Ala, Leu, $Glu(NH_2)$ and Arg were released.

Peptide T9a (4·5 μmoles) was digested with pepsin, and the peptides produced were separated by paper electrophoresis and chromatography. The amino acid composition of the peptides was measured quantitatively (Table 9).

Peptide T9aP5 was shown to differ from peptide T9aP4 only by the absence of the *N*-terminal

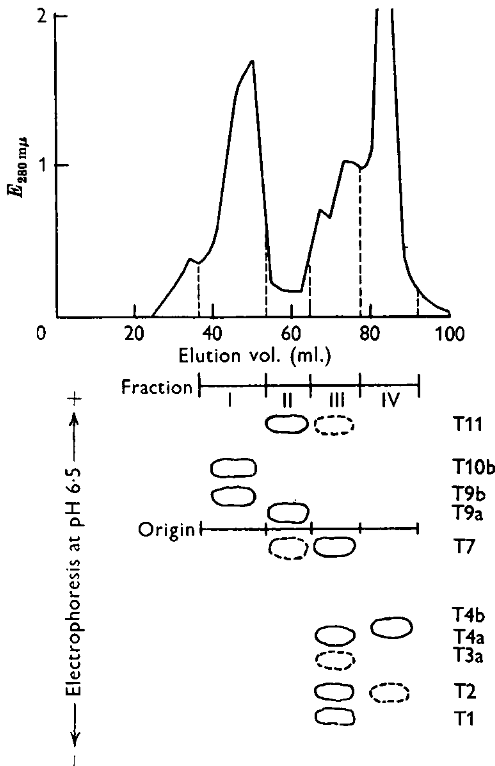


Fig. 5. Separation of the products of tryptic digestion of *Pseudomonas* cytochrome *c*-551 (10 μmoles) by gel-filtration followed by electrophoresis. Gel-filtration was on a column (120 cm. × 1 cm. diam.) of Sephadex G-25, electrophoresis at pH 6·5 [pyridine–acetic acid–water (25:1:225, by vol.)] at 60 v/cm. for 1 hr. Definition of the symbols is given in the text.

Table 6. *Properties of peptides formed by tryptic digestion of* Pseudomonas *cytochrome* c-551

The peptides in the lower section of the Table are either minor components of the digest or peptides derived from major components as by-products. Experimental details and definition of symbols are given in the text.

Peptide	Electrophoretic mobility (*m*)	Purification procedure	Yield (%)	Amino acid composition: Gly	Ala	Val	Leu	Ileu	Ser	Thr	Asp	Glu	Phe	Tyr	Try	Cys	Met	Pro	Lys	His	Arg	No. of residues
T1	+0·65–+0·69	G6B3	13	.	.	.	.	0·98	.	.	.	.	.	.	.	.	.	.	1·02	.	.	2
T4a	+0·36–+0·37	G6G	43	1·00	1·02	1·01	0·03	0·02	0·05	0·03	.	0·03	.	0·88	.	.	0·91	1·04	0·94	.	.	7
T4b	+0·32–+0·34	G6G	29	.	.	1·00	0·98	.	0·99	.	.	1·00	.	.	1·0†	.	.	.	1·03	.	.	6
T7	0	G63B3	44*	.	2·02	0·99	.	.	.	.	1·00	.	.	.	.	.	.	.	0·98	.	.	5
Th8	−0·09	Cm63	11	1·09	2·11	1·04	.	1·02	.	1·01	1·97	0·05	.	.	.	1·72	.	.	2·08	1·03	.	13
T9a	−0·19	G63	44	2·04	4·98	0·03	0·99	.	.	.	0·03	4·05	0·94	.	.	.	.	.	0·02	.	0·96	14
T9b	−0·20–−0·23	G63	21	3·07	3·17	1·96	1·04	1·00	1·88	1·03	3·86	3·14	.	.	+‡	.	1·00	4·01	0·97	.	.	27
T11	−0·50–−0·53	G63	45	.	0·02	0·96	0·94	.	.	.	1·02	2·08	1·00	.	.	.	.	0·99	1·03	.	.	8
Total			.	7	13	7	4	3	3	2	8	10	2	1	(2)	2	2	6	8	1	1	82
Whole protein§	—	—	—	6·9	12·9	7·1	4·2	3·0	2·8	1·9	8·0	10·3	2·0	1·0	2	1·5	1·9	6·1	8·3	1·0	1·0	
T2	+0·57–+0·60	G6B3	8*	.	.	.	.	.	.	.	0·99	.	.	.	.	.	.	.	1·01	.	.	2
T3a‖	+0·45	G63	< 1	.	.	.	.	.	0·97	.	1·18	.	.	.	.	.	.	0·97	3·18	.	.	6
Th10	−0·29	Cm63	8	1·03	2·03	0·96	.	1·01	0·03	1·02	1·01	0·03	.	.	.	1·71	.	.	0·97	0·92	.	11
T10b	−0·33–−0·36	G63	17	3·12	3·10	1·92	0·98	0·99	1·91	0·99	3·85	3·14	.	.	+‡	.	0·99	4·00	0·98	.	.	27
T10c	−0·35	G63	4	2·99	3·14	1·95	1·04	1·01	2·02	1·01	3·86	3·37	.	.	+‡	.	0·83	3·76	0·88	.	.	27

* Yield calculated for the peptide before the final electrophoretic purification.
† Tryptophan content determined by the ultraviolet-absorption spectrum of the purified peptide.
‡ Tryptophan detected by staining reaction of peptide on paper (see also Table 10).
§ From Ambler (1963).
‖ See the Discussion section.

phenylalanine residue, as phenyl isothiocyanate degradation converted peptide T9aP4 into a peptide T9aP4ψ1 that had the same mobility on electrophoresis as peptide T9aP5, and both peptide T9aP5 and peptide T9aP4ψ1 produced peptides with the same mobilities when digested with subtilisin B. Peptide T9aP4ψ1 contained no phenylalanine.

The sequences of peptides T9aP4 or T9aP5 were not determined completely, as it was more convenient to determine the amino acid sequence of this difficult region from the chymotryptic peptide C9 (Table 24 and Fig. 11). Peptides C9 and T9a must be derived from the same region of the protein as they both have the same characteristic amino acid composition. The correspondence was con-

Table 7. *Peptides formed by subtilisin B digestion of peptide* T4b

The digest (0·3 μmole) was fractionated by gel-filtration and electrophoresis. Experimental details and definition of the symbols are given in the text.

Peptide	Electrophoretic mobility (m)	Purification procedure	Relative yield	Amino acid composition	Other details
T4bS1	+0·62	G6	1	Glu (+), Lys (+)	Glu(NH_2), not Glu, because of m value (T4bS1 and T4bS2)
T4bS2	+0·47	G6	5	Ser (+), Glu (+), Lys (+)	
T4bS4	0	G	5	Val (+), Leu (+), Try (+)*	CP: Leu (++), Val (+) and Try (trace) released; residual peptide, (Val,Try), formed LAP: Leu (++), Val (++) and Try (+) released

* Tryptophan detected by the ultraviolet-absorption spectrum of the peptide, and by the colour reaction on paper.

Table 8. *Peptides formed by partial acid hydrolysis of peptide* T7

The hydrolysate (0·5 μmole) was separated by electrophoresis at pH 6·5. Experimental details and definition of the symbols are given in the text.

Peptide	Electrophoresis mobility (m)	Relative yield	Amino acid composition	Other details
T7π1	+0·74	8	Ala (+), Lys (+)	Same mobility as T9bC1/T10bC1 (see Table 11)
T7π2	−0·43	1	Ala (+), Val (+), Asp (+)	Ninhydrin on paper: stable yellow colour
T7π3	−0·51	1	Ala (+), Val (+), Asp (+)	
T7π4	−0·61	8	Ala (+), Val (+), Asp (+)	CP: Ala released; residual peptide (m = −0·71) formed
T7π5	−0·71	1	Val (+), Asp (+)	

Table 9. *Peptides formed by peptic digestion of peptide* T9a

The digest (4·5 μmoles) was separated by electrophoresis and paper chromatography. Experimental details and definition of the symbols are given in the text.

Peptide	Electrophoretic mobility (m)	Purification procedure	Relative yield	Amino acid composition	Other details
T9aP1	+0·56	63	8	Ala (0·97), Glu (1·03), Arg (1·00)	Glu(NH_2), not Glu, because of m value PTC degradation: (i) T9aP1ψ1, (Glu, Arg) (m = +0·59); (ii) T9aP1ψ2, Arg (m = +0·90). Sequence: Ala-Glu(NH_2)-Arg
T9aP3	0	62	4	Phe (+)	
T9aP4	−0·33	6B3	4	Gly (2·03), Ala (2·98), Glu (2·07), Phe (0·93)	LAP: Phe and Ala released PTC degradation: see the text
T9aP5	−0·37	6B3	4	Gly (2·07), Ala (3·04), Glu (1·88)	LAP: Ala released; same residual peptide as from T9aP4 formed
T9aP6	−0·55	63	8	Ala (0·96), Leu (1·00), Glu (1·03)	Glu, not Glu(NH_2), because of m value PTC degradation: (i) T9aP6ψ1, (Leu,Glu) (m = −0·63); (ii) T9aP6ψ2, Leu (m = 0)

firmed by the finding that peptide C9P1 (Table 24) had the same mobility as that of peptide T9aP5, and that the peptides derived from subtilisin B digestion of peptide T9aP5 all corresponded in mobility and composition to peptides obtained by the same digestion of peptide C9. (The peptides from T9aP5 corresponded to peptides C9S1b, C9S1c, C9S3 and C9S4.)

Peptide P8, with the same mobility and amino acid composition as those of peptide T9aP5, was isolated in good yield from peptic digests of the whole protein (Table 25).

The presence of arginine in peptide T9aP1 located it as the *C*-terminus of peptide T9a, and the phenylalanine in peptide T9aP4 showed that this peptide formed the *N*-terminus. The composition of both peptides T9a (P4+P6+P1) and peptides T9a (P3+P5+P6+P1) equalled the composition of the whole peptide, and the relative yields (Table 9) fitted this finding.

The sequence of the peptide T9a is therefore:

Phe-Ala(Gly$_2$,Ala$_2$,Glu$_2$)Ala-Glu-Leu-Ala-Glu(NH$_2$)-Arg

Peptides T9b *and* T10b. The first fraction eluted during gel-filtration of the tryptic digest ('trypsin fraction I': see Fig. 5) was separated by electrophoresis into three peptides, T9b, T10b and (in very much smaller yield) T10c. All these peptides were found to have the same amino acid composition (Table 10). A sample of 'trypsin fraction I' was also found to have the same amino acid composition. The acid and alkaline ultraviolet-absorption spectra of 'trypsin fraction I' were very similar to those of tryptophan, and so the tryptophan content of this fraction was determined by the amount of the ultraviolet absorption. This method could not be used for the peptides T9b or T10b, as, during electrophoresis or elution, degradation of tryptophan occurred and the shape of the ultraviolet-absorption spectrum was altered.

During electrophoretic purification, peptide T9b was partially converted into T10b. When peptides T9b and T10b were digested by chymotrypsin (Table 11), most of the peptides produced were common to both digests. There was no peptide formed from peptide T10b that corresponded to peptide T9bC3, but when peptide T9bC3 was purified some of a peptide identical with peptides T9bC4 and T10bC4 was formed. Examples of this type of alteration were observed in other peptides derived by enzymic degradation of peptide T9b (Fig. 15). The cause of the electrophoretic differences in these peptides is believed to be due to the unusual behaviour of the amino acid at the *N*-terminus of peptides T9b and T10b. This residue is present as asparagine in chymotryptic peptides C2 and C7c (Fig. 7), but is altered after tryptic digestion.

The sequence evidence for peptides T9b and T10b was as follows: dinitrophenylation and hydrolysis of both peptide T9b and peptide T10b gave DNP-Asp. With leucine aminopeptidase, no effect was obtained. With carboxypeptidase B, Lys was released from both peptide T9b and peptide T10b. With carboxypeptidase, Ala (++), Leu (+), Thr (+), Glu(NH$_2$) (+) and Lys (+) were released from peptide T9b.

