Isolation and Amino Acid Composition of Chymotryptic Peptides from Horse Heart Cytochrome c*

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Mammalian cytochrome c is a protein of low molecular weight (approximately 25000) containing one heme per mole of protein. Through the efforts of many investigators, our knowledge of its biological function and over-all physicochemical properties is well documented (see reviews by Paul (4), Keilin and Slater (5), and George and Lyster (6)); however, there is relatively little information as yet concerning the structure of this protein. The only section of the amino acid sequence that has been reported is the portion immediately adjacent to the heme (7-9). For horse heart cytochrome c this sequence (10) is

Val-GluNH₂-Lys-Cys-Ala-GluNH₂-Cys-His-Thr-Val-Glu-Lys

The prosthetic group is covalently linked to the protein by thioether bonds from the two cysteinyl residues in this sequence to the vinyl side chains of the heme (11-13). This “hemopeptide” or “core” contains approximately 10% of the amino acid residues of the entire protein.

Studies of such cytochrome c “cores” have led to various suggestions as to the basic groups in the protein that coordinate with the central iron atom in the heme (hemochromogen-forming groups) as well as of the relationship of the “core” to the remainder of the protein (14-16). Moreover, it has been demonstrated that the properties of the entire molecule in its native state are involved in conferring to the prosthetic group the particular behavior required to make it biologically active (15). It is evident that a precise understanding of the behavior of cytochrome c, as well as of its reactions with other units of the terminal oxidation chain, must be based on knowledge of its three-dimensional structure. A study of the amino acid sequence was undertaken as a first step toward the elucidation of the complete structure.

The composition of horse heart cytochrome c (17) as determined in this laboratory (17) is: Try, Lys₂, His, Arg₂, Asp₂, (AspNH₂)₂, Thr₁, Glu₂, (GluNH₂)₃, Pro₁, Gly₂, Ala₁, Cys₂, His₁, Val₁, Met₁, Ile₁, Leu₁, Phe₁, Tyr₁.

This paper presents the isolation and amino acid composition of the peptides obtained by hydrolyzing horse heart cytochrome c with chymotrypsin. Preliminary reports of part of this work have appeared (18, 19).

EXPERIMENTAL PROCEDURE

Digestion of Cytochrome c by Chymotrypsin—Ethanol-denatured horse heart cytochrome c (3) (100 μmoles) was dissolved in 120 ml of deionized water and adjusted to pH 7.8. A three times crystallized α-chymotrypsin preparation (2% by weight) (Worthington Biochemical Corporation) was added. This preparation was free from tryptic activity in tests that would have detected less than 0.05% contamination, as judged with benzoyl-L-argininamide as substrate and the alcohol titration method of Grassmann and Heyde (20). The hydrolysis was performed at room temperature (22°), and the reaction mixture was kept at pH 7.8 by continuous titration with 2.16 N NH₄OH with the aid of a recording Radiometer pH-Stat. Further additions of 2% chymotrypsin were made at 9 and 20 hours. The digestion was stopped at 29 hours by freezing the reaction mixture and storing it at -20°. An aliquot of the digestion mixture was titrated between pH 6.0 and 10.5 with 10 mM NaOH. From this titration curve and the amount of base added during the digestion, the number of peptide bonds hydrolyzed could be estimated.

Peptide maps (21) (see below) of the total chymotryptic digest were obtained, and the peptides containing some of the amino acids for which specific color reactions are available (histidine, arginine, tyrosine, tryptophan, and methionine) were located by techniques cited below.

Column Chromatographic Separation of Peptides—The chymotryptic digest of cytochrome c was chromatographed on a column (3.7 x 150 cm) of Dowex 50-X2 (Dow Chemical Company) at 40° at a flow rate of 220 ml per hour. Fractions of 20 ml were collected with the aid of a Technicon fraction collector. The flow rate was maintained constant by a constant volume delivery pump (Mini-pump, Milton Roy Company, Philadelphia). The resin was prepared and sized essentially as described by Moore, Spackman, and Stein (22). The mesh size was finer than 400 mesh. The detailed procedure for this chromatographic method is described elsewhere (23).

The buffers used for elution were 0.2 M pyridine-acetic acid at pH 3.1 (80.5 ml of pyridine and 1395 ml of glacial acetic acid diluted to 5 liters) and 2.0 M pyridine-acetic acid at pH 5.0 (805 ml of pyridine and 716 ml of acetic acid diluted to 5 liters). The resin was equilibrated with the 0.2 M buffer, and after it was packed under pressure on the column, it was washed for 4 days.
with the same buffer before the peptide mixture was applied. Column volume was approximately 1 liter. Thirteen column volumes of the 0.2 M pH 3.1 buffer were passed, and then a linear gradient of ionic strength and pH was established between the more dilute and the more concentrated buffers. pH 5.0 was reached after a further 23 column volumes had been passed. The eluting solution was then changed to 3.0 M trimethylamine, and 2.5 column volumes were passed. This removed only a fraction of the heme-containing peptide, the bulk of which remained adsorbed to the top of the column. It could not be eluted except with 1 N NaOH. Since under these conditions one would expect drastic chemical changes, such as peptide bond hydrolysis and amino acid racemization, the main heme peptide obtained from chymotryptic digests of cytochrome c was prepared in separate experiments described below.

Elution of the peptides was followed by the ninhydrin method both directly and after alkaline hydrolysis as described by Moore and Stein (24) and by Hirs, Moore, and Stein (25). Aliquots of 500 μl, taken from every third tube, were examined. In those fractions containing trimethylamine, only the ninhydrin reaction after alkaline hydrolysis could be used, since the trimethylamine itself gave high blank values.

Appropriate fractions under each chromatographic peak were pooled, taken to dryness in a rotary evaporator at 40°C, transferred to vials, lyophilized, and stored at −20°C. This procedure resulted in fractions that were essentially free from nonpeptide material.

