The amino acid sequences of cytochromes from many species have been shown to be homologous, the number of residues which differ depending on the biological relationship of the species being compared (2, 3). At the time the present study of dog heart cytochrome c was undertaken, only the sequences of horse (4) and human (5) cytochromes c were known. It was essential, therefore, to determine the over-all sequence in some detail. At present, this is the only known sequence of a cytochrome c which has been reported for a member of the carnivores.

Methods and Materials

Isolation of Dog Heart Cytochrome c—Cytochrome c was isolated from frozen dog hearts and purified by the method of Hagihara et al. (6). Batch elution from IRC-50 by buffers of 0.15 ionic strength was found to be too slow, and 0.18 ionic strength was used instead. The course of purification was followed at each step by measurement of the absorbance ratio of the oxidized form at 278 μm, and of the reduced form at 550 μm. The ratio A550/A278 for dog and horse proteins which proved to have the same tyrosine and tryptophan content should be the same, viz. 1.28 (6).

From dog hearts (4.1 Kg, ground defatted tissue), 60 μmoles of cytochrome c were isolated after column chromatography. More than 60% of the eluate had an absorbance ratio of 1.25. A second preparation yielded cytochrome c with an absorbance ratio of 1.28.

Both preparations of dog heart cytochrome c were found to be homogeneous on free electrophoresis and in the ultracentrifuge at pH 7.35. The sedimentation constant was identical to those for other mammalian cytochromes c, indicating that the molecule was essentially the same size.

Amino Acid Analysis—Dialyzed solutions of cytochrome c were hydrolyzed for 20 and hours in glass-distilled (three times) 6 M HCl. A known portion of the hydrolysates was analyzed in duplicate on a Spinco automatic amino acid analyzer.

Chymotryptic Digestion—Ethanol-denatured dog heart cytochrome c (34 μmoles dissolved in 40 ml of water) was digested with chymotrypsin at 27-28°C over a period of 29 hours. The pH was maintained near 7.8 by periodic additions of sodium hydroxide (0.1 M). Additions of chymotrypsin (2% by weight) were made at 0, 13 and 21 hours. The digest was acidified by addition of acetic acid to pH 2.4 and chromatographed on Dowex 50-X2 as described below.

Tryptic Digestion—Ethanol-denatured dog heart cytochrome c (29 μmoles dissolved in 30 ml of water) was digested with trypsin at 30°C over a period of 24 hours. The pH was maintained at 7.8 continually by the addition of NaOH (0.2 M) with an automatic titrator. Additions of trypsin (2% by weight) were made initially and at 1 1/2 hours (1%). The uptake of alkali at 2 1/2 hours corresponded to an estimated cleavage of 16 bonds. About 80% of the total digestion occurred during the first 40 min. The digestion was terminated by addition of acetic acid to pH 2.5. The digest was chromatographed on Dowex 50-X2 as described below.

Column Chromatographic Separation of Peptides—The tryptic and chymotryptic digests of cytochrome c were resolved on jacketed columns (2 × 175 cm) of Dowex 50-X2 (Dow Chemical), wet sized through 100 mesh, less fines, at 40°C and at a flow rate of 40 or 50 ml per hour. The flow rate was maintained constant by a constant volume delivery pump. Fractions of 10 ml were collected.

Elution buffers of pyridine and acetic acid, 0.2 M in pyridine at pH 3.1 and 2 M at pH 5.1, were used. The resin, equilibrated with the pH 3.1 buffer, was degassed under vacuum before pouring the column.

The digest was applied to the column at pH 2.5 to 2.7 and washed into the resin bed with small amounts of 30% acetic acid followed by pH 3.1 buffer. After passage of an appropriate volume of pH 3.1 buffer, a gradient of pH and ionic strength was set up between buffers of pH 3.1 at 0.2 M and pH 5.1 at 2.0 M. The gradient was divided into two or more sections by operating between various mixtures of these two buffers in order of increasing pH and ionic strength. In this way advantage could be taken of the analogous published elution patterns of the enzymic digests of human (5) and horse (7) heart cytochromes c to improve the resolution of the present digests within a certain total elution volume by varying the gradient as needed. The elution was followed by the colorimetric ninhydrin method (8) after alkaline hydrolysis of 0.5 ml aliquots of every second fraction. Tubes were pooled corresponding to peaks in the elution diagram and pooled fractions were examined as described previously (5, 7).

Paper Electrophoresis—Electrophoresis (9) was performed in pyridine-acetic acid buffers of ionic strength 0.06 M at a mean potential gradient of 30 volts per cm. These buffers gave better
resolution and had greater capacity for peptides than 0.03 M buffers, as well as having greater solvent power. Also, pH 5.6 buffer was used in preference to the pH 6.5 buffer to conserve purified pyridine and to reduce the atmospheric concentration of pyridine vapor.