Tryptic fraction I (0·3 μmole) was treated with carboxypeptidase, and the reaction mixture separated by the automatic amino acid analyser. The yields of amino acids (mole/mole of peptide) were: Ala (1·22), Leu (0·90), Thr (0·84), Glu(NH$_2$) (0·62) and Lys (0·88).

The peptides T9b and T10b were digested with chymotrypsin, and the peptides shown in Table 11 isolated by electrophoresis at pH 6·5 and pH 3·5. Of these peptides, the largest was peptide T9bC5/T10bC5 identical with peptide C8; the evidence for sequence is given in the next section (Tables 20–23, and Figs. 9 and 10).

The peptides T9bC3, T9bC4 and T10bC4 all had the same composition, and peptides T9bC4 and T10bC4 were indistinguishable. Each peptide was digested with subtilisin B, and the peptides shown in Table 12 were isolated by electrophoresis. The sequence of peptide T10bC4S3 was determined by partial acid hydrolysis, the peptides shown in Table 13 being isolated. The evidence for the

Table 10. *Amino acid composition of components of fraction I, obtained by separation by gel-filtration of tryptic digest of* Pseudomonas *cytochrome* c-551 (*Fig.* 5)

Experimental details and definition of the symbols are given in the text.

	Whole fraction I	T9b	T10b	T10c
Gly	3·09	3·07	3·12	2·99
Ala	3·21	3·17	3·10	3·14
Val	2·01	1·96	1·92	1·95
Leu	1·19	1·04	0·98	1·04
Ileu	1·01	1·00	0·99	1·01
Ser	1·53	1·89	1·91	2·02
Thr	0·81	1·03	0·99	1·01
Asp	4·06	3·86	3·85	3·86
Glu	3·41	3·14	3·14	3·37
Try	0·85*	+†	+†	+†
Met	0·98	1·00	0·99	0·83
Pro	3·74	4·01	4·00	3·76
Lys	1·00	0·98	1·00	0·88
Yield (%)	90	21	17	4

* Tryptophan determined by the ultraviolet-absorption spectrum of the fraction in acid and alkali.

† Tryptophan detected by the staining reaction of the peptide on paper.

sequence of peptides T9bC3/T9bC4/T10bC4 is given in Table 14. The behaviour of the N-terminal 'aspartic acid' residue is considered in the Discussion section.

Peptides T9bC3/T9bC4/T10bC4 were the only ones derived by chymotryptic digestion of peptides T9b/T10b that contained N-terminal aspartic acid, and so must be formed from the N-terminal sequence of the main peptides. Similarly, peptides T9bC1/T10bC1, containing lysine, must form the C-terminus. The amino acid composition of peptides T9b (C3/C4+C5+C1) and of peptides T10b

Table 11. *Peptides formed by chymotryptic digestion of peptides* T9b *and* T10b

Experimental details and definition of the symbols are given in the text.

Peptide	Electrophoretic mobility (m)	Purification procedure	Relative yield	Amino acid composition	Other details
T9bC1 / T10bC1	+0·74	63	8 / 8	Ala (+), Lys (+)	Same mobility as T7π1 (see Table 8)
T9bC3	−0·07	6	5*	Gly (2·03), Val (0·93), Ser (0·95), Asp (0·97), Glu (1·12), Try (+)†	DNP: Asp CP: Val and Try released; residual peptides (m = −0·11 and −0·49) formed
T9bC4	−0·28	6	3*	Gly (++), Val (+), Ser (+), Asp (+), Glu (+), Try (+)†	CP: Val and Try released; residual peptide (m = −0·49) formed
T10bC4	−0·28	6	8	Gly (1·96), Val (0·70), Ser (1·05), Asp (0·97), Glu (1·02), Try (+)†	CP: Val and Try released; residual peptide (m = −0·49) formed
T9bC5	−0·39	63	8	Gly (0·99), Ala (1·96), Val (1·00), Leu (0·96), Ileu (0·96), Ser (0·93), Thr (0·95), Asp (3·14), Glu (2·05), Met (0·80), Pro (3·95)	Same mobility and composition as C8 (see Table 18); also produced same mixture of peptides when digested with pepsin (see Table 20) Ninhydrin on paper: yellow going blue on standing (as does C8)
T10bC5	−0·39	63	8	Gly (+), Ala (++), Val (+), Leu (+), Ileu (+), Ser (+), Thr (+), Asp (+++), Glu (++), Met (+), Pro (++)	Same peptide as T9bC5 and C8

* The relative yields of peptides T9bC3 and T9bC4 varied from digestion to digestion.
† Tryptophan detected by the staining reaction of the peptide on paper, and by the fluorescent component in the hydrolysate.

Table 12. *Peptides formed by subtilisin B digestion of peptides* T9bC3, T9bC4 *and* T10bC4

The peptides were purified by electrophoresis at pH 6·5 and 3·5. Experimental details and definition of the symbols are given in the text.

Peptide	Electrophoretic mobility (m)	Relative yield	Amino acid composition	Other details
T9bC3S1 / T9bC4S1 / T10bC4S1	0	8	Gly (+), Val (+), Try (+)*	Ninhydrin on paper: faint colour CP: Try and a fluorescent compound that did not react with ninhydrin released; residual peptide did not contain Try, and had m = 0
T9bC3S2	−0·09	6	Gly (+), Ser (+), Asp (+), Glu (+)	CP: Glu(NH_2) released; residual peptides (m = −0·09 and −0·68) formed
T9bC3S3	−0·51	†		
T9bC4S3	−0·51	8	Gly (1·00), Ser (0·97), Asp (1·07), Glu (1·00)	CP: Glu(NH_2) released; residual peptide (m = −0·68) formed
T10bC4S3	−0·51	8	Gly (+), Ser (+), Asp (+), Glu (+)	CP: Glu(NH_2) released; residual peptide (m = −0·68) formed

* Tryptophan detected by the staining reaction of the peptide on paper and by the fluorescent component in the hydrolysate.
† Peptide present in small amount: neither hydrolysed nor yield estimated.

Table 13. *Peptides formed by partial acid hydrolysis of peptide* T10bC4S3

The peptides were separated by electrophoresis at pH 6·5. The free amino acids Gly (relative yield 2), Ser (1), Asp (2) and Glu (1) were also formed. Experimental details and definition of the symbols are given in the text.

Peptide	Electrophoretic mobility (m)	Relative yield	Amino acid composition	Other details
T10bC4S3π2	−0·52	2	Gly (+), Asp (+)	Ninhydrin on paper: stable yellow colour
T10bC4S3π3	−0·69	6	Ser (+), Glu (+)	PTC degradation: Glu formed after one step
T10bC4S3π4	−0·80	2	Gly (+), Asp (+)	.

Table 14. *Amino acid sequence of peptides* T9bC3/T9bC4/T10bC4

Definition of the symbols is given in the text.

Peptide		Sequence
T10bC4S3π3	Table 13	Ser-Glu
T10bC4S3π2, T10bC4S3π4	Table 13	(Asp,Gly)
T9bC3S2, T9bC4S3, T10bC4S3	Table 12	(Asp,Gly,Ser)Glu(NH_2)
T9bC3S1, T9bC4S1, T10bC4S1	Table 12	(Gly,Val)Try
T9bC3	Table 11	Asp(Gly,Ser,Glu,Gly)(Val,Try)
Deduced sequence for T9bC3/T9bC4/T10bC4		*Asp-Gly-Ser-Glu(NH_2)-Gly-Val-Try

* For the state of this aspartic acid residue in the protein see the Discussion section.

(C4+C5+C1) each add up to the compositions of the whole peptides. The amino acids released from peptides T9b/T10b by carboxypeptidase were those known to be formed from peptide C8 (identical with peptides T9bC5 and T10bC5) together with alanine and lysine from peptide T9bC1 or peptide T10bC1.

Peptide T11. The composition was (Val,Leu,Asp,-Glu_2,Phe,Pro,Lys). Dinitrophenylation and hydrolysis gave DNP-Glu; partial acid hydrolysis gave DNP-Glu-Asp and DNP-Glu. With leucine aminopeptidase, small amounts of Glu were released, and a residual peptide ($m = -0{\cdot}38$) was formed; most of the peptide was unaltered. With carboxypeptidase B, Lys was released, and a residual peptide ($m = -0{\cdot}82$) was formed. With carboxypeptidase: (*a*) when 10 μg. of enzyme/0·07 μmole of peptide was used for 1 hr. at 37°, Leu, Phe, Lys and a trace of Val were released, and residual peptides T11κ1 ($m = -1{\cdot}00$) and T11κ2 (trace) ($m = -1{\cdot}12$) were formed; (*b*) when 20 μg. of enzyme/0·07 μmole of peptide was used for 5 hr. at 37°, Val, Leu, Phe and Lys were released, and residual peptides T11κ2 and T11κ1 (trace) were formed.

The peptide (2·5 μmoles) was degraded by partial acid hydrolysis, and the peptides shown in Table 15 were isolated. From these peptides it was possible to deduce the sequence of peptide T11, but not possible to determine the presence or position of amide groups. The absence of amide groups had been indicated by the effect of leucine aminopeptidase on the peptide. This had shown that the *N*-terminal residue was present as glutamic acid, and that the residual peptide had a negative mobility at pH 6·5, showing the presence of two acidic groups in addition to the basic lysyl residue in the residual peptide. The absence of amide groups was confirmed by Pronase digestion of peptide T11, after which the peptides shown in Table 16 were isolated. Peptide T11Q4, containing all the aspartic acid and glutamic acid residues from peptide T11, was subjected to phenyl isothiocyanate degradation. The composition and electrophoretic mobilities of the residual peptides formed at each stage were determined (Table 16). The mobilities decreased at each step, showing that acidic residues were released.

The sequence of peptide T11 is therefore:

Glu-Asp-Pro-Glu-Val-Leu-Phe-Lys

Peptides from chymotryptic digestion of Pseudomonas *cytochrome*-c-551

The number of peptides present in the non-haem portion of chymotryptic digests was determined from peptide maps (Fig. 6). The peptide mixture was separated by gel-filtration, and an elution pattern analogous to that shown in Fig. 5 obtained. Table 17 shows the distribution of peptides from the whole chymotryptic digests of the cytochrome: if the haem fraction had previously been removed by adsorption on to carboxymethylcellulose, the only qualitative difference in the pattern was the absence of fraction C1.

The peptides were separated and purified by electrophoresis and chromatography, and the yield and composition determined by quantitative amino acid analysis (Table 18).

In the remainder of this section the sequence evidence for the chymotryptic peptides is given.

Peptides C1, C2, C6 *and* C7c. The whole protein contained only one arginine residue/molecule: hence the three chymotryptic peptides (C1, C2 and C6) that contain arginine must all come from the same region of peptide chain. These peptides all occur in low yields in chymotryptic digests, and

insufficient material has been available for accurate analyses to be performed. The results obtained are shown in Table 18. Peptide C1 was the smallest; peptide C2 had a similar composition, but contained in addition an aspartic acid residue. To account for the similarities in electrophoretic mobility between peptides C1 and C2 the aspartic acid would have to be present as the amide, and this was confirmed by carboxypeptidase digestion, which released asparagine from peptide C2 and left a residual peptide with the same mobility as that of peptide C1.

Difficulty was experienced in purifying peptide C6, very small recoveries being obtained after electrophoresis at pH 3·5. It was finally found that the peptide could be purified by chromatography on carboxymethylcellulose, by using the same column and starting buffer as was used for separating haem peptides from the remainder of the peptide mixture: at pH 4·0, in ammonium acetate (0·05N with respect to acetic acid), peptide C6 was adsorbed, whereas the impurities were unretarded. The peptide was then eluted by pH 6·5 buffer. Peptide C6 thus prepared contained all the amino acids of peptide C2, together with six more residues (Table 18). Peptide C7c contained just these six residues. Because of the low yields of the peptides it was not possible to carry out any more experiments to determine their sequence, but their analyses, taken in conjunction with knowledge of the sequence of tryptic peptides, can agree only with the sequences shown in Fig. 7.