It is important to note that in many cases fractions were pooled and processed although they were not under a distinct chromatographic peak. This permitted the recovery of some peptides in low yield, the composition and partial sequence of which showed clearly that they represented overlaps of two or more other peptides obtained in high yields. If these or similar experiments are to be repeated, the authors recommend the appropriate pooling of all fractions collected between distinct chromatographic peaks and their further examination before the ones containing peptides in yields considered to be too low to be useful are rejected.

Further Purification of Peptides—Each of the 37 fractions obtained by column chromatography was examined on paper by electrophoresis in one direction, followed by chromatography in the other by the procedure of Ingram (21), as used in this laboratory (23). Aliquots of 0.05 to 0.5% of the total material were used. For electrophoresis a pyridine-acetic acid buffer, pH 0.4, was employed (26) at a potential of 16 volts per cm for 2½ hours. The solvent routinely used for the descending paper chromatography was a freshly prepared n-butanol-glacial acetic acid-water (200:30:75 by volume) mixture, and it was run for 18 hours at room temperature. The electrophoretic and chromatographic movement of all peptides was examined under these standard conditions. In some cases a mixture containing n-butanol-pyridine-glacial acetic acid-water (600:400:120:480 by volume) was used for the preparative separation of peptides.

The peptide maps were developed with ninhydrin spray (0.1% ninhydrin in 95% ethanol-glacial acetic acid-collidine (50:15:2 by volume)) and in some cases with reagents for histidine (27), arginine (28), tyrosine (29), tryptophan (30), and sulfur-containing amino acids (31). In most cases the peptide maps permitted a judgment as to which peptides were present in the highest yield in the fraction examined and which peptides contained the amino acids detected by specific reagents. It was also possible to estimate the overall charge of each peptide, to identify which peptide in each fraction corresponded to particular peptides first observed in the peptide map of the original unfractioned enzymic hydrolysate, and, most important, to decide what procedure or combination of procedures would be best for the final purification of each peptide.

After these indications, the important components of each fraction were purified by unidimensional ionophoresis or electrophoresis or by successive use of these techniques on Whatman No. 3MM paper and the solvents described above. A quantity of 2 to 8 μmoles of peptide could be loaded on a single sheet of paper (22½ × 18½ inches). The more complex the mixture and the more difficult the separation of the components by the procedures employed, the less peptide was put on each sheet of paper. After each strip, a center guide strip was cut in the paper to locate the peptides with 0.1% ninhydrin or with specific reagents. Appropriate areas were cut out, and the peptides were eluted with glass-distilled water over periods of at least 24 hours by collecting 20 to 40 ml from each strip. The eluates were lyophilized. In some cases repeated separations were employed, but in no case were more than three steps necessary. When the separations were not clear cut, intermediate strips containing the overlapping components were repurified or discarded.

Most peptides used for amino acid sequence studies were purified to the point where they contained less than 5 to 10% impurity in terms of the stoichiometry of the component amino acids.

Amino Acid Composition of Peptides—A 0.3 to 10% aliquot of each purified peptide was hydrolyzed at 110°C with 5 ml of three times glass-distilled 6 N HCl in sealed tubes in a vacuum for 40 hours. The hydrolysates were taken to dryness in a rotary evaporator at 40°C, and the evaporation was repeated twice after addition of 15 ml of glass-distilled water. The hydrolysate was dissloved in 2.5 ml of 0.2 N sodium citrate buffer at pH 2.1. By applying aliquots of 1.0 ml to each column, the composition was determined on the Spinco automatic amino acid analyzer (32). Peptides containing tryptophan were hydrolyzed to amino acids with leucine aminopeptidase (33). The digestion mixture contained 0.5 μmole of peptide, 10 μl of 0.5 M Tris-HCl buffer at pH 8.5, 10 μl of 25 mm MgCl₂, and 20 μl of leucine aminopeptidase (10 mg per ml, C₁ = 35 to 40) in a total volume of 250 μl. The digestion mixture was adjusted to pH 8.5, and the hydrolysis ran for 18 hours at 40°C. The mixture was diluted to 2.5 ml with the pH 2.1 citrate buffer and analyzed.

Yields were calculated from the analyses of the purified peptides. Thus these figures represent only minimal yields, in some cases possibly as much as 20 to 30% below the actual yields, since variable losses were incurred during the purification procedures on paper as well as from the incorrect pooling of the fractions collected in the original column chromatography.

Nomenclature—Peptides are designated in Roman numerals corresponding to the column chromatographic fraction from which they were prepared, with letters after these numbers to distinguish different peptides obtained from the same fraction. The "C" placed before the fraction number indicates that the peptide was obtained from a chymotryptic digest.

The symbol, ε, will be used to indicate the electrophoretic movement on paper given in centimeters, + referring to mobility toward the anode, −, toward the cathode, and 0 indicating no electrophoretic movement. Similarly, the symbol, χ, will in-
Heme Peptides from Chymotryptic Digest of Cytochrome c—The digestion of ethanol denatured cytochrome c (100 µmole) with chymotrypsin was performed at room temperature and at pH 7.8 for 9 hours with only a single addition of chymotrypsin (4% by weight).

Amberlite IRC-50 (100 to 200 mesh) (Rohm and Haas Company, Philadelphia) was cycled four times with 2 N NaOH and 2 N HCl and washed eight times with acetone during the second Pi cycle. The final Pi form was converted to the NH4 form (2 x NH4OH), washed eight times with water, and equilibrated with a pH 8.4 ammonium acetate (0.267 M)-ammonium hydroxide (0.01 M) buffer. Deionized water was used throughout. The total digestion mixture was adsorbed onto a column of this resin (4.3 X 60 cm) and developed with the same buffer. A small heme colored band traveled rapidly down the column and was discarded. The main heme colored band moved slowly, and some heme colored material remained at the top of the column. When the main band had moved 20 to 25 cm, the eluting solution was changed to a pH 9.5 buffer (0.3 M ammonium acetate-0.19 M ammonium hydroxide), and only the colored eluate was collected in a volume of 1.4 liters. This solution was dialyzed in cellophane tubing (4 X 10 liters of water) for 3 days, concentrated to 100 ml in a rotary evaporator, dialyzed overnight, and lyophilized after filtering off a fine precipitate; yield, 215 mg. A spectrophotometric estimate of the molecular weight of this material, with an ε₉₅ of 27.7 (550 µµ, pH 11.0, reduced form) (15), gave a value of approximately 4000, corresponding to an average chain length of 29 to 30 amino acids, in addition to the heme.