**Paper Chromatography**—Fractions were chromatographed in descending manner with Solvent I, butanol-acetic acid-water, 40:6:15, by volume, or Solvent II, butanol-pyridine-acetic acid-water, 15:10:3:12, by volume.

Pyridine was purified by stirring vigorously under gentle reflux for 1 hour with 2% by volume of concentrated H$_2$SO$_4$ and allowed to cool. The supernatant pyridine was decanted from the dark lower layer which may have solidified, filtered through glass wool to remove any suspended crystalline material, and then distilled through a fractionating column. The first 5% and the last 20% (residue) were rejected. The product was colorless or distilled through a fractionating column. The first 5% and the last 20% (residue) were rejected. The product was colorless or distilled through a fractionating column.

**Amino Acid Composition of Peptides**—Peptides were hydrolyzed with 6 M HCl or with leucine aminopeptidase (see below) and analyzed qualitatively or quantitatively. For sequence studies, peptides were purified until the amino acid analysis showed less than 0.5 or 10% deviation from stoichiometry. Mean residue figures and net and gross yields of peptides were calculated as described previously (5, 7).

**Enzymic Hydrolysis of Peptides**—Peptides were hydrolyzed at 1 M concentration and at 25°, or occasionally, where specified in the text, at 40°. The micromolar concentrations of enzymes used in each experiment are given in the text; the higher levels of carboxypeptidase A represent micromoles of protein added per ml of reaction mixture, rather than the actual concentration in solution, because of the low solubility of the enzyme. Hydrolysis by purified trypsin or chymotrypsin was performed at pH 8.

Limited hydrolysis by purified leucine aminopeptidase (10), C$_1$ - 21, at a final protein concentration of 10 µg per ml, was performed at pH 8.5 in 2.5 mM MgCl$_2$. Complete hydrolysis was carried out at protein concentration 250 to 400 µg per ml for 16 to 24 hours at 40°. Hydrolysis by carboxypeptidase A (Worthington, recrystallized twice) and carboxypeptidase B (11) was carried out at pH 8; these enzymes were treated with diisopropylphosphorofluoridate before use. Peptic digestion was performed in 0.01 M HCl.

**Degradation of Peptides by the Edman Procedure**—Peptides were degraded sequentially from the amino terminus by the procedure of Edman and Sjöquist (12). PTH-amino acids were identified by chromatography on Whatman No. 1 paper, usually with two different solvent systems (12, 13). In most cases quantitative analysis of the residual peptide was also performed.

**Nomenclature**—Column fractions are numbered consecutively with arabic numerals. Fractions derived from the trypsin and chymotryptic digests of dog heart cytochrome c are prefixed T- and C-, respectively.

Peptides isolated from a given column fraction take the number of that fraction, except that when more than one peptide was isolated from the fraction, the distinguishing suffixes a, b, etc., are used. Peptides derived from the original peptides by further degradation are similarly distinguished by appending -T, -C, -P (pepsin), or -L (leucine aminopeptidase) to the symbol for the parent peptide. Residual peptides from partial leucine aminopeptidase degradation are denoted -L1, -L2, etc., for the number of residues removed from the original peptide. Each peptide is distinguished also by noting the position of the residues in the sequence of the protein, the amino-terminal residue being number 1.

**Limited Chymotryptic Digest**—Cytochrome c (15 µmoles, ethanol-denatured, in 15 ml) was digested with chymotrypsin (4% by weight of substrate) at pH 7.8 and 28° for 40 min with continuous addition of 0.1 M NaOH. The digest was fractionated on Sephadex G-25 (175 x 2.0 cm) with aqueous pyridine (40%) at 30°. Column fractions were examined by direct ninhydrin colorimetric analysis and, after spotting on paper, by the Ehrlich reagent for tryptophan and the iodine-azide reagent for methionine peptides. On the basis of this examination, appropriate fractions were pooled as described below, and two-dimensional peptide maps were prepared. A few selected peptides were isolated and purified from these fractions.