Table 15. *Peptides formed by partial acid hydrolysis of peptide* T11

The peptides were purified by electrophoresis at pH 6·5 and 3·5. Experimental details and definition of the symbols are given in the text.

Peptide	Electrophoretic mobility (*m*)	Relative yield	Amino acid composition	Other details
T11π1	+0·58	4	Phe (+), Lys (+)	.
T11π2	+0·40	2	Val (+), Leu (+), Phe (+), Lys (+)	
T11π3a	0	8	Val (+), Leu (+), Glu (+), Phe (+), Pro (+), Lys (+)	CP: Val, Leu, Phe and Lys released; T11π9 and T11π10 formed as residual peptides
T11π3b	0	4	Val (+), Leu (+)	Ninhydrin on paper: very weak colour
T11π3c	0	2	Val (+), Leu (+), Phe (+)	.
T11π4	−0·35	1	Val (+), Leu (+), Glu (+), Phe (+), Pro (+)	*N*-Terminal Pro (isatin colour)
T11π5	−0·42	1	Val (+), Leu (+), Glu (+), Phe (+)	.
T11π6	−0·44	6	Val (+), Leu (+), Glu (+), Pro (+)	*N*-Terminal Pro (isatin colour) CP: Val and Leu released; T11π10 formed as residual peptide
T11π7	−0·50	8	Val (+), Leu (+), Glu (+)	CP: Leu released; T11π9 formed as residual peptide
T11π8	−0·57	1	Val (+), Glu (+), Pro (+)	*N*-Terminal Pro (isatin colour) CP: Val released: T11π10 formed as residual peptide
T11π9	−0·63	1	Val (+), Glu (+)	.
T11π10	−0·66	8	Glu (+), Pro (+)	*N*-Terminal Pro (isatin colour)
T11π11	−0·73	2	Asp (+), Glu (+), Pro (+)	Ninhydrin on paper: stable pink colour
T11π14	−1·15	10	Asp (0·99), Glu (1·01)	.

Table 16. *Peptides formed by Pronase digestion of peptide* T11

The peptides were purified by electrophoresis at pH 6·5 and 3·5. The free amino acids Val, Leu, Phe and Lys were also formed. Experimental details and definition of the symbols are given in the text.

Peptide	Electrophoretic mobility (*m*)	Relative yield	Amino acid composition	Other details
T11Q1	+0·59	4	Phe (+), Lys (+)	.
T11Q2a	0	2	Val (+), Leu (+)	.
T11Q3	−1·0	1	Val (+), Asp (+), Glu (++), Pro (+)	.
T11Q4	−1·12	8	Asp (1·07), Glu (1·90), Pro (1·04)	Partial acid hydrolysis formed T11π10, T11π11 and T11π14 (see Table 15) PTC degradation: (i) T11Q4ψ1, (Asp,Glu,Pro) ($m = -1·00$); (ii) T11Q4ψ2, (Glu,Pro) ($m = -0·66$). *N*-Terminal Pro (isatin colour)

Peptide C3a. The composition was (Ala,Try,Lys). The tryptophan content was determined from the ultraviolet-absorption spectrum (in acid and alkali) of the purified peptide. Dinitrophenylation and hydrolysis gave DNP-Ala. With carboxypeptidase, Try was released and a residual peptide, (Ala,Lys) ($m = +0{\cdot}77$), was formed. The residual peptide was non-fluorescent, and had the same mobility as peptides T9bC1 and T7π1, both believed to be alanyl-lysine.

The sequence of the peptide is therefore:

Ala-Lys-Try

Peptide C3b. The composition was (Ser,Glu,Lys). Dinitrophenylation and hydrolysis gave DNP-Ser. With leucine aminopeptidase, the peptide was

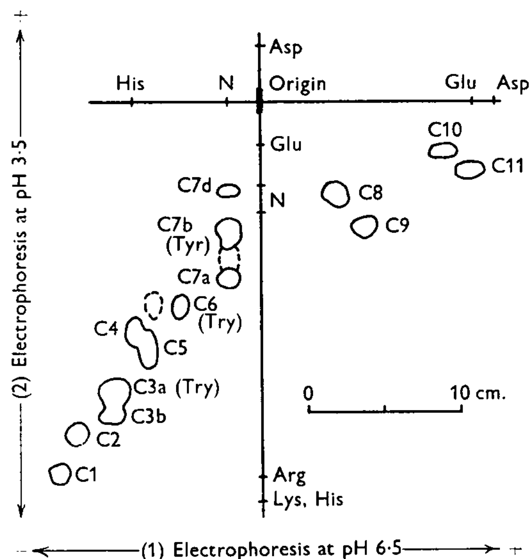


Fig. 6. Products of chymotryptic digestion of *Pseudomonas* cytochrome *c*-551. Separation in the first dimension was by electrophoresis at pH 6·5, and in the second dimension at pH 3·5. Buffers, conditions and abbreviations are the same as for Fig. 3. Definition of the symbols is given in the text.

Table 17. *Fractions from gel-filtration of chymotryptic digest of* Pseudomonas *cytochrome* c-551

Experimental details and definition of the symbols are given in the text.

Fraction	Elution volume (ml.)	Peptides present
CI	22–45	Ch (haem attached)
CII	45–58	C8
CIII	58–86	C1, 2, 3b, 4, 5, 6*, 7a, 7b, 9, 10, 11
CIV	86–96	C3a*, 7c*, 7d
CV	96–109	C3a*

* Peptides containing tryptophan.

Table 18. *Properties of peptides formed by chymotryptic digestion of* Pseudomonas *cytochrome* c-551

Experimental details and definition of the symbols are given in the text.

Peptide	Electrophoretic mobility (m)	Purification procedure	Yield (%)	Amount analysed (μmole)	Amino acid composition: Gly	Ala	Val	Leu	Ileu	Ser	Thr	Asp	Glu	Phe	Tyr	Try	Cys	Met	Pro	Lys	His	Arg	No. of residues
C1	+0·74	G63	2	*	.	+	.	.	+	.	.	.	+	.	.	.	.	.	.	+	.	+	(5)
C2	+0·68	G63	4	0·04	.	0·94	.	.	0·86	.	.	1·22	1·06	.	.	.	.	.	.	1·11	.	0·81	6
C3a	+0·51	G6G	33	> 0·1	.	1·00	.	.	.	.	.	.	.	.	.	1·2†	.	.	.	1·00	.	.	3
C3b	+0·51– +0·54	G63	25	> 0·1	0·03	.	.	.	.	0·98	.	.	1·03	.	.	.	.	.	.	0·99	.	.	3
C4	+0·41	G6Cm6	3	0·08	.	.	0·96	0·99	.	1·00	.	.	1·07	.	.	.	.	.	.	1·00	.	.	5
C5	+0·34– +0·37	G63	26	> 0·1	.	2·08	1·02	.	.	.	.	0·99	.	0·97	.	.	.	.	.	1·93	.	.	7
C6	+0·22	G6Cm6	2	0·05	2·08	0·85	1·00	0·21	0·89	1·24	0·19	1·09	2·11	.	.	+‡	.	.	0·17	0·83	.	0·94	12
C7a	0	G63B3	10	> 0·1	.	.	0·94	1·06	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2
C7b	0	G63B3	14	> 0·1	1·00	1·02	0·97	.	.	0·02	.	.	0·04	.	0·96	.	.	0·08	1·05	.	.	.	5
C7c	0	G63	2	*	++	.	+	.	.	+	.	.	+	.	.	+‡	.	.	.	.	.	.	(6)
C7d	0	G63	3	*	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	(1)
C8	−0·39– −0·44	G63	32	> 0·1	0·96	2·00	1·06	1·07	1·08	0·94	0·92	2·75	1·97	.	.	.	.	0·95	4·29	.	.	.	18
C9	−0·51	G63	41	> 0·1	1·95	3·97	.	1·01	.	.	.	.	3·07	.	.	.	.	.	.	.	.	.	10
C10	−0·82	G63	23	> 0·1§	.	.	1·01	1·00	.	.	.	1·01	2·00	1·01	.	.	.	.	0·93	.	.	.	7
C11	−0·92	G63	13	> 0·1	.	.	0·81	0·81	.	.	.	0·97	1·97	.	.	.	.	.	1·03	.	.	.	6
Cha	+0·16	Cm63	8	0·04	1·25	1·96	1·02	.	1·10	0·10	1·00	2·11	0·10	.	.	.	2·01‖	0·08‖	.	2·74	0·87	.	14
Chb	+0·16	Cm63	8	> 0·05	1·12	2·02	1·06	.	1·04	0·04	1·00	2·02	0·08	.	.	.	1·96‖	0·76‖	.	2·74	0·86	.	15

* Composition determined by paper electrophoresis.
† Tryptophan determined by ultraviolet-absorption spectrum of purified peptide.
‡ Tryptophan detected by staining reaction of peptide on paper.
§ Peptide hydrolysed for 48 hr.
‖ Oxidized peptide: Cys as $CySO_3H$, Met as $MetSO_2$.

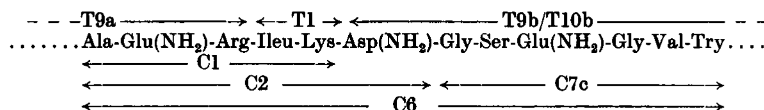


Fig. 7. Amino acid sequence of peptides C1, C2, C6 and C7c. Definition of the symbols is given in the text.

Table 19. *Peptides formed by subtilisin B and pepsin digestion of peptide* C5

The peptides were purified by electrophoresis at pH 6·5 and 3·5. Experimental details and definition of symbols are given in the text.

Peptide	Electrophoretic mobility (m)	Relative yield	Amino acid composition	Other details
C5S1	+1·0	2	Lys (+)	
C5S2	+0·79	8	Ala (+), Lys (+)	CP: no action Same composition and mobility as T7π1 (see Table 8)
C5S3a	0	8	Ala (+), Val (+), Asp (+), Lys (+)	CP: Ala and trace of Val released PTC degradation: C5S3aψ1 had same mobility (−0·59) as C5S4
C5S3b	0	8	Phe (+)	
C5S4	−0·59	1	Ala (+), Val (+), Asp (+)	CP: Ala released; residual peptide (m = −0·70) formed Same composition and mobility as T7π4 (see Table 8)
C5P1	+0·66	2	Ala (+), Phe (+), Lys (+)	CP: Phe released, residual peptide (m = +0·79) formed
C5P2	+0·37	8	Ala (+ +), Val (+), Asp (+), Phe (+), Lys (+ +)	CP: Ala, Phe and Lys released Unchanged C5
C5P3	0	4	Ala (+), Val (+), Asp (+), Lys (+)	Same composition and mobility (electrophoresis at pH 3·5) as C5S3a CP: Ala and trace of Val released

largely unaffected; traces of Ser, Glu(NH_2) and Lys were released and a peptide (m = +0·65) was formed. With carboxypeptidase, Lys was released and a residual peptide, (Ser,Glu) (m = 0), was formed. From the mobility of the peptide and of that of the carboxypeptidase residual peptide, the peptide must contain glutamine rather than glutamic acid.

The sequence of the peptide is therefore:

Ser-Glu(NH_2)-Lys

Peptide C4. The composition was (Val,Leu,Ser,-Glu,Lys). With leucine aminopeptidase, Val and Leu were released, and a residual peptide with the same mobility as that of peptide C3b (m = +0·51) was formed; small amounts of another peptide (m = +0·65) were also formed.

The sequence of the peptide is therefore:

(Val,Leu)(Ser,Glu(NH_2),Lys)

The peptide was present only in small amounts in the chymotryptic digest.

Digestion of peptide C4 with chymotrypsin produced a neutral peptide (m = 0, but not further characterized), unchanged peptide and a peptide with the same mobility as that of peptide C3b. This is what would be expected if peptide C4 appeared in chymotryptic digests because of incomplete splitting of the Leu-Ser (or Val-Ser) bond; this

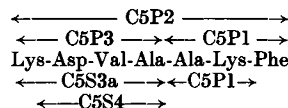


Fig. 8. Amino acid sequence of peptide C5 (see Table 19). Definition of the symbols is given in the text.

hypothesis was confirmed by the finding of the peptide Val-Leu in chymotryptic digests (see peptide C7a, below).