Amino acid analyses of the product (see Table III) showed that this peptide was impure. Several attempts at further purification by using a cation exchanger for chromatography, paper chromatography, and paper electrophoresis did not yield significantly purer products, as judged by amino acid analysis. The preparation was therefore used for tryptic digestion as follows: 12.2 µmole were dissolved in 10 ml of water by adding the minimal quantity of 1 M NH₄OH, the solution was neutralized to pH 7.8, 5% by weight of twice crystallized trypsin (Worthington Biochemical Corporation) was added, and digestion was allowed to proceed at room temperature for 5 hours, the pH being kept constant by continuous titration (0.033 M NH₄OH) with a Radiometer pH-Stat. The digest was lyophilized.

Aliquots (0.1 to 0.2 µmole) of the digest were subjected to paper electrophoresis in one direction, followed by paper chromatography in the other (21) as described above. Peptides were located (see Fig. 4) by spraying with 0.01% ninhydrin in 95% ethanol, followed by color development at room temperature. The appropriate areas were cut out, the paper was washed three times with acetone to remove excess ninhydrin, and the peptides were eluted with water containing 0.05% (by volume) glacial acetic acid. Eluates from corresponding areas of 15 peptide maps were pooled and lyophilized. Aliquots of each peptide were hydrolyzed with 6 N HCl and analyzed as described above. Because of the numerous manipulations and the losses due to incomplete elution from the paper, it was impossible to calculate yields for the tryptic peptides. The account presented below (see “Results”) is therefore restricted to those peptides recovered in amounts sufficient to give unequivocal amino acid analyses and that presumably represent the main peptides released by tryptic digestion.

Identification of the amino-terminal residue of the heme peptide preparation by the diisopropylfluorophosphoryl procedure of Sanger and Thompson (34) and digestion with carboxypeptidase for the identification of the carboxyl-terminal residue were performed as described in the following paper (35).

RESULTS

Hydrolysis of Cytochrome c with Chymotrypsin—Fig. 1 shows the digestion curve of ethanol-denatured cytochrome c with chymotrypsin. It should be noted that the digestion was not allowed to go to completion and that hydrolysis was still proceeding slowly even after 29 hours. Moreover, after each addition of fresh chymotrypsin the rate of hydrolysis increased for a short period. When the digestion was stopped, approximately 16 peptide bonds had been hydrolyzed of an estimated 17 to be expected (Try, Met, Leu, Tyr, Phe) (17). An additional chymotrypsin susceptible band was subsequently found at an asparagine residue (see the following paper (35)) so that the total hydrolysis obtained was no more than 88% complete.

Column Chromatography—Fig. 2 shows the elution pattern of peptides from the chymotryptic digest of cytochrome c and indicates the portions of the eluate that were combined into the fractions used for the purification of the peptides described below. With the possible exception of Fraction XIX, none of the peaks appeared to be symmetrical; this suggested that each fraction contained more than one component. Early peaks, such as those separated into Fractions XII, XIII, and XIV, tended to be broad and proved to be complex mixtures of di- and tripeptides. Intermediate peaks were sharper and relatively well separated, whereas peaks that emerged late contained mainly larger peptides having several positively charged groups and were eluted by more than one column volume of solution.

Purification of Fractions—The following is a summary of the procedures used for the purification of peptides. These are listed in the order of emergence of the fractions from the column. All fractionations were done on paper, and, unless otherwise stated, the ionophoresis and chromatography were performed under the standard conditions described above.

Fraction I: one neutral component, Peptide C-Ia (el, 0; ch, 21 cm; + reaction for methionine), was prepared by chromatography.

Fraction II: one neutral component, Peptide C-IIa (el, 0; ch, 21 cm; + reaction for methionine), was prepared by chromatography.

Fraction III: one neutral component, Peptide C-IIIa (el, 0; ch, 21 cm; + reaction for methionine), was prepared by chromatography.

Fraction IV: one neutral component, Peptide C-IVa (el, 0; ch, 25.5 cm), was prepared by chromatography.

Fraction V: one neutral component, Peptide C-Va (el, 0; ch, 16 cm; initially yellow with ninhydrin), was prepared by chromatography.

Fraction VI: one neutral component, Peptide C-VIa (el, 0; ch, 40 cm), was prepared by chromatography.

Fraction XII: two peptides were prepared by chromatography. Peptide C-XIIa (el, -6 cm; ch, 19 cm; + reaction for tyrosine) was acidic, whereas Peptide C-XIIb (el, 0; ch, 30.5 cm; initially yellow with ninhydrin) was neutral.

Fraction XIII: four neutral peptides and one acidic peptide were isolated from this fraction. One of the neutral peptides and the acidic peptide were identical with the two peptides prepared from Fraction XII, whereas another neutral peptide was identical with Peptide C-XIVa. Since the main yields of the aforementioned three peptides were obtained from the contig-
FIG. 1. Digestion curve of ethanol-denatured horse heart cytochrome c with chymotrypsin. Cytochrome c (1.2 g, dry weight) in 100 ml of water at pH 7.8 was digested with 3 X 24 mg of chymotrypsin. The pH was kept at 7.8 by the use of an automatic titration apparatus. The method of calculating the number of peptide bonds hydrolyzed is described in the text.

FIG. 2. Elution pattern of peptides from a chymotryptic digest of horse heart cytochrome c. Digest (100 μmoles) was chromatographed on a column of Dowex 50-X2 (3.7 X 150 cm) with pyridine-acetic acid buffers. The details are described in the text. The solid lines on the abscissa indicate the fractions pooled from which the peptides described in this paper were prepared.

uous fractions, these will not be discussed further under the present heading. Fraction XIII was separated by electrophoresis into a neutral and an acidic band, and the eluted material from each band was chromatographed, yielding the following peptides: Peptide C-XIIIa (el, 0; ch, 19.5 cm; initially yellow with ninhydrin, + reaction for tyrosine) and Peptide C-XIIIb (el, 0; ch, 27.5 cm; + reaction for tyrosine).