**Preparation of Tryptic Hemopeptide T-H**—Ethanol-denatured dog heart cytochrome c (15 µmoles, 1% in water) was digested with trypsin (2% by weight) at 30° and pH 7.8 for 5 hours. The digest was concentrated on the rotary evaporator and dissolved in pH 4.6 pyridine-acetate buffer (0.7 M). After some insoluble material had been removed by centrifugation, the material was adsorbed on a column of Dowex 50-X2 (90 x 0.9 cm) and eluted at 40° with 1.4 M pyridine-acetate buffer at pH 4.6. The fractions were sampled for ninhydrin analysis without alkaline hydrolysis. The location of red hemopeptide components was observed visually. The main hemopeptide fraction eluted after most of the ninhydrin-positive material. This fraction was oxidized by performic acid, the residual performic acid was removed at the rotary evaporator, and the product was chromatographed on an identical column of Dowex 50-X2 with 0.2 M pyridine-acetate buffer at pH 3.1. The oxidized hemopeptide emerged at the column volume and was checked for purity by electrophoresis at pH 5.6. It contained only one ninhydrin-positive, Pauly-positive (orange-red) component.

**RESULTS**

**Amino Acid Composition**—The amino acid composition of dog cytochrome c is given in Table I. Although the number of residues is calculated on the basis of the arginine content of 2 residues, these values are in accord with the amount of protein used for hydrolysis as calculated from the absorbance at 550 nm of the reduced protein. The fourth column gives the assumed number of residues. By assuming the presence of 2 half-cystine residues, confirmed by analysis of the oxidized tryptic hemopeptide T-H, and including the unique tryptophan residue of Peptide C-22, we found that the total number of residues is 104, identical with that of other mammalian cytochromes c (2, 3).

At the time these results were obtained, only the sequences of horse and human cytochromes were known. It was evident that dog cytochrome c showed at least 8 differences in composition from that of the horse (14), and 12 differences from that of man (15). It follows that there must be at least 4 and 6 substitutions in comparing the sequence of dog cytochrome and those of horse and man, respectively. As shown below, the actual numbers of sequence differences are greater than indicated by the amino acid composition.

**Purification and Composition of Peptides**—The elution patterns of the chymotryptic and tryptic digests of dog heart cytochrome...
c on Dowex 50-X2 columns are shown in Figs. 1 and 2. The methods of purification of peptides isolated from these column fractions, the paper electrophoretic and paper chromatographic properties, and the ninhydrin colors of the tryptic and chymotryptic peptides are summarized in Tables II and III. The amino acid compositions, the number of residues, and the estimated yields for the purified peptides are given in Tables IV and V.

![Figure 1](image1.png)

**Figure 1.** Fractionation of a chymotryptic digest of dog heart cytochrome c (34 pmoles) on a column of Dowex 50-X2 (175 x 2 cm). Fractions of 10 ml were collected, and aliquots of 0.5 ml were analyzed by the ninhydrin method after alkaline hydrolysis. The pH gradient, obtained with pyridine-acetate buffers, is shown by the continuous line.

**Figure 2.** Fractionation of a tryptic digest of dog heart cytochrome c (29 pmoles) on a column of Dowex 50-X2 (175 x 2 cm). The pH gradient, shown by the continuous line, was obtained with pyridine-acetate buffers. Fractions of 10 ml were collected, and aliquots of 0.5 ml were analyzed after alkaline hydrolysis by the ninhydrin method.

### Amino Acid Composition of Dog Heart Cytochrome c

The numbers of residues were calculated on the basis of the presence of 2 residues of arginine in the protein. Duplicate analyses were performed after hydrolysis for 20 and 70 hours in 6 N HCl.

<table>
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<th>Amine Acid</th>
<th>20 hours</th>
<th>70 hours</th>
<th>Average</th>
<th>Assumed and Calculated by Linear Extrapolation to Zero Time</th>
<th>Assumed Value, Confirmed by Analysis of Tryptic Heme Peptide</th>
<th>Includes Values for Methionine Oxidation Products</th>
<th>Assumed Value, Confirmed by Analysis of Peptides</th>
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</table>

* Calculated by linear extrapolation to zero time.
* Assumed value; confirmed by analysis of tryptic heme peptide.
* Includes values for methionine oxidation products.
* Assumed value; confirmed by analysis of peptides.

### Amino Acid Sequences of Peptides

The derivation of the sequences of the peptides from the chymotryptic and tryptic digestion is described below. The location of a single basic residue at the carboxyl terminus of a tryptic peptide and a single aromatic residue at the carboxyl terminus of a chymotryptic peptide in high yield will be assumed without further comment. Sufficient sequence work was done on either the tryptic or the corresponding chymotryptic peptides derived from the same sequence to establish both the overlaps and the complete sequence of the molecule. The sole exception involves the 9-residue tryptic heme peptide. Since the composition of this peptide is identical to that obtained from horse cytochrome c, it was assumed that the sequence is the same.