Peptide C5. The composition was (Ala_2,Val,Asp,-Phe,Lys_2). Dinitrophenylation and hydrolysis gave bis(DNP)-Lys. With carboxypeptidase, Ala, Phe and Lys were released. Peptide C5 was digested by subtilisin B and by pepsin, and the resultant peptides were purified and investigated. The properties of these peptides are shown in Table 19, and the sequence deduced for peptide C5 is given in Fig. 8.

Peptide C6. This is considered with peptide C1, above.

Peptide C7a. The composition was (Val,Leu). The N-terminal amino acid was removed by phenyl isothiocyanate degradation. The residue had the same electrophoretic mobility at pH 2·0 as that of leucine, both before and after acid hydrolysis. The peptide gave only a very faint colour with ninhydrin after paper electrophoresis or chromatography.

The sequence of the peptide is therefore:

Val-Leu

Peptide C7b. The composition was (Gly,Ala,Val,-Tyr,Pro). The peptide was hydrolysed in 6N-hydrochloric acid containing 2% (v/v) of thioglycollic acid. Hydrolysis without thioglycollic acid resulted in a much lower tyrosine recovery. With leucine aminopeptidase, Val was released, and a residual peptide was formed; this peptide contained tyrosine and initially gave a yellow colour when heated on paper with ninhydrin. With carboxypeptidase, Tyr was released, and a residual peptide (not containing tyrosine) was formed. Peptide C7b was then treated with a mixture of leucine aminopeptidase and carboxypeptidase: only one peptide was formed, which had the same electrophoretic mobility at H 3·5 as that of peptide T4a$\lambda\kappa$4, the same transient yellow colour with ninhydrin, and also yielded only glycine, alanine and proline on hydrolysis.

The sequence of the peptide C7b is therefore:

Val(Gly,Ala,Pro)Tyr

Peptide C7c. This is considered with peptide C1, above.

'*Peptide*' C7d. This component had the same electrophoretic mobility at pH 2·0 as that of phenylalanine, both before and after acid hydrolysis, and was therefore free phenylalanine.

Peptides C10 and C11 (see below) have identical compositions and sequences, except for an additional phenylalanine residue at the *C*-terminus of peptide C10. 'Peptide' C7d was therefore formed by further chymotryptic attack on C10.

Peptide C8. The composition was (Gly,Ala_2,Val,-Leu,Ileu,Ser,Thr,Asp_3,Glu_2,Met,Pro_4). This peptide was identical with peptides T9bC5/T10bC5 (Table 11), as judged by amino acid composition, mobility and products of peptic digestion. Material from either source was therefore used to gain the evidence described below. The peptides all gave a transient yellow when treated with ninhydrin on paper. Dinitrophenylation and hydrolysis gave DNP-Gly. With carboxypeptidase, about 1 mol.-prop. of each of Ala, Leu, Thr and Glu(NH_2) was released.

The peptide was then digested with pepsin, and the peptides produced were separated by electrophoresis at pH 6·5 and then at pH 3·5. The properties of the major peptides are shown in Table 20. Some peptides present in amounts less than that of peptide C8P3 were not characterized.

Peptide C8P1a contained the only glycine residue, and was also the only one of the C8P

Table 20. *Peptides formed by peptic digestion of peptide* C8

The peptides were purified by electrophoresis at pH 6·5 and 3·5. Experimental details and definition of the symbols are given in the text.

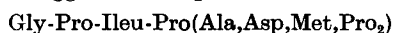
Peptide	Electrophoretic mobility (*m*)	Relative yield	Amino acid composition	Other details
C8P1a	0	7	Gly (0·92), Ala (1·01), Ileu (0·96), Asp (1·04), $MetSO_2$ (1·05)*, Pro (4·00)	Ninhydrin on paper: yellow going blue on standing; CP: Ala released; For other properties see Tables 21 and 22
C8P1b	0	4	Ala (0·95), Leu (0·98), Thr (0·94), Glu (1·12); [impurities: Gly (0·20), Ser (0·12), Val (0·06), Asp (0·06)]	DNP: Ala; CP: Leu and Thr released
C8P2	−0·37	2	Ala (1·08), Leu (1·01), Thr (0·99), Glu (1·93)	CP: Leu (1·0), Thr (0·6), and Glu(NH_2) (0·2) released†: residual peptides C8P2κ1, [Ala (+), Thr (+), Glu (++)] (m = −0·47), C8P2κ2, [Ala (+), Glu (++)] (m = −0·54), and C8P2κ3, [Ala (+), Glu (+)] (m = −0·70), formed
C8P3	−0·68	1	Ala (+), Leu (+), Thr (+), Glu (++), Asp (+)	
C8P4	−0·82	2	Val (1·01), Ser (1·00), Asp (1·98); [impurities: Glu (0·07)]	Ninhydrin on paper: very weak colour; DNP: Val; CP: no action; LAP: see Table 23
C8P5	−1·02	3	Val (1·00), (Ser), Asp (1·97), Glu (1·02)‡	Ninhydrin on paper: very weak colour; DNP: Val; CP: no action; LAP: see Table 23

* Analysis after performic acid oxidation.

† Glutamine (not glutamic acid) identified by high-voltage paper electrophoresis; the sample was then acid-hydrolysed before quantitative analysis.

‡ Serine peak lost during analysis, owing to machine fault.

peptides to give the transient yellow ninhydrin colour. The first step of phenyl isothiocyanate degradation produced a peptide that did not contain glycine. Peptide C8P1a was therefore located at the *N*-terminus of peptide C8. Peptide C8P1a was very resistant to degradation by the enzymes available. Carboxypeptidase removed alanine in good yield, but subtilisin B and Pronase had very little effect. Partial acid hydrolysis did not produce a satisfactory mixture of peptides. The sequence of the peptide was therefore determined by phenyl isothiocyanate degradation (Table 21) and by cleavage at the methionine residue with cyanogen bromide (Table 22). The evidence from Table 21 suggests the sequence:


Gly-Pro-Ileu-Pro(Ala,Asp,Met,Pro_2)

and that from Table 22 the sequence:

Gly-Pro(Ileu,Pro)Met-Pro-Pro(Ala,Asp)

Alanine (and no other amino acid) was removed from the peptide by carboxypeptidase.

As the peptide had $m = 0$, the aspartic acid must have been present as the amide. Hence the sequence of the peptide is:

Gly-Pro-Ileu-Pro-Met-Pro-Pro-Asp(NH_2)-Ala

The specificity of carboxypeptidase gives supporting evidence to the *C*-terminal sequence, as this enzyme does not hydrolyse bonds involving a proline residue (Smith, 1954). (Further evidence as to the sequence of this peptide is given in the Addendum by W. R. Gray & B. S. Hartley.)

Peptide C8 contained only 1 leucine and 1 threonine residue/molecule, and, as these amino acids were released by carboxypeptidase, they must be near the *C*-terminus of the peptide. Three peptic peptides, C8P1b, C8P2 and C8P3 (Table 20), contained these amino acids, and so must all be

Table 21. *Phenyl isothiocyanate degradation of peptide* C8P1a

The peptides were purified by electrophoresis at pH 3·5. Experimental details and definition of symbols are given in the text.

Peptide	Amino acid composition	Colour produced with ninhydrin and isatin on paper
C8P1a	Gly (+), Ala (+), Ileu (+), Asp (+), $MetSO_2$ (+), Pro (+ + +)	Ninhydrin: yellow colour turning blue on standing Isatin: no colour
C8P1aψ1	Ala (+), Ileu (+), Asp (+), $MetSO_2$ (+), Pro (+ + +)	Ninhydrin: stable faint-yellow colour Isatin: blue colour
C8P1aψ2	Ala (+), Ileu (+), Asp (+), $MetSO_2$ (+), Pro (+ +)	Ninhydrin: no colour Isatin: no colour Detected by Reindel & Hoppé (1954) reagent
C8P1aψ3	Ala (+), Asp (+), $MetSO_2$ (+), Pro (+ +)	Ninhydrin: no colour Isatin: blue colour

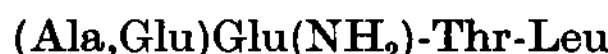
Table 22. *Peptides formed by cyanogen bromide cleavage of peptide* C8P1a

Experimental details and definition of the symbols are given in the text. The properties of products of phenyl isothiocyanate degradation of these peptides are also shown.

Peptide	Electrophoretic mobility (m)	Purification procedure	Relative yield	Amount for analysis (μmole)	Amino acid composition	Other details
C8P1aX1	+0·54	6	1	0·03	Gly (0·98), Ileu (1·13), Pro (1·89), 'Glu' (0·45)*	Ninhydrin on paper: yellow colour going blue on standing
C8P1aX1ψ1	+0·62	6	—	—	(Not determined)	*N*-Terminal Pro (isatin colour)
C8P1aX2a	0	63	2	0·05	Ala (1·10), Asp (1·04), Pro (1·88), [Ileu (0·09)]	Ninhydrin on paper: stable yellow colour *N*-Terminal Pro (isatin colour)
C8P1aX2aψ1	0	3	—	0·02	Ala (1·5), Asp (1·2), Pro (0·6)	Ninhydrin on paper: stable yellow colour *N*-Terminal Pro (isatin colour)
C8P1aX2b	0	63	3	0·07	Gly (0·85), Ala (1·00), Ileu (1·04), Asp (1·11), Met (0·50)†, Pro (4·03)	Unchanged C8P1a Ninhydrin on paper: yellow colour going blue on standing

* Homoserine formed from methionine (Gross & Witkop, 1961).
† Sample not oxidized before hydrolysis: some methionine sulphoxide present, but not included in analysis.

derived from this region of the molecule. The evidence from carboxypeptidase degradation showed that peptide C8P2 has the sequence (Table 20 and Fig. 10):


(Ala,Glu)Glu(NH_2)-Thr-Leu

whereas peptide C8P1b has the sequence:

Ala-Glu(Leu,Thr)

Hence the sequence must be as shown in Fig. 9.

The sequences of peptides C8P4 and C8P5 were determined by leucine-aminopeptidase degradation (Table 23). The residual peptide (C8P4λ2) from exhaustive treatment with leucine aminopeptidase of peptide C8P4 contained only aspartic acid, but had a greater electrophoretic mobility than free aspartic acid. Peptide C8P4 contained 2 residues of this amino acid/molecule, and none had been released by the enzyme, so peptide C8P4λ2 must be:

Asp-Asp

Peptide C8P5 had the same amino acid composition as that of peptide C8P4 except for containing an extra glutamic acid residue/molecule. The leucine-aminopeptidase-limit peptide, C8P5λ2, contained only glutamic acid and aspartic acid, and so, if peptides C8P4 and C8P5 are both derived from the same region of peptide C8, peptide C8P5λ2 must have the sequence:

Asp-Asp-Glu

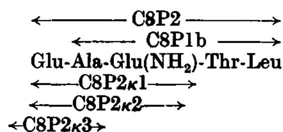


Fig. 9. Amino acid sequence of peptides C8P1b and C8P2 (see Table 20). Definition of the symbols is given in the text.

The amino acid composition of peptides C8P (1a+1b+5) and C8P (1a+2+4) both add up to the composition of the whole peptide, and their sequences overlap to form the sequence for the whole peptide shown in Fig. 10. Peptide C8P3, present in small amounts in the peptic digest, has a composition that fits into this sequence.

Peptide C9. The composition was (Gly_2,Ala_4,Leu,-Glu_3). Dinitrophenylation and hydrolysis gave DNP-Ala. With carboxypeptidase, Leu, traces of Glu and Ala were released, and a residual peptide ($m = -0{\cdot}54$) was formed. Peptide C9 was digested with subtilisin B and with pepsin. The peptides formed were separated by paper electrophoresis at pH 6·5 and pH 3·5, and had the properties shown in Table 24. The compositions of peptides C9S (1b+2+3), the major subtilisin B peptides, added up to the composition of peptide C9. Of these peptides C9S2, containing leucine, must be at the *C*-terminus of the molecule, and C9S1b, with *N*-terminal alanine, must be at the *N*-terminus of peptide C9. The electrophoretic mobilities of peptides C9S2 and C9S3 shows that the glutamic acid residues in these peptides are present as glutamic acid, whereas the zero mobility of peptide C9S1b (and the effect of carboxypeptidase) show the presence of glutamine in this peptide.