Fraction XIV: neutral Peptide C-XIVa (el, 0; ch, 37 cm) was prepared by chromatography.

Fraction XV: neutral Peptide C-XVa (el, 0; ch, 5 cm) and the
acetic Peptide C-XXVI (el, +5.5 cm; ch, 6 cm) were isolated by electrophoresis.

Fraction XVII: two acidic peptides were separated from numerous impurities by isolating electrophoretically the acidic band, which was then chromatographed to yield Peptide C-XXVIIa (el, +5.5 cm; ch, 12 cm) and Peptide C-XXVIIb (el, +4.5 cm; ch, 14 cm; + reaction for methionine). An intermediate strip of the chromatogram containing both of these peptides was discarded. The main component, Peptide C-XXVIIb, was purified further by an additional electrophoretic separation under the same conditions.

Fraction XVIII: one acidic and one basic peptide were purified by electrophoresis, followed by chromatography of the acidic and basic bands. The pure peptides obtained were Peptide C-XXVIIIa (el, -5 cm; ch, 14 cm; + reaction for methionine) and Peptide C-XXVIIIb (el, +5.5 cm; ch, 13.5 cm; + reactions for methionine and tyrosine).

Fraction XIX: neutral Peptide C-XIXa (el, 0; ch, 8.5 cm) was prepared by chromatography. Fraction XX: this fraction contained numerous peptides in very low yield. A single neutral peptide, Peptide C-XXa (el, 0; ch, 3.5 cm; + reaction for histidine) was prepared by electrophoresis followed by chromatography of the neutral band with the butanol-pyridine-acetic acid-water solvent. With this solvent, Peptide C-XXa moved 23 cm in 34 hours.

Fraction XXI: basic Peptide C-XXIa (el, -4.5 cm; ch, 6 cm; + reaction for histidine) was purified by electrophoresis. Fraction XXII: two basic components, Peptide C-XXIIa (el, -2.5 cm; ch, 4 cm) and Peptide C-XXIIb (el, -2.5 cm; ch, 5 cm; + reaction for tyrosine), were prepared by chromatography with the butanol-pyridine-acetic acid-water solvent. With this solvent, Peptide C-XXIIa moved 9.5 cm and Peptide C-XXIIb moved 16 cm in 60 hours. An intermediate area containing both components was discarded.

Fraction XXIV: basic Peptide C-XXIVa (el, -2.5 cm; ch, 7 cm; + reaction for tyrosine) was purified by chromatography. Fraction XXV: two basic components, Peptide C-XXVa (el, -4.5 cm; ch, 10.5 cm; + reaction for histidine) and Peptide C-XXVb (el, -4.5 cm; ch, 10.4 cm; + reaction for tryptophan), were separated by a 40-hour chromatography. With this prolonged chromatography, Peptide C-XXVa moved 22 cm and Peptide C-XXVb moved 33 cm.

Fraction XXVI: Peptide C-XXVIa (el, -6.5 cm; ch, 3 cm; initially yellow with ninhydrin; + reaction for arginine) and neutral Peptide C-XXVIb (el, 0; ch, 2 cm; + reactions for arginine and tyrosine) were separated by electrophoresis. Peptide C-XXVIb was further purified by chromatography with the butanol-pyridine-acetic acid-water solvent for 17 hours. Under these conditions it moved 16 cm.

Fraction XXVII: two basic components, Peptide C-XXVIIa (el, -5.5 cm; ch, 4.5 cm; + reaction for arginine) and Peptide C-XXVIIb (el, -14 cm; ch, 1 cm), were separated by electrophoresis. Peptide C-XXVIIb was further purified by chromatography with the butanol-pyridine-acetic acid-water solvent for 38 hours. Under these conditions it moved 8 cm.

Fraction XXVIII: basic Peptide C-XXVIIIa (el, -3.5 cm; ch, 3.5 cm; + reactions for arginine and tyrosine) was isolated from this fraction by electrophoresis, followed by chromatography with the butanol-pyridine-acetic acid-water solvent. With this solvent the peptide moved 18 cm in 38 hours.

Fraction XXVII: this fraction contained the heme peptide which was eluted from the chromatographic column with 3 M trimethylamine. It was dialyzed for 3 days against six changes of water and lyophilized. However, since the yield of this peptide was only 2% and the main heme peptide present in chymotryptic digest of cytochrome C was prepared separately by entirely different procedures, it will not be considered further.

In summary, none of the fractions prepared by column chromatography contained a single peptide component. Even fractions that contained mainly one peptide in high yield (see Table I) also contained small amounts of other peptides, which were readily detected on the peptide maps, although they could not be prepared in significant yield. Thus, at least one further purification step was required in every case. Of the 37 fractions collected by the column chromatography, 20 eventually were used for the preparation of peptides. Of the latter, only one peptide in yields above 25% was prepared from each of four fractions, one fraction contained two peptides in high yields, seven fractions gave one peptide in yields above 25% as well as one or more peptides in lower yields, and from eight fractions only peptides in low yields could be prepared.

Amino Acid Composition of Purified Peptides—Tables I and II list the amino acid composition of the peptides obtained in yields of more than 25% and those obtained in lower yields, respectively.