The sequence of the peptide C9 is therefore as shown in Fig. 11, which is also consistent with the sequence of the minor subtilisin B peptides and the peptic peptides.

Peptide C10. The composition was (Val,Leu,-Asp_2,Glu,Phe,Pro). Dinitrophenylation and hydrolysis gave DNP-Glu. With carboxypeptidase, Phe (+ +), Leu (+ +) and Val (+) were released, and two residual peptides ($m = -1{\cdot}0$ and $-1{\cdot}12$) were formed.

Peptide C11. The composition was (Val,Leu,-Asp_2,Glu,Pro). Dinitrophenylation and hydrolysis

Table 23. *Action of leucine aminopeptidase on peptides* C8P4 *and* C8P5

The peptides were purified by electrophoresis at pH 6·5. Experimental details and definition of symbols are given in the text.

		Residual peptides produced			
Peptide	Amino acids released by leucine aminopeptidase	Peptide	Electrophoretic mobility (m)	Amino acid composition	Effect of further enzyme treatment
C8P4	Val (+ +), Ser (+)	C8P4λ1	−1·02	Ser (+), Asp (+ +)	Ser + C8P4λ2
		C8P4λ2	−1·27	Asp	No effect
C8P5	Val (+ +), Ser (+)	C8P5λ1	−1·21	Ser (+), Asp (+), Glu (+)	Ser + C8P5λ2
		C8P5λ2	−1·38	Asp (+ +), Glu (+)	No effect

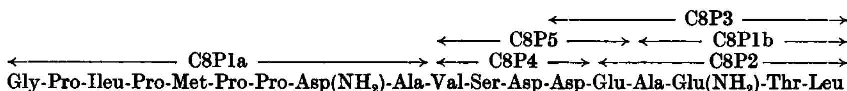


Fig. 10. Amino acid sequence of peptide C8 (see Tables 20–23). Definition of the symbols is given in the text.

Table 24. *Peptides formed by subtilisin B and pepsin digestion of peptide* C9

The peptides were purified by electrophoresis at pH 6·5 and 3·5.

Peptide	Electrophoretic mobility (*m*)	Relative yield	Amino acid composition	Other details
C9S1a	0	1	Gly (+), Ala (+)	
C9S1b	0	8	Gly (1·00), Ala (1·98), Glu (1·02)	CP: Ala and Glu(NH_2) released LAP: Ala released; residual peptide C9S1bκ (m = 0) formed. C9S1bκ treated with CP: Ala released, and residual peptide, Gly,-Glu(NH_2), formed Sequence: Ala-Gly-Glu(NH_2)-Ala
C9S1c	0	2	Gly (1·70), Ala (1·96), Glu (1·03)*	CP: no action
C9S2	−0·49	8	Ala (0·98), Leu (0·99), Glu (1·04)	Same composition and mobility as T9aP6 (see Table 9) and P10 (see Table 25)
C9S3	−0·57	5	Gly (0·93), Ala (0·96), Glu (1·10)	Ninhydrin on paper: yellow colour changing to blue on standing PTC degradation: (i) C9S3ψ1, (Ala,Glu) (m = −0·66); (ii) C9S3ψ2, Glu (m = −0·91) Sequence: Gly-Ala-Glu
C9S4	−0·66	2	Ala (+), Glu (+)	PTC degradation: Glu formed at first step
C9S5	−0·68	2	Gly (+), Ala (++), Leu (+), Glu (++)	CP: Leu released; residual peptide (m = −0·80) formed
C9S6	−0·81	1	Ala (+), Leu (+), Glu (++)	
C9P1	−0·35	8	Gly (++), Ala (+++), Glu (++)	Same mobility as T9aP5 (see Table 9) and P8 (see Table 25)
C9P2	−0·50	8	Ala (+), Leu (+), Glu (+)	Same mobility as C9S2 (see this Table), T9aP6 (see Table 9) and P10 (see Table 25)

* The sample of peptide C9S1c analysed was contaminated with about 30% of peptide C9S1b.

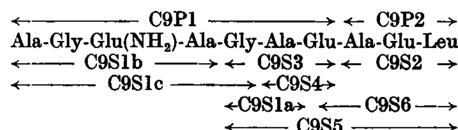


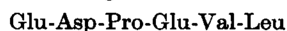
Fig. 11. Amino acid sequence of peptide C9 (see Table 24). Definition of the symbols is given in the text.

gave DNP-Glu. With carboxypeptidase, Leu (++) and Val (+) were released, and two residual peptides (m = −1·0 and −1·12) were formed.

Peptides C10 and C11 had very similar properties, the only difference being the presence of phenylalanine as additional residue on the *C*-terminus of peptide C10. Free phenylalanine was found in chymotryptic digests of the whole protein (see 'peptide' C7d, above), which would have been formed from further chymotryptic digestion of peptide C10.

When subjected to partial acid hydrolysis peptide C10 formed peptides identical, in mobility, composition and reaction with carboxypeptidase, with those of peptides T11π4, T11π5, T11π10, T11π11 and T11π14 (Table 15), whereas peptide C11 formed peptides the same as T11π6, T11π7, T11π10, T11π11 and T11π14. The formation of these peptides is consistent with peptide C10 being:

and peptide C11 being:

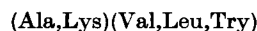


Peptides from peptic digestion of Pseudomonas *cytochrome*-c-551

The number of peptides present in the non-haem portion of peptic digests was determined from peptide maps (Fig. 12). The digest was fractionated by gel-filtration and paper electrophoresis, but few of the constituent peptides were purified completely. Table 25 shows the amino acid composition, with impurities, of many of these peptides.

The haem peptides (Tables 1 and 5), and peptides P3, P8, P10 and P11a, were investigated further.

*Peptide P*3. The composition was (Ala,Val,Leu,-Try,Lys). The tryptophan content was not determined quantitatively. With carboxypeptidase, Val, Leu and Try were released, and a residual peptide, (Ala,Lys) [m = +0·74, the same as that of peptide T9bC1, (Ala,Lys)], was formed. The sequence of the peptide is therefore:



Peptide P8. The composition was (Gly_2,Ala_3,-Glu_2). The composition and mobility of this peptide were the same as those of peptide T9aP5 (Table 9). Dinitrophenylation and hydrolysis gave DNP-Ala. With carboxypeptidase, no action was obtained. Digestion with subtilisin B gave the same peptide pattern as that obtained by similar digestion of peptide T9aP5.

Peptide P10. The composition was (Ala,Leu,Glu). Dinitrophenylation and hydrolysis gave DNP-Ala. With carboxypeptidase, Leu was released, and a

residual peptide, (Ala,Glu) ($m = -0{\cdot}66$), was formed.

The peptide must contain glutamic acid rather than glutamine, because of the electrophoretic mobility, and the sequence is therefore:

Ala-Glu-Leu

which is identical with peptide T9aP6 (Table 5) and peptides C9S2 and C9P2 (Table 24).

Peptide P11a. The composition was (Val,Leu,-Asp,Glu_2,Pro). Dinitrophenylation and hydrolysis gave DNP-Glu. With carboxypeptidase, Val and Leu were released, and two residual peptides ($m = -1{\cdot}0$ and $-1{\cdot}12$), were formed.

The peptide had the same composition and terminal groups as those of peptide C11, and gave the same peptides after partial acid hydrolysis; the sequence is therefore:

Glu-Asp-Pro-Glu-Val-Leu

The location in the protein amino acid sequence (Fig. 13) of the remainder of the peptides in Table 25 was based on amino acid composition and the position of bonds in tryptic and chymotryptic peptides known to be sensitive to pepsin.

Deduction of the complete amino acid sequence (*Fig.* 13)

The only *N*-terminal amino acid detected in the intact protein was glutamic acid (Ambler, 1963). Peptide T11 is the only tryptic peptide that contained this amino acid as *N*-terminus, and so must

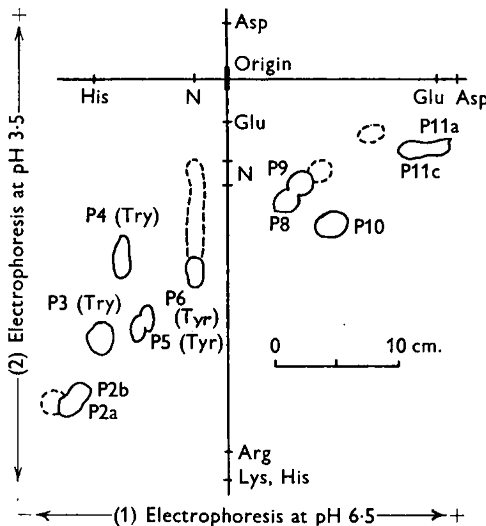


Fig. 12. Products of peptic digestion of *Pseudomonas* cytochrome *c*-551. Separation in the first dimension was by electrophoresis at pH 6·5, and in the second dimension at pH 3·5. Buffers,conditions and abbreviations are the same as for Fig. 3. Definition of the symbols is given in the text.

Table 25. *Properties of peptides formed by peptic digestion of* Pseudomonas *cytochrome* c-551

Experimental details and definition of symbols are given in the text.

Peptide	Electrophoretic mobility (m)	Amount for analysis (μmole)	Amino acid composition: Gly	Ala	Val	Leu	Ileu	Ser	Thr	Asp	Glu	Phe	Tyr	Try	Cys	Met	Pro	Lys	His	Arg	Identical peptides
P2a	+0·55	*	.	+	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	+	T9aP1
P2b	+0·53	*	.	.	.	.	.	+	.	.	+	.	.	.	.	.	.	+	.	.	C3b
P3	+0·40	> 0·1	0·13	0·99	1·01	0·98	.	0·06	0·09	0·10	0·07	.	.	+†	.	0·07	.	1·03	.	.	.
P4a	+0·32	0·02	3·0	1·9	1·0	.	1·9	1·0	.	2·5	2·0	.	.	+†	.	0·2	3·8	+	.	+	.
P4b	+0·30	0·06	3·1	1·1	0·8	.	1·7	1·1	.	2·1	1·2	.	.	+†	.	0·9	4·1	+	.	.	.
P5a	+0·28	0·03	1·1	2·1	1·9	.	.	0·3	.	1·0	0·2	.	1·3	.	.	0·7	0·7	+ +	.	.	.
P5b	+0·24	0·02	1·1	2·1	2·3	.	.	.	0·7	1·3	.	.	0·8	.	.	0·8	0·8	+ +	.	.	.
P6a	0	0·06	2·2	4·1	0·07	.	.	.	.	0·14	2·1	0·9	.	.	.	.	.	0·8	.	.	.
P6b	0	> 0·1	1·07	1·99	1·78	0·19	0·10	0·15	0·19	0·98	0·19	.	0·4‡	.	.	.	1·14	0·97	.	.	.
P8	−0·35	> 0·1	2·09	3·12	.	.	.	.	.	.	1·79	.	.	.	.	.	.	.	.	.	T9aP5
P9	−0·41	0·08	0·27	1·11	0·13	0·98	.	0·14	0·91	0·18	2·00	.	.	.	.	.	.	.	.	.	C8P2
P10	−0·53	*	.	+	.	+	.	.	.	.	+	.	.	.	.	.	.	.	.	.	T9aP6
P11a	−0·94	> 0·1	.	.	1·01	0·86	.	0·17	.	1·28	1·92	.	.	.	.	.	0·93	.	.	.	C11
P11c	−0·84	0·06	.	.	1·01	.	.	0·95	.	2·04	.	.	.	.	.	.	.	.	.	.	C8P4
Ph	−0·02	0·07	1·02	2·06	1·05	.	0·99	.	0·34	2·00	.	0·92	.	.	1·88	.	.	2·08	0·99	.	.