With the exception of Peptide C-XIXa (Table I), the amino acid composition of all of these peptides is compatible with their electrophoretic movement on paper given in the previous section. Peptide C-XIXa, which contains 3 lysyl residues and 1 residue each of glutamic acid and aspartic acid, behaves as a neutral compound at pH 6.4. Similarly, insofar as one can judge from the known chromatographic mobility of free amino acids, the paper chromatographic movement of these peptides is compatible with their composition. Of the peptides listed in Table I, all but two contain residues at which chymotrypsin is known to cleave peptide bonds, e.g., tryptophan, leucine, tyrosine, phenylalanine, and methionine. The exceptions are Peptides C-XV and C-XXIa. One of these probably represents the carboxyl-terminal sequence of the protein. Of the peptides listed in Table II, only C-XVb and C-XXVIIb do not contain amino acids forming peptide bonds susceptible to chymotryptic hydrolysis. One of these, Peptide C-XVb, was identical in composition with Peptide C-XVa. Peptide C-XVb contained 3 aspartic acid residues and 1 lysine; since it behaved electrophoretically as a neutral compound, 2 of the aspartic acid residues must have been in the form of the amide. Peptide C-XVb was acidic and in much lower yield than C-XVa. Therefore, in all probability, it has the same sequence in the protein, one or both of the asparagine residues having been deamidated during the course of the preparation. Evidence that the deamination had probably occurred before the column chromatography of the peptides was afforded by the shape of chromatographic peak XV from which these peptides were prepared. This peak showed a distinct hump (see Fig. 2), which was apparently due to Peptide C-XVb, since Fraction C-XV did not contain any components detectable on the peptide map other than the two peptides prepared from it.

Because of their unique composition, a number of the peptides in small yield arc clearly fragments of peptides obtained in higher yield. Thus C-IIIa, free methionine (Table II), was probably derived from Peptide C-XVIIb, and C-Ia, free leucine, from Peptide C-XXIVa. Peptide C-VIIa has the com-
### Table I

**Amino acid composition of peptides obtained in yields above 25%**

The composition of each peptide is given as the molar ratios of the amino acids calculated without correction for destruction during acid hydrolysis or for the presence of impurities. In the case of Peptide C-XXVb, tryptophan was present as judged by qualitative test, and analyses of both an acid and an enzyme hydrolysate were performed. Values for major constituents are given in bold-face type, and the assumed number of residues is given in parentheses. Values for residues present of less than 0.01 mole are omitted.

The peptides are numbered as in the text.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>C-XIIa</th>
<th>C-XIIa</th>
<th>C-XIIIa</th>
<th>C-XIVa</th>
<th>C-XVIIa</th>
<th>C-XVIIIa</th>
<th>C-XIXa</th>
<th>C-XXa</th>
<th>C-XXIa</th>
<th>C-XXIIa</th>
<th>C-XXIIIa</th>
<th>C-XXIVa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan</td>
<td>0.94(1)</td>
<td>0.97(1)</td>
<td>1.08(1)</td>
<td>3.00(3)</td>
<td>1.00(1)</td>
<td>2.23(2)</td>
<td>2.30(2)</td>
<td>0.96(1)</td>
<td>1.05(1)</td>
<td>0.88(1)</td>
<td>0.92(1)</td>
<td>1.05(1)</td>
</tr>
<tr>
<td>Lysine</td>
<td>3.03(3)</td>
<td>1.00(1)</td>
<td>0.98(1)</td>
<td>0.93(1)</td>
<td>0.86(1)</td>
<td>1.00(1)</td>
<td>0.99(1)</td>
<td>1.05(1)</td>
<td>1.04(1)</td>
<td>0.97(1)</td>
<td>1.06(1)</td>
<td>0.06</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.04(1)</td>
<td>2.17(2)</td>
<td>1.08(1)</td>
<td>0.99(1)</td>
<td>0.90(1)</td>
<td>0.07</td>
<td>0.07</td>
<td>0.11</td>
<td>0.11</td>
<td>0.07</td>
<td>0.07</td>
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</tr>
<tr>
<td>Arginine</td>
<td>1.02(1)</td>
<td>1.00(1)</td>
<td>2.05(2)</td>
<td>2.05(2)</td>
<td>0.22</td>
<td>0.08</td>
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<tr>
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<td>0.06</td>
<td>1.08(1)</td>
<td>2.05(2)</td>
<td>0.90(1)</td>
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<tr>
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<td>1.00(1)</td>
<td>1.00(1)</td>
<td>0.96(1)</td>
<td>0.96(1)</td>
<td>0.96(1)</td>
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<tr>
<td>Glutamic acid</td>
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<td>1.00(1)</td>
<td>0.97(1)</td>
<td>1.00(1)</td>
<td>0.04</td>
<td>0.07</td>
<td>0.07</td>
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</tr>
<tr>
<td>Proline</td>
<td>0.96(1)</td>
<td>1.00(1)</td>
<td>0.97(1)</td>
<td>1.00(1)</td>
<td>0.04</td>
<td>0.07</td>
<td>0.07</td>
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<td>Glycine</td>
<td>1.01(1)</td>
<td>1.00(1)</td>
<td>1.00(1)</td>
<td>1.00(1)</td>
<td>0.04</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
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<tr>
<td>Alanine</td>
<td>0.96(1)</td>
<td>1.00(1)</td>
<td>0.96(1)</td>
<td>1.00(1)</td>
<td>0.04</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
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</tr>
<tr>
<td>Valine</td>
<td>0.93(1)</td>
<td>0.07</td>
<td>1.02(1)</td>
<td>1.04(1)</td>
<td>1.00(1)</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
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<tr>
<td>Methionine</td>
<td>0.97(1)</td>
<td>0.06</td>
<td>1.08(1)</td>
<td>2.05(2)</td>
<td>0.22</td>
<td>0.08</td>
<td>0.08</td>
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<td>0.08</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.05(1)</td>
<td>1.00(1)</td>
<td>1.00(1)</td>
<td>1.00(1)</td>
<td>0.04</td>
<td>0.07</td>
<td>0.07</td>
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<td>0.07</td>
<td>0.07</td>
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</tr>
<tr>
<td>Leucine</td>
<td>0.96(1)</td>
<td>1.00(1)</td>
<td>1.00(1)</td>
<td>1.00(1)</td>
<td>0.04</td>
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<td>0.07</td>
<td>0.07</td>
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</tr>
<tr>
<td>Tyrosine</td>
<td>1.00(1)</td>
<td>1.00(1)</td>
<td>1.00(1)</td>
<td>1.00(1)</td>
<td>0.04</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.01(1)</td>
<td>1.00(1)</td>
<td>1.00(1)</td>
<td>1.00(1)</td>
<td>0.04</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>Total residues</td>
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<td>3</td>
<td>2</td>
<td>2</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>10</td>
<td>7</td>
<td>6</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Yield</td>
<td>35%</td>
<td>39%</td>
<td>38%</td>
<td>33%</td>
<td>35%</td>
<td>27%</td>
<td>47%</td>
<td>94%</td>
<td>52%</td>
<td>44%</td>
<td>49%</td>
<td>34%</td>
</tr>
</tbody>
</table>

### Table II

**Amino acid composition of peptides obtained in yields below 25%**

See Table I for method of calculating the results and symbols. In the case of Peptide C-XXa, tryptophan was present as judged by qualitative test, and 1 residue was assumed to be present. In the case of Peptide C-XXIVa, the analytical results were corrected for the known contamination with Peptide C-XXIIIa.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>C-XXIa</th>
<th>C-XXIIa</th>
<th>C-XXIIIa</th>
<th>C-XXIVa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.03</td>
<td>0.07</td>
<td>0.09</td>
<td>0.09</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.07</td>
<td>0.09</td>
<td>1.01(1)</td>
<td>1.00(1)</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.05</td>
<td>0.10</td>
<td>2.02(2)</td>
<td>2.02(2)</td>
</tr>
<tr>
<td>Proline</td>
<td>1.24(1)</td>
<td>0.09</td>
<td>1.01(1)</td>
<td>1.00(1)</td>
</tr>
<tr>
<td>Glycine</td>
<td>3.03(2)</td>
<td>0.17</td>
<td>1.01(1)</td>
<td>1.00(1)</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.24(1)</td>
<td>1.01(1)</td>
<td>0.96(1)</td>
<td>0.96(1)</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.00(1)</td>
<td>0.07</td>
<td>0.83(1)</td>
<td>0.05</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.00(1)</td>
<td>0.10</td>
<td>0.96(1)</td>
<td>0.02</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.00(1)</td>
<td>0.09</td>
<td>0.98(1)</td>
<td>0.05</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.00(1)</td>
<td>0.08</td>
<td>0.97(1)</td>
<td>0.07</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.55(1)</td>
<td>0.11</td>
<td>0.89(1)</td>
<td>0.02</td>
</tr>
<tr>
<td>Total residues</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Yield</td>
<td>8%</td>
<td>19%</td>
<td>5%</td>
<td>4%</td>
</tr>
</tbody>
</table>
chymotryptic hydrolysis, and the leucine in Peptide C-XXIVa is, therefore, probably amino-terminal.

Other peptides in low yield show a composition that is exactly the sum of the compositions of two other peptides obtained in high yield. Thus, Peptide C-XVIIIb represents the sum of Peptides C-XIa and C-XVIIb; Peptide C-XXa, the sum of Peptides C-XVa and C-XXVb; and Peptide C-XXVa, the sum of Peptides C-XIIa and C-XXVb. Such overlapping peptides readily permit the assignment of the adjacent positions of the smaller peptides of which they are composed (see the following paper (35)).

Finally, a group of peptides obtained in low yield clearly bears no relationship to any of the peptides obtained in high yield but rather appear to be overlapping portions of the same 15 amino acid sequence in the protein. Peptide C-XXVIIIa includes the entire sequence, whereas Peptides C-XIIIb, C-XXVIb, C-XXVIIa, and C-XXVIIb contain various overlapping portions from the same area. Summation of the yields of those peptides derived from the same central part of that sequence gives an over-all yield of 29%, which is rather lower than the yields from other portions of the protein sequence.

In summary, not counting repeatedly those residues that belong to the same sequence but that appeared in more than one peptide, the peptides prepared from the chymotryptic digest of horse heart cytochrome c and listed in Tables I and II account for the following residues: Try, Lys1G, His, Arg, Asp, Thr, Gly, Ala, Val, Met, Ile, Leu, Phe, Tyr. These residues, together with those occurring in the smallest heme-containing peptide present in the chymotryptic digest described below, account for the entire amino acid composition of the protein.

**Peptide Map of Chymotryptic Digest of Horse Heart Cytochrome c**—Fig. 3 is a typical peptide map of the chymotryptic digest of horse heart cytochrome c. The electrophoresis was performed in pyridine-acetate buffer at pH 6.4 for 24 hours at a potential drop of approximately 16 volts per cm. The chromatographic solvent was n-butanol-acetic acid-water (200:30:75 by volume) and was run for 18 hours at room temperature. The numbers of the peptides are those employed in the text. Peptides showing an initial yellow ninhydrin color are marked Y. Peptides giving specific color reactions are noted as follows: arginine, $\text{Arg}$; histidine, $\text{His}$; aspartic acid, $\text{Asp}$; threonine, $\text{Thr}$; glutamic acid, $\text{Glu}$; proline, $\text{Pro}$; glycine, $\text{Gly}$; alanine, $\text{Ala}$; valine, $\text{Val}$; methionine, $\text{Met}$; isoleucine, $\text{Ile}$; leucine, $\text{Leu}$; phenylalanine, $\text{Phe}$; tryptophan, $\text{Tyr}$. The area circled by a dotted line was that occupied by the main heme peptide present in the digest. Except for this heme peptide, only those peptides obtained in pure form are indicated on this peptide map.
Amino acid composition of heme peptide preparation and of peptides obtained from it by tryptic digestion.

See Table I for method of calculating the results and symbols. Under the heading, “Color,” those peptides that contained heme are marked, “Heme,” whereas for the other peptides, the symbols refer to the color after reaction on paper with the ninhydrin-collidine reagent (see “Experimental Procedure”).