* Composition by paper electrophoresis. † Tryptophan detected by staining reaction of peptide on paper. ‡ As tyrosine X.

be the N-terminal peptide of the whole protein. This is confirmed by finding peptides C10, C11 and P11 with an N-terminal sequence identical with that of peptide T11, and also agreed with the results for the N-terminal sequence of the intact protein (Ambler, 1963). Several proteins, including horse-heart cytochrome *c* (Margoliash *et al.* 1961), contain an acetylated N-terminus. The possibility of partial acetylation of the N-terminus of P-cytochrome-551 was excluded by the good recoveries of peptide T11 and of peptides C10 plus C11 that were obtained (Tables 6 and 18), and by the absence of any peptides more acidic than peptide T11 in tryptic digests of the protein.

The next tryptic peptide to T11 must be the haem peptide, Th8 (T2+Th10), as the sequence -Phe-Lys-, C-terminal in peptide T11, is associated with the haem region, being the N-terminal sequence of the peptic haem peptides (Fig. 2). In the whole molecule there are only two residues of phenylalanine; one occurs as the N-terminus of the large peptide T9a, and is therefore incompatible with the haem region.

Methionine (as the sulphone) is found as the C-terminus of one of the chymotryptic haem peptides. There are only two methionine residues in the whole protein, and methionine is found in peptides Chb, C8, T9b/T10b and T4a. Peptide C8, which has a very characteristic amino acid composition, can be obtained by chymotryptic digestion of peptides T9b/T10b, and so the methionine of peptide Chb must be the same as that at the N-terminus of peptide T4a. Peptide T4a has the sequence:

Met-Val-Gly-Pro-Ala-Tyr-Lys

and contains the only residue of tyrosine in the protein. From the known specificity of chymotrypsin, it would be expected that in the intact protein the Tyr-Lys bond would be hydrolysed by this enzyme. Peptide C7b is found, and has the sequence:

Val(Gly,Ala,Pro)Tyr

Therefore the next chymotryptic peptide must have an N-terminal lysine residue. Of all the chymotryptic peptides isolated, only peptide C5 has

S. Sh [Gly-$CySO_3H$(Val,Ala)$CySO_3H$-His-Ala]
P. P11a [Glu-Asp-Pro-Glu-Val-Leu][Phe(Asp, Lys_2)(Ph Gly, Ala_2, Val, Ileu, Thr, Asp, $CySO_3H_2$,
C. C10 [Glu-Asp-Pro-Glu-Val-Leu-Phe][Lys(Gly, Ala_2, Val, Ileu, Thr, Ch Asp_2, $CySO_3H_2$, Lys, His)Lys-Met]
T. T11 [Glu-Asp-Pro-Glu-Val-Leu-Phe-Lys][Asp(NH_2)-Lys-Gly-$CySO_3H$-Val-Ala($CySO_3H$,His)Th8 Ala-Ileu-Asp-Thr-Lys]

P. ←— P6b —→ ←— P6a —→
←— P5b —→
His)] ←— P5a —→ P8 [Ala(Gly_2, Ala_2, Glu_2)] [Ala-Glu-Leu]
C. C7b [Val(Gly,Pro,Ala)Tyr][C5 Lys-Asp-Val-Ala-Ala-Lys-Phe][C9 Ala-Gly-Glu(NH_2)-Ala-Gly-Ala-Glu-Ala-Glu-Leu]
T. T4a [Met-Val-Gly-Pro-Ala-Tyr-Lys][T7 Asp-Val-Ala-Ala-Lys][T9a Phe-Ala(Gly_2, Ala_2, Glu_2,) Ala-Glu-Leu-

P. ←— P4a —→
←— P2a —→ ←— P4b —→
←— C6 —→
←— C1 —→
C. C2 [(Ala,Glu,Arg,Ileu,Lys)Asp(NH_2)] ←— C7c —→ [Gly-Pro-Ileu-Pro-Met-Pro-Pro-Asp(NH_2)-Ala
T. T9a -Ala-Glu(NH_2)-Arg][T1 Ileu-Lys][Asp-Gly-Ser-Glu(NH_2)-Gly-Val-Try-Gly(Ala_2, T9b/T10b Val, Leu, Ileu, Ser, Thr,

P. ←— P11c —→ ←— P9 —→ P3 [(Ala,Lys)(Try,Val,Leu)] ←— P2b —→
C7a C3b [Val-Leu][Ser-Glu(NH_2)-Lys]
C. C8 -Val-Ser-Asp-Asp-Glu-Ala-Glu(NH_2)-Thr-Leu][C3a Ala-Lys-Try][C4 (Val,Leu)Ser-Glu(NH_2)-Lys]
T. Asp_3, Glu_2, Met, Pro_4) T4b Ala-Lys][(Try,Val)Leu-Ser-Glu(NH_2)-Lys]

Fig. 13. Deduction of complete amino acid sequence of *Pseudomonas* cytochrome *c*-551. Definition of the symbols is given in the text.

N-terminal lysine, and this peptide, from composition and sequence, must overlap peptide T7. The *C*-terminal sequence of peptide C5 is . . . Lys-Phe, and the only phenylalanine residue not accounted for is present as the *N*-terminus of peptide T9a. The phenylalanine residues in peptides C5 and T9a are therefore the same, and peptide T9a comes next to peptide T7.

Peptide T9a contains, as *C*-terminus, the only residue of arginine present in the protein: peptides C1, C2 and C6 also contain arginine, and so must be derived from this region. Peptides C1, C2, C6 and C7c were all obtained only in low yield. Because of this it was not possible to carry out detailed studies of their sequences, but the amino acid compositions were determined. Of the amino acids present in small amounts in the whole protein, tryptophan was found in peptides C6 and C7c, and isoleucine in peptides C1, C2 and C6. There are only three residues of isoleucine in the protein: one occurs in the haem peptides Th8 and Ch, another in the related peptides T9b/T10b and C8, and so the isoleucine residues in peptides C1, C2 and C6 must be the same as that in peptide T1. Peptide C2 overlaps beyond peptide T1 by one asparaginyl residue. Peptides T9b/T10b contain either *N*-terminal asparagine or aspartic acid, and the composition of the longer overlapping peptide C6 extends along peptides T9b/T10b as far as the first bond in the tryptic peptide that is sensitive to chymotrypsin. Peptide C8 is derived from the middle portion of peptides T9b/T10b, leaving the sequence -Ala-Lys- from peptides T9b/T10b to overlap a chymotryptic peptide.

The only remaining tryptic peptide is T4b. Peptides C3a and C4 are found which have the sequence expected of peptides overlapping peptides T9b/T10b and T4b, and a peptic peptide, P3, has also been found that overlaps. Peptide T4b is therefore the *C*-terminal portion of the whole protein, and lysine the *C*-terminal residue.

The order of the tryptic peptides in the protein is therefore:

T11–Th8–T4a–T7–T9a–T1–T9b/T10b–T4b

and the complete amino acid sequence of the protein is as shown in Fig. 14. All the tryptic, chymotryptic and peptic peptides isolated (except the trace peptide T3a discussed below) fit into this sequence, confirming that the protein consists of only a single peptide chain.

Glu(1)-Asp(2)-Pro(3)-Glu(4)-Val(5)-Leu(6)-Phe(7)-Lys(8)-Asp(NH_2)(9)-Lys(10)-Gly(11)-Cys(12)-Val(13)-Ala(14)-Cys(15)-His(16)-Ala(17)-Ileu(18)-Asp(19)-Thr(20)-Lys(21)-Met(22)-Val(23)-Gly(24)-Pro(25)-Ala(26)-Tyr(27)-Lys(28)-Asp(29)-
(Haem linked to Cys 12 and Cys 15)

Val(30)-Ala(31)-Ala(32)-Lys(33)-Phe(34)-Ala(35)-Gly(36)-Glu(NH_2)(37)-Ala(38)-Gly(39)-Ala(40)-Glu(41)-Ala(42)-Glu(43)-Leu(44)-Ala(45)-Glu(NH_2)(46)-Arg(47)-Ileu(48)-Lys(49)-Asp(NH_2)(50)-Gly(51)-Ser(52)-Glu(NH_2)(53)-Gly(54)-

Val(55)-Try(56)-Gly(57)-Pro(58)-Ileu(59)-Pro(60)-Met(61)-Pro(62)-Pro(63)-Asp(NH_2)(64)-Ala(65)-Val(66)-Ser(67)-Asp(68)-Asp(69)-Glu(70)-Ala(71)-Glu(NH_2)(72)-Thr(73)-Leu(74)-Ala(75)-Lys(76)-Try(77)-Val(78)-Leu(79)-Ser(80)-Glu(NH_2)(81)-Lys(82)

Fig. 14. Complete amino acid sequence of *Pseudomonas* cytochrome *c*-551.

DISCUSSION

Comments on experimental methods. *P*-cytochrome-551 was readily digested by all the proteolytic enzymes tried. The products of digestion were easy to fractionate as all were soluble. No 'cores'

were produced such as have complicated some sequence investigations [e.g. chymotrypsin (Hartley, 1959) and sperm-whale myoglobin (Edmundson & Hirs, 1961)]. The protein contained no cystine, and the only cysteine residues were those forming the thioether links to the haem. When the haem peptides had been removed the remainder of the digest contained no sulphydryl groups to be protected or disulphide bridges to be broken. The difficulties that were encountered are discussed below. Most of the sequence was elucidated by enzymic methods, but the molecule contained one region with four prolyl residues in a sequence of six amino acids, and chemical methods had to be used here.

The haem peptides were separated from the remainder of the digest by adsorption on to carboxymethylcellulose, followed by elution at a higher pH. This method was better suited to the separation of small amounts of material than the isoelectric-precipitation method used by Tsou (1951*a*, *b*). The talc-adsorption method (Tuppy & Bodo, 1954*a*, *b*) was not used, because of the bad flow properties of columns of this material. It proved difficult to remove the haem from *P*-cytochrome-551. Margoliash *et al.* (1959) found that performic acid oxidation alone was adequate for horse-heart cytochrome *c*, but with the *Pseudomonas* protein this method only gave 40 % recovery of cysteic acid. The silver salt method (Paul, 1951) was also ineffectual. The mercuric chloride method was more satisfactory, and 75 % recoveries of cysteine as cysteic acid have been achieved (Ambler, 1963). The final yields of cysteic acid peptides have been low, but only 55 % recovery of a peptide containing 2 residues of cysteine/molecule would be expected if the recovery of cysteic acid after total hydrolysis was only 75 %.

The percentage yields of tryptic and chymotryptic peptides are shown in Tables 6 and 18. The yield was calculated from the analytical values for the final pure peptide, and no corrections were made for losses during purification, as only some of these could be determined. The material lost by staining marker strips for the detection of peptides after electrophoresis was large, amounting to up to a 25 % loss in a two-stage purification. Several of the tryptic peptides had recoveries of about 45 %, but this was partly fortuitous, as the number of paper steps for purification was not the same for each peptide. The recoveries of the tryptophan-containing peptides T4b and T9b/T10b were low, especially that of the former, in which the tryptophan was present as the *N*-terminal residue. The low yield of peptide T1 may have been connected with the purification by paper chromatography (see below). The yields of chymotryptic peptides were lower. In many cases this was due to the same region of the protein molecule being digested to form two or more peptides.

The subtractive phenyl isothiocyanate degradation method, as developed by Hirs, Moore & Stein (1960), could not be used, as it is both extravagant with material and requires repeated quantitative amino acid analyses. In the present investigation a simplified procedure was used to determine the sequence of di- and tri-peptides by a subtractive method. The residual peptide was repurified at each step, and only qualitative determinations were made of the compositions of the residual peptides. This method used less material than was required for the fluorodinitrobenzene method for *N*-terminal-group analysis, and additional information was gained about the remainder of the peptide. No attempt was made to isolate the phenylthiohydantoins formed. This method was not found to work for peptides that contained lysine; after the first step the residual peptide was not homogeneous, possibly because of instability of the ϵ-phenylthiocarbamate group on the lysine residue.