![Table III](https://www.jbc.org/content/237/7/2158)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Heme peptide</th>
<th>Tryptic peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td></td>
<td>T-1</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.99(1)</td>
<td>2.16(2)</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.69</td>
<td>0.97(1)</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.32</td>
<td>0.08</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.94</td>
<td>0.88(1)</td>
</tr>
<tr>
<td>Serine</td>
<td>0.06</td>
<td>0.09</td>
</tr>
<tr>
<td>Glutamate acid</td>
<td>3.31</td>
<td>1.85(2)</td>
</tr>
<tr>
<td>Proline</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>4.40</td>
<td>0.25</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.65</td>
<td>1.02(1)</td>
</tr>
<tr>
<td>Half-Cystine</td>
<td>1.67</td>
<td>1.30(2)</td>
</tr>
<tr>
<td>Valine</td>
<td>1.83</td>
<td>0.99(1)</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.72</td>
<td>0.02</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.30</td>
<td>0.21</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td>Total residues</td>
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<td>12</td>
</tr>
<tr>
<td>Color</td>
<td>Heme</td>
<td>Heme</td>
</tr>
</tbody>
</table>

that such peptide maps appear to be readily reproducible. The chymotryptic digests of cytochrome c prepared from both the native and the denatured protein, as well as with different concentrations of enzyme, and for different times gave peptide maps that were indistinguishable.

**Heme Peptides**—Fig. 4 represents a peptide map of the tryptic digest of the heme peptide preparation in which only those peptides recovered in amounts sufficient to yield an unequivocal analysis are indicated. Table III lists the compositions of the original heme peptide preparation and of the tryptic peptides derived from it.

Peptide T-1 has the amino acid composition of the heme peptide that can be isolated from tryptic digests of horse heart cytochrome c (36), whereas Peptide T-2 has the composition of the peptic heme peptide (15) with an additional lysyl residue. After dinitrophenylation of the chymotryptic heme peptide preparation used in this study, and after total acid hydrolysis, 0.83 mole of dinitrophenylvaline was isolated per mole of heme peptide. The only other dinitrophenylamino acids identified were e-dinitrophenyllysine and imidazoledinitrophenylhistidine. Therefore, since the peptic heme peptide contains 2 valyl residues (15), one of which is amino-terminal, and the third valyl residue in horse heart cytochrome c is located in Peptide C-XIXa (see Table I), the amino-terminal sequence of the chymotryptic heme peptide must be that of the peptic heme peptide (15). The basic peptide T-5 containing (Lys, Glu, Val) represents the amino-terminal sequence, Val-GluNH$_2$-Lys, and the tryptic peptides other than T-1, T-2, and T-5 must derive from the portion of the chymotryptic heme peptide that is carboxy-terminal to the 12-amino acid sequence included in the peptic and tryptic heme peptides.

The amino acid compositions of the tryptic peptides, T-6 (Lys, Gly), T-7 (Lys, His), and T-8 (Lys, His, Gly), indicate that these peptides fit into the sequence, Gly-Gly-Lys-His-Lys. Peptides T-6 and T-8 give an initial yellow ninhydrin coloration on paper, confirming that the amino-terminal residue in each is glycine. The recovery of Peptide T-8, which has the sequence, Gly-Gly-Lys-His, as well as of free histidine (T-4) shows that these two peptides could not be the result only of a tryptic hydrolysis, since a cleavage at a His-Lys bond is required. Therefore, it is probable that part of the heme peptide preparation consisted of chains extending from the amino-terminal valine only as far as the above histidyl residue and that the hydrolysis at the His-Lys bond was due to chymotrypsin. The recovery of Peptide C-XXIa in 62% yield from the chymotryptic digest of cytochrome c (see Table I), and its final assignment of position in the over-all sequence of the protein (37) as a continuation of the Gly-Gly-Lys-His sequence discussed above, confirms that this particular His-Lys bond is in fact susceptible to chymotryptic attack. A similar chymotryptic cleavage at a histidyl residue was later found in Peptide C-XXIa (see the following paper (35)).

Peptide T-3, the only other peptide recovered from the tryptic digest of the chymotryptic heme peptide preparation, has the composition (His, Asp, Thr, Pro, Gly, Leu, Phe), which is identical with that of Peptide C-XXVIIa (see Table II) except for the absence of 1 lysyl residue. Peptide T-3 is slightly basic, indicating that at pH 6.4 the only operative charge is due to the histidyl residue and that the aspartyl residue is in the form of asparagine. On digestion of the chymotryptic heme peptide preparation with carboxypeptidase, the free amino acids remaining in the original heme peptide preparation and of the tryptic peptides recovered in amounts sufficient to yield an unequivocal analysis are indicated. Table III lists the compositions of the original heme peptide preparation and of the tryptic peptides derived from it.
only traces of tyrosine, methionine, glycine, alanine, threonine, glutamine, and asparagine being found. This shows that Peptide T 3 represents the carboxyl terminal sequence of the main heme peptide and that the sequence, Gly-Gly-Lys-His-Lys, is immediately adjacent to the tryptic heme peptide sequence.

In conclusion, the partial amino acid sequence of the chymotryptic heme peptide is

Val-GluNH₂–Lys–CyS–Ala–GluNH₂–CyS–His–Thr–Val–Glu–Lys–Val–Glu–Lys–His–Lys

This sequence is compatible with the composition of the heme peptide given in Table III but clearly does not correspond to the minimal chymotryptic heme peptide, which would extend only to the histidy1 residue in position 16. Indeed, because of its unique amino acid composition, the section including residues 17 to 26 clearly represents the same sequence as Peptides C-XXIa, C-XXIIb, and C-XXVIa (see Tables I and II), which were recovered in high yield from the chymotryptic digest of the whole protein.

**DISCUSSION**

**Experimental Procedure**—The purified peptides prepared from the chymotryptic digest of cytochrome c probably represent nearly all of the peptides present in yields above 5 to 10%, excluding the main heme-containing peptide, which could not be eluted from the Dowex 50 column. The relative ease with which these peptides were isolated can be ascribed to the choice of experimental procedures as well as to the properties of cytochrome c itself. In this connection, the following points appear to be noteworthy.