The only peptide found in any digest with a composition that could not be reconciled to the final sequence shown in Fig. 14 was peptide T3a (Table 6). This peptide was present in very small amounts, but showed up quite strongly on the peptide maps. It would appear that this peptide was formed from a protein contaminant of the cytochrome preparation, and was only detected because of the strong ninhydrin colour caused by the high lysine content.

Specificity of proteolytic enzymes used. In tryptic digestions of the whole protein, all the lysyl and arginyl bonds were hydrolysed. No other bonds were broken in detectable amounts. The only sensitive bond that was not completely hydrolysed was the Lys-Gly bond between residues 10 and 11 (Fig. 14).

Chymotrypsin hydrolysed completely all the bonds on the *C*-terminal side of aromatic residues. Two of the leucyl bonds (both Leu-Ala) were completely hydrolysed, and the remaining two were incompletely split. One of the two methioninyl bonds was partly hydrolysed, but the other one, occurring in the sequence -Pro-Met-Pro-Pro-, was unaffected. Two lysyl bonds were partly hydrolysed, suggesting that the trypsin contained in the chymotrypsin preparation used had not been completely inactivated by the soya-bean trypsin inhibitor. One of the three asparaginyl bonds in the molecule (that between residues 50 and 51) was also partly hydrolysed. This apparent arbitrary sensitiveness of a small proportion of asparaginyl bonds has been noted in other proteins [chymotrypsin (B. S. Hartley, personal communication); ribonuclease (Hirs *et al.* 1960); horse-heart cytochrome *c* (Margoliash, 1962)].

The number of bonds hydrolysed by pepsin was about the same as that hydrolysed by chymotrypsin. The peptic peptide map was made more complicated by the large proportion of bonds that were only partly hydrolysed. All the bonds on the *C*-terminal side of leucine residues were split rapidly. Seven out of the thirteen bonds on the *N*-terminal side of alanine residues were hydrolysed detectably.

Subtilisin B digestion produced peptides with sizes of about 2–5 residues/molecule, but did not produce many free amino acids. Elastase digestion produced peptides in the same size range, but split different bonds in forming them. Pronase digestion resulted in the production of many dipeptides and free amino acids, but larger peptides were formed from regions containing prolyl, aspartyl or glutamyl residues.

Under the conditions described, leucine aminopeptidase did not extensively degrade peptides. Glutamic acid, aspartic acid, glycine and proline were released very slowly, and their presence effectively stopped further degradation. Removal of amino acids by carboxypeptidase was also stopped when the *C*-terminal amino acid was aspartic acid or glutamic acid. There was only one exception to this generalization: carboxypeptidase released small amounts of aspartic acid, isoleucine and alanine from peptide Phb (*C*-terminal sequence -Ala-Ileu-Asp), whereas no amino acid was released from peptide Pha (*C*-terminal sequence -Ala-Ileu-Asp-Thr).

Difficulties encountered in elucidation of the sequence. The sequence investigation showed that *P*-cytochrome-551 contained 2 residues of tryptophan/molecule. Gel-filtration proved to be of great use for the study of sequences containing this amino acid, as it was found that small tryptophan-containing peptides could be separated from all other peptides by this method alone. This method was applied to peptides T4b (Fig. 5), T4bS4 (Table7), C3a and P3. Gel-filtration was also used to free tryptophan-containing peptides from ultraviolet-absorbing material with which they had become contaminated during electrophoresis in pyridine–acetate buffers. After gel-filtration, the tryptophan content could be determined from the ultraviolet-absorption spectrum of the peptide. During electrophoretic purification, it was always observed that peptides containing tryptophan were associated with fluorescent material, which had almost, but not exactly, the same mobility on electrophoresis as that of the material that reacted with ninhydrin. After electrophoretic separation at pH 3·5, peptides containing tryptophan were found to be degraded. The ultraviolet absorption at 280 mμ was very much less than the theoretical value for the amount of peptide present, and the spectrum did not have the characteristic tryptophan shape. No peptide bonds had been broken, and chymotrypsin still appeared to hydrolyse the peptide in the same way. If the peptide was degraded by exopeptidases, any tryptophan that was released was accompanied by two other compounds that had lower mobilities than that of tryptophan on electrophoresis at pH 2·0.

The amino acid analyses of some peptide preparations were not compatible with other properties of these peptides. For instance, peptide T7 had zero mobility at pH 6·5, but, in analyses of different samples of the peptide, the ratios of aspartic acid to lysine (the only amino acids in the peptide with ionizable side chains) were 0·27 and 0·33. Again, phenylalanine was released in good yields by the action of leucine aminopeptidase on peptide T9aP4, but very little of this amino acid (0·03 and 0·06 mole/mole of peptide) was detected by amino acid analysis. Inconsistent results were also observed with peptide T1, the ratios of isoleucine to lysine being (for three analyses) 0·54, 0·85 and 0·46. The common factor between all these peptide samples was that they had been prepared by electrophoresis in pyridine–acetate buffers, followed by paper chromatography. The paper chromatography, in the butan-1-ol–acetic acid–water (3:1:1, by vol.) system, had been the last step before hydrolysis and analysis. When the same samples that had given the inconsistent analyses were again separated by electrophoresis in pyridine–acetate buffer, the peptides on hydrolysis gave stoicheiometric results. As the cause of this phenomenon was not known, paper chromatography was avoided wherever there was an alternate method available for purifying peptides. Where it was used, it was always followed by an electrophoretic separation.

The electrophoretic mobilities of peptides isolated during this investigation were measured at pH 6·5, and the mobilities used as evidence for the location of amide groups. The only cases where the observed mobility was inconsistent with other evidence involved peptides that contained aspartic acid as the *N*-terminal residue.

Two cases involved aspartyl peptides formed by partial acid hydrolysis of larger peptides. In both cases, peptides T7π2 ('Asp'-Val-Ala; Table 8) and T10bC4S3π3 ('Asp'-Gly, Table 13), the peptides reacted with ninhydrin on paper to give a stable yellow colour, and had mobilities that were lower than would be expected for peptides of their composition. The hydrolysates also contained peptides of the same composition with normal mobilities and purple or blue ninhydrin colour. The aberrant peptides may have been $\alpha\beta$-aspartyl peptides, formed during acid hydrolysis (Naughton, Sanger, Hartley & Shaw, 1960). Swallow & Abraham (1958) reported that $\alpha\beta$-aspartyl iso-

hexylimide gave a yellow–brown colour with ninhydrin on paper, and described the electrophoretic mobility of this compound at pH 7. The mobility was about that for a compound of the molecular weight of $\alpha\beta$-aspartyl isohexylimide, and with about one-quarter of a net positive charge/molecule. The mobilities of peptides T7π2 and T10bC4S3π3 were consistent with their assumed molecular weights, and a net charge of about three-quarters/molecule. Peptide T11π11 (Table 15), which gave a stable pink colour with ninhydrin on paper, was also possibly a peptide of this type, $\alpha\beta$-Asp-Pro-Glu.

The spontaneous formation of peptide T10b and derivatives from peptide T9b and derivatives was mentioned in the Results section. The conversions observed are shown in Fig. 15. The N-terminal residue of all these peptides was either aspartic acid or asparagine, from dinitrophenylation studies on several of the peptides. The mobilities of the peptides of the T10b series were consistent with them containing this residue in the form of aspartic acid, though the same residue (no. 50 in Fig. 14) had been identified as being present as asparagine in the chymotryptic peptide C2. The status of this residue in the peptides of the T9b series was not determined. Leucine aminopeptidase did not release asparagine from the peptide, as would be expected were this amino acid present at the N-terminus, and the slightly acidic mobilities of peptides T9bC3, T9bC3S2 and T9bC3S2κ1 were not such as had been found for other peptides containing only monoaminomonocarboxylic acids. Leach & Lindley (1954) have reported the dissociation constants for various synthetic asparagine-containing peptides, including asparaginylglycine, which had the anomalously low pK_2 value of 7·21. This would explain the observed electrophoretic mobilities of the peptides of the series T9b, and the conversion into peptides of the peptides of the T10b series would be due to the loss of the amide group from this N-terminal asparagine residue. If this hypothesis is correct, there is still no explanation of why this particular amide group is unstable under the mild conditions of electrophoresis and elution. LeQuesne & Young (1952) reported that β-aspartyl peptides gave blue rather than purple colours with ninhydrin on paper. Attempts to degrade peptide T10bC4S3 by the phenyl isothiocyanate method were unsuccessful. These observations suggest that the peptides of the T10b series may contain some β-aspartyl peptides, which would probably not have separated from the α-aspartyl peptides during electrophoresis at pH 6·5.

Some small peptides isolated during the present investigation gave such weak colours with ninhydrin on paper that the peptides were only detected by the Reindel & Hoppé (1954) method. The peptides were C8P4, C8P5 and PhS9. All three were acid peptides with N-terminal valine or isoleucine. Peptide PhS9 (Table 5) was not detected during the initial part of the present investigation; this failure, together with the difficulties mentioned with carboxypeptidase in this region, were the reason for the erroneous report that residues 18 and 19 had the order -Asp-Ileu- (Ambler, 1962) instead of -Ileu-Asp-.

In the present investigation it was not possible, for reasons of time, material and availability of apparatus, to investigate each peptide with each of the techniques used. It was considered better to determine which of the available techniques to use for a particular peptide (after exploratory experiments), rather than to attempt to follow a more rigid approach. The aim has been to obtain evidence from two different sources for each peptide bond in the molecule, rather than to use all the available material for a single, but very precise, experiment.

Comparison of sequence with that of other cytochromes. Tuppy (1959) believed that a characteristic feature in the structure of cytochrome c was the presence of two porphyrin-linked cysteine residues, separated in the sequence by two amino acids, and with a haem-iron-bound histidine residue in the adjoining position. The sequence of P-cytochrome-551 had these features, but not others that had been noticed in the sequences of all cytochromes previously investigated. These absent features were the threonine residue at position H (Table 26; alanine

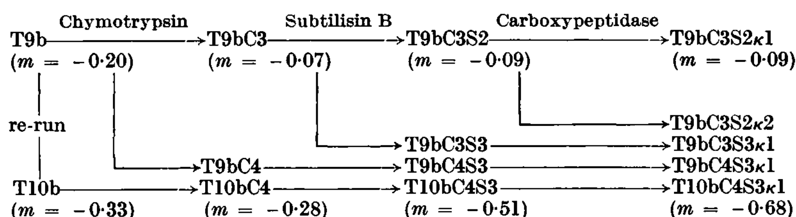


Fig. 15. Peptides formed by enzymic degradation of peptides T9b and T10b. Definition of symbols is given in the text. Only the peptides from the N-terminal part of peptides T9b/T10b are shown. Re-run: some peptide T10b was formed from peptide T9b during elution from paper or subsequent electrophoresis.

in *P*-cytochrome-551) and the basic residue at position B (glycine in *P*-cytochrome-551). They have been found to be present in one of the haem sequences of the 'RHP' from *Chromatium* spp. (Dus *et al.* 1962).

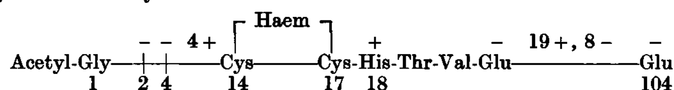
It is still not known what groups in the protein of horse-heart cytochrome *c* combine with the haem iron and cause the haemochromogen spectrum. Theorell & Åkeson (1941*a–d*) concluded that at least one of the links was from the imidazole side chain of a histidine residue. Ehrenberg & Theorell (1955) used a model to demonstrate that the cysteine residues were suitably spaced to form thioether bonds, and the histidine residue (position G; see Table 26) was in a suitable position to co-ordinate with the iron, if the peptide chain were coiled to form an α-helix. With this model the same workers showed that the basic residue at position B could not be the other haemochromogen-forming group unless the helix were extended. They also produced physicochemical evidence that a second histidine residue elsewhere in the peptide chain was the second co-ordinating group. Margoliash *et al.* (1959) believed that the occurrence of a basic residue in position B of all the *c*-type cytochromes that had been examined justified proposing the alternative hypothesis that it was this residue, together with the position G histidine, that were the haemochromogen-forming groups. Margoliash (1962) noted in support of this theory that one out of the nineteen lysyl residues in horse-heart cytochrome *c* had an unreactive ε-amino group. The spectrum of *P*-cytochrome-551 is very similar to that of mammalian cytochrome *c* (Horio *et al.* 1960), but there is only one histidine residue in the bacterial protein. The protein also does not contain a basic residue at position B, though there is a lysyl residue in the adjoining position A. This finding suggests either that the coiling of the peptide chains and hence the side chains that are in suitable positions to combine with the iron to form the haemochromogen differs between different cytochromes or that both the Theorell and the Margoliash hypotheses are incorrect.