Digestion was taken nearly to completion by the use of a thoroughly denatured preparation of cytochrome c, by three successive additions of chymotrypsin, and by continuing the digestion for as long as 29 hours. Complete digestion of a protein after a single addition of an endopeptidase appears to be the exception (see Hirs, Stein, and Moore (38) for oxidized ribonuclease, for example) rather than the rule. The advantages of dealing with the relatively simple peptide mixture obtained on complete hydrolysis of a long peptide chain rather than the complex mixtures present in partial digests are obvious.

The use of volatile buffers in the initial column chromatographic procedure obviated the necessity for any desalting procedure. The advantages of such a technique cannot be over-stressed. There is no one method available as yet that can be used for desalting any peptide or amino acid mixture, as indicated by the numerous procedures proposed (25, 39–44). These methods are often tedious, have to be adapted in each case to the properties of the particular peptides in each fraction, and, more important, often lead to considerable losses of material or partial decomposition of labile peptides.

The examination of the fractions obtained from the column chromatography by the peptide map techniques of Ingram (21), in addition to giving some of the characteristics of each peptide, also indicated which procedures would most readily yield a purified peptide. In the present work, except for the occasional use of a different solvent, it was not found necessary to employ purification procedures other than those directly indicated by the peptide maps, i.e. ionophoresis or chromatography on paper.

![Image of chymotryptic heme peptide](https://www.jbc.org/content/240/4/2159.f1)

The desirability of a quantitative approach to amino acid sequence studies has been amply stressed (45). In the present work, quantitative criteria, in terms of the stoichiometry of the component amino acids, were used to check the purity as well as the yield of each isolated peptide.

Finally, cytochrome c, which contains 104 amino acids in a single chain, has only 17 major points of susceptibility to chymotryptic digestion and has no disulfide bonds. The only difficulty that arose was due to the high content of basic amino acids, 10 lysine and 2 arginine residues. This resulted in numerous positively charged peptides, which, in some cases, were not too easily separable from each other. The use of a basic solvent (n-butanol-pyridine-acetic acid-water) in which the charge on these residues was partly repressed, permitted, on the whole, satisfactory fractional extractions.

**Yields of Peptides and Overlapping Sequences**—The yields of pure peptides derived from most areas of the protein were relatively high, varying from 27 to 94%. Such yields were sufficient for a single column chromatographic run with 100 μmoles of digest on a 3.7 X 100 cm column to provide enough material to permit an unequivocal determination of the amino acid sequence of all of the important peptides (see the following paper (35)). However, from the portion of the protein represented by Peptides C-XXVIb, C-XXVIIa, C-XXVIIb, and C-XXVIIIa, the yields of individual peptides were only 7 to 15%. There appear to be several possible reasons for these unusually small yields. Because of their relatively high content of basic amino acids and of residues having large hydrophobic side chains, these peptides emerged late during the column chromatography in rather ill defined peaks that were difficult to pool accurately. The same characteristics probably caused large losses during the further purification of these peptides because of irreversible adsorption on paper. Moreover, the multiplicity of peptide bonds only poorly susceptible to enzymatic attack in this area of the protein (35) probably led to the formation, in low yields, of more peptides than were actually isolated. In particular, in the sequence, Ileu-Lys-Lys-Lys-Thr, hydrolysis of the second peptide bond was required to give rise to Peptide C-XXVIb, whereas hydrolysis of the fourth peptide bond led to the formation of Peptide C-XXVIIb (see the following paper (35)); it is readily conceivable that cleavages at other similarly poorly susceptible bonds in the same sequence could produce in low yields many more peptides than those described above.

All of the other peptides recovered in low yield represented either smaller fragments of peptides that had more than one possible point of chymotryptic hydrolysis and were obtained in high yield or, conversely, longer sequences encompassing two peptides that had been prepared in high yields. These two groups of low yield peptides were most useful in sequence studies (see the following paper (35)), since they gave the possibility of studying independently smaller fragments of larger sequences and of establishing the relative positions of two contiguous sequences.

Because of the presence of small numbers of certain residues in cytochrome c, the amino acid composition of the chymotryptic peptides was in itself sufficient to determine which peptides represented the same sequences in the protein. Such conclusions were confirmed in every case when the amino acid sequences of the peptides were established.

**Peptide Maps**—The peptide map of the chymotryptic digest of horse heart cytochrome c was readily reproducible with differ-
cut digests. It is, therefore, likely to be of value in investigating differences among cytochrome c preparations from various sources. Certain difficulties are apparent, however. The area of the positively charged peptides, which moved slowly in the chromatographic solvent, contained relatively numerous peptides, as would be expected from the high content of basic residues in the protein. The area of the neutral peptides, which moved little, is similarly complex. Minor differences in the peptides in these areas could easily be overlooked; this emphasizes one of the dangers of relying solely on peptide maps to identify differences or similarities in amino acid sequence between two similar proteins.

**Summary**

Twenty-eight peptides present in chymotryptic digests of horse heart cytochrome c have been prepared in pure form by column chromatography on Dowex 50, followed by electrophoresis or chromatography on paper or by various combinations of these procedures. The operations that permitted simple and effective separation of these peptides were the use of a chymotrypsin preparation free from trypsinic activity to carry out an enzymic digestion of the denatured protein to a point approaching completeness, the use of volatile buffers for column chromatography, the use of peptide maps as guides for further purification, and the monitoring of each purification procedure by quantitative amino acid analyses.

The amino acid composition of these pure peptides has been established. Excluding those residues that arose from the same area in the protein but were present in more than one peptide, and including the amino acids in the minimal heme-containing peptide found in chymotryptic digests of the protein, these peptides account for the entire amino acid composition of horse heart cytochrome c.

A partially purified preparation of the heme peptides present in chymotryptic digests of cytochrome c was obtained by chromatography on Amberlite IRC-50. The amino acid composition and partial sequence of these peptides was determined by analysis of peptides obtained by digestion with trypsin.

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**References**

Isolation and Amino Acid Composition of Chymotryptic Peptides from Horse Heart Cytochrome c
E. Margoliash and Emil L. Smith


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