There are few similarities between other parts of the sequences of *P*-cytochrome-551 and horse-heart cytochrome *c*. In both molecules the haem is attached to the peptide chain near the *N*-terminus of the protein, and there are similarities in charge distribution over this *N*-terminal region (Fig. 16). Margoliash (1962) observed that amino acids with hydrophobic side chains were clumped into certain sections of the sequence of horse-heart cytochrome *c*. No such clumping was detected in the *P*-cytochrome-551 sequence. There was some concentration of the acidic residues, and a region of eighteen residues (no. 50–no. 67) in which none of the amino acids had charged side chains. In this part of the molecule there was also a region of six amino acids of which four were prolyl residues

Table 26. *Amino acid sequence of haem region of* c*-type cytochromes from various sources and the 'RHP' from* Chromatium

Cytochrome *c*	Residue A B C D E F G H	Reference
Ox, horse, pig	...Val-Glu(NH$_2$)-Lys-Cys-Ala-Glu(NH$_2$)-Cys-His-Thr-Val-Glu...	Tuppy & Bodo (1954*b*, *c*)
Chicken	...Val-Glu(NH$_2$)-Lys-Cys-Ser-Glu(NH$_2$)-Cys-His-Thr(Val,Glu)...	Tuppy & Paleus (1955)
Salmon	...Val-Glu(NH$_2$)-Lys-Cys-Ala-Glu(NH$_2$)-Cys-His-Thr(Val,Glu)...	Tuppy & Paleus (1955)
Silkworm	...Val-Glu(NH$_2$)-Arg-Cys-Ala-Glu(NH$_2$)-Cys-His-Thr(Val,Glu)...	Tuppy (1957)
Baker's yeast	...Lys- Thr -Arg-Cys-Glu- Leu- -Cys-His-Thr(Val,Glu)...	Tuppy & Dus (1958)
Rhodospirillum rubrum cytochrome c_2	...(Lys)Cys-Leu Ala -Cys-His-Thr-Phe-Asp...	Paleus & Tuppy (1959)
Chromatium 'RHP'	...Ala- Gly- -Lys-Cys-Ser-Glu(NH$_2$)-Cys-His-Thr-Leu-Val... and ...Lys-Cys-His-Thr-Phe-Asp...	Dus *et al.* (1962)
Pseudomonas fluorescens cytochrome *c*-551	...Asp(NH$_2$)-Lys-Gly-Cys-Val- Ala -Cys-His-Ala-Ileu-Asp...	This investigation

(i) Horse-heart cytochrome *c*



(ii) *Pseudomonas* cytochrome *c*-551

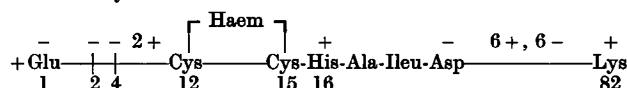


Fig. 16. Comparison of amino acid sequences of horse-heart cytochrome *c* (Margoliash *et al.* 1961) and of *Pseudomonas* cytochrome *c*-551.

(no. 58–no. 63). Williams, Clegg & Mutch (1961) have drawn attention to the dangers of giving undue emphasis to what may appear to be highly non-random sequences of amino acids.

SUMMARY

1. The complete amino acid sequence of *Pseudomonas* cytochrome *c*-551 has been determined. It consists of 82 amino acids forming a single peptide chain.

2. The haem is attached to cysteine residues in positions 12 and 15 in the protein sequence, and the only histidine residue of the protein is at position 16. The *N*-terminal residue of the protein is glutamic acid, and the *C*-terminal residue lysine.

3. There is a similarity in charge distribution between the *N*-terminal portion (residues 1–20) of *Pseudomonas* cytochrome *c*-551 and that of horse-heart cytochrome *c*. No similarities were noticed between the remainders of the molecules.

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ADDENDUM

The Structure of a Chymotryptic Peptide from *Pseudomonas* Cytochrome *c*-551

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A micromethod involving the use of fluorescent derivatives of 1-dimethylaminonaphthalene-5-sulphonyl chloride (Gray & Hartley, 1963) has been used in a parallel study of the structure of peptide C8P1a (as defined by Ambler, 1963) of the *Pseudomonas* cytochrome *c*-551. The basis of the method was a modification of the phenyl isothiocyanate procedure for the removal of the *N*-terminal residue (Edman, 1950*a*, *b*), followed by investigation of the free amino groups of the residual peptide The very high sensitivity of the fluorescent end-group reagent used enabled the subtractive procedure to be applied over six consecutive steps, commencing with approx. 0·02 μmole of peptide. The partial sequence

Gly(Pro$_2$,Ileu)-Met-Pro-Pro-Asp(NH$_2$)-Ala

had already been determined by cyanogen bromide cleavage and partial acid hydrolysis (Ambler, 1963).

The peptide (0·02 μmole) was oxidized with performic acid, to convert methionine into its sulphone. The oxidized peptide is referred to below as CPO, and in subsequent stages of degradation as CPOψ1, CPOψ2, according to the convention adopted by Ambler (1963). Interaction of peptides with the sulphonyl chloride attaches the fluorescent dimethylaminonaphthalenesulphonyl residue to free amino groups.

Abbreviations. The 1-dimethylaminonaphthalene-5-sulphonyl derivatives are indicated by the term DNS-.

Phenyl isothiocyanate degradation. The oxidized peptide (CPO) was dissolved in 0·1 ml. of water in a 5 ml. stoppered tube, and 10 μl. was removed for end-group studies. To the remainder was added 0·2 ml. of a solution of phenyl isothiocyanate in pyridine (5 %, v/v). The resulting single-phase mixture was incubated overnight at room temperature, and, after the addition of 0·2 ml. of water, the pyridine and excess of phenyl isothiocyanate were removed by four extractions with 3 ml. of benzene. The aqueous solution was then freeze-dried *in situ*, and 0·2 ml. of acetic acid saturated with anhydrous hydrogen chloride was added. After 30 min. at room temperature, the acid was removed *in vacuo* and the residual peptide dissolved in 0·1 ml. of water. A portion corresponding to one-tenth of the original material was removed for end-group studies, and the remainder resubmitted to the degradation procedure. At each successive cycle a similar portion was removed.

N-*Terminal studies.* Samples from each cycle of the phenyl isothiocyanate degradation, corresponding to approx. 2 μm-moles, were placed in small fermentation tubes containing 10 μl. of 0·1 M-sodium hydrogen carbonate in ammonia-free water. To each of these was added 15 μl. of a 1 % (w/v) solution of 1-dimethylaminonaphthalene-5-sulphonyl chloride in acetone, forming a single-phase reaction mixture. After 2 hr. at room temperature, during which time all excess of reagent had been hydrolysed to sulphonic acid, the mixture was applied as a 2 cm. band to a strip of Whatman no. 3 MM paper. High-voltage ionophoresis was then carried out for 1·5 hr. at 120 v/cm., with the apparatus described by Gross (1961). The buffer used was 10 % (v/v) of pyridine in 0·4 % acetic acid, pH 6·5, in which all of the DNS-peptides move towards the anode. The labelled peptides were located by their yellow–green fluorescence under an ultraviolet lamp, and their mobilities measured relative to that of 1-dimethylaminonaphthalene-5-sulphonic acid, which has a blue fluorescence on paper. The true origin is indicated by the position of the sulphonamide, which is formed from ammonia contained in the reagents and the sulphonyl chloride, and which is neutral at this pH. The DNS-peptides were eluted with 6 N-hydrochloric acid directly into capillary tubes, which were then sealed and heated at 105° overnight. The hydrolysates were dried *in vacuo*, and the *N*-terminal DNS-amino acid residues identified by high-voltage ionophoresis at pH 4·40 (0·4 % of pyridine in 0·8 % acetic acid) and at 80 v/cm. for 2½ hr. (Gray & Hartley, 1963).

CPO, after the labelling, gave a single band of DNS-CPO, having a mobility of 0·42 relative to that of the sulphonic acid. After the first and second cleavages, single bands were again obtained, the mobilities of DNS-CPOψ1 and DNS-CPOψ2 being 0·43 and 0·47 respectively. At the third stage two bands were obtained, one having a mobility identical with that of DNS-CPOψ2, the

other moving faster (mobility 0·52). This suggested incomplete cleavage at the second stage, resulting in the production of CPOψ2 together with CPOψ3 at the third stage. Cleavage appeared to be complete at all subsequent stages, although two peptides were always obtained, owing to the incomplete cleavage of the second stage. The DNS-CPOψ4 (mobility 0·56) and DNS-CPOψ5 (mobility 0·78) were obtained in reasonable yield. The DNS-CPOψ6 was identified as a very weak band (mobility 0·94), moving in the tail of the dimethylaminonaphthalenesulphonic acid band. Subsequent stages were unsuccessful, since labelling was inhibited by the presence of an accumulation of oxidized pyridine products.

After hydrolysis of the bands, each gave rise to a single fluorescent spot at pH 4·40, corresponding to the *N*-terminus of the peptide. In order, the residues identified were DNS-Gly, DNS-Pro, DNS-Ileu, DNS-Pro, DNS-MetSO$_2$ and DNS-Pro. DNS-CPOψ6 did not give an identifiable product. Thus the *N*-terminal sequence was established as Gly-Pro-Ileu-Pro-Met-Pro-, in complete agreement with the conclusions reached by the larger-scale methods used by Ambler (1963).

Generally it is not necessary to purify the DNS-peptides before hydrolysis, and this may be undesirable when only very small amounts (1 μmole) are available. Here the large number of proline residues present made it desirable to obtain additional information, such as extent of cleavage and mobilities of the DNS-peptides. These mobilities were inversely proportional ($\pm 5\%$) to the molecular weights of the DNS-peptides, so that useful confirmation of the proposed structures was obtained in this way.

SUMMARY

1. A micro-technique for stepwise sequential analysis of small quantities of peptide is outlined.

2. By applying this method to 0·02 μmole of a chymotryptic peptide from *Pseudomonas* cytochrome *c*-551, the *N*-terminal sequence

Gly-Pro-Ileu-Pro-Met-Pro-

was determined.

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The Sensitivity of the Neuraminosidic Linkage in Mucosubstances towards Acid and towards Neuraminidase

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Neuraminic acid may readily be split from substances containing it either with 0·1 N acid at 80° or with the enzyme neuraminidase. The rate and extent of hydrolysis may conveniently be followed by means of the thiobarbituric acid reaction (Warren, 1959; Aminoff, 1961), which estimates only the free acid. Generally, acidic hydrolysis is rapid; after about 1 hr. it is maximal and has usually been assumed to be quantitative. Likewise the liberation of neuraminic acid by neuraminidase is often found to go essentially to completion, though in some substances, particularly those from epithelial mucus, there is some residual neuraminic acid relatively resistant to this enzyme (Warren & Spicer, 1960).

This investigation originated with the observation that bovine cervical mucopolysaccharide behaved anomalously on hydrolysis in 0·1 N acid at 80°; the shape of the hydrolysis curve (amount of neuraminic acid estimated by thiobarbituric acid against time) was not that predicted for the second member of a sequential series of first-order irreversible reactions; the time of maximal hydrolysis was 2·5 hr., and the maximum amount released, as estimated by thiobarbituric acid with *N*-acetylneuraminic acid as standard, was only 65 % of the amount estimated by the direct Ehrlich's reaction (Werner & Odin, 1952). Furthermore, only an apparent 38 % of the total neuraminic acid was removed at all readily with neuraminidase.