#### Chymotryptic Peptides

**Peptide C-1 (Residues 60 through 66): Gly-Glu-Glu-Thr-Leu-Met—**This peptide has the composition of Peptide C-2 plus 1 residue of methionine which therefore must be terminal. Digestion of Peptide C-1 with Carboxypeptidase A (0.02 mg) at 40°C yielded in 18 hours: Thr, 0.29; Leu, 1.02; Met, 0.96, establishing the carboxyl-terminal sequence -Thr-Leu-Met, or -Thr-Leu-Met since methionine must be terminal. From the yellow color of Peptide C-1 with ninhydrin, glycine is amino-terminal as shown below for Peptide C-2. Since Peptide C-1 has almost the same electrophoretic mobility as Peptide C-2 (Table III), it must also have 2 glutamyl residues.

**Peptide C-2 (Residues 60 through 64): Gly-Glu-Glu-Thr-Leu—**Complete hydrolysis with the aminopeptidase showed the presence of 2 residues of glutamic acid and the absence of glutamine, whereas limited hydrolysis for 18 hours at 25°C gave Gly, 0.26; Glu, 0.07; Thr, 0.05; Leu, 0.02, showing that glycine is amino-terminal, in agreement with the yellow ninhydrin color. Hydrolysis with carboxypeptidase (0.05 mg) at 40°C for 1 hour (Thr, trace; Leu, ++ + + +) and for 18 hours (Thr, + + + +; Leu, ++ + + +) indicated that the carboxyl-terminal sequence is -Thr-Leu. The positions of the 2 glutamyl residues are therefore established.

1 Denotes essentially quantitative release of the residue as shown by virtual absence of the original peptide from the peptide pattern of the digest.
**Peptide C-3 (Residues 66 and 67): Gla Tyr**  The presence of a glutamyl residue is evident from the anodic electrophoretic mobility of this peptide.

**Peptide C-4b (Residues 47 and 48): Ser-Tyr.**

**Peptide C-6b (Residues 81 and 82): Ile-Phe.**

**Peptide C-4a (Residues 1 through 7):** R—CO—(Gly, Asp, Val, Gla, Lys, Gla)Gla—This peptide contains 1 of the 3 valyl residues and is evidently derived from the amino terminus by a weak hydrolysis between 2 lysine residues. This portion of the sequence was established by two steps of the Edman degradation. Step 1: PTH-Gly (Systems A, F); residue: Gly, 0.13; Leu, 1.00; Phe, 0.11; Ala, 1.03; Lys, 0.97; Step 2: PTH-Asp (Systems C, F); residue: Thr, 0.05; Asp, 3.15; Ala, 1.02; Lys, 0.94; Step 3: PTH-Glu (Systems A, F); residue: Glu, 2.27; Ala, 1.03; Lys, 0.97; Step 4: PTH-Asp (Systems A, F); residue: Asp, 2.27; Ala, 1.03; Lys, 0.97; Step 5: PTH-Leu (Systems A, F); residue: Leu, 0.11; Phe, 1.00.

**Peptide C-4b (Residues 47 and 48): Ser-Tyr.**

**Peptide C-6b (Residues 81 and 82): Ile-Phe.**

**Peptide C-4c (Residues 34 through 36): Gly-Leu-Phe.** This peptide contains 1 of the 3 valyl residues and is evidently derived from the carboxyl terminus by a weak hydrolysis with the amine peptidase for 18 hours. The sequence was established by two steps of the Edman degradation. Step 1: PTH-Thr (Systems A, F); residue: Thr, 0.05; Asp, 3.15; Ala, 1.02; Lys, 0.97; Step 2: PTH-Leu (Systems A); residue: Gly, 0.06; Leu, 0.11; Phe, 1.00.

**Peptide C-5a (Residues 49 through 54): Thr-Asp-Ala-Asn—This peptide has the same composition as Peptide C-6a, but from its net negative charge at pH 5.6 (Table III), it must have fewer amide groups. Threonine is probably aminoterminal; like Peptide C-6a, the peptide yields an initial yellow color with ninhydrin. Asparagine should be carboxyl-terminal because of chymotryptic specificity. Thus the deamidated residue must be internal.

**Peptide C-5b (Residues 55 through 57): Ile-Asp-Ala.** The sequence was established with one step of the Edman degradation. Step 1: PTH-Ile (Systems A, F); residue: Ile, 0.02; Ala, 1.00; Tyr, 0.67; Step 2: PTH-Leu (System A); residue: Gly, 0.06; Leu, 0.11; Phe, 1.00.

**Peptide C-5c (Residues 58 through 60):** Thr-Asp-Ala-Asn—The peptide is neutral at pH 5.6 (Table III) and hence must have 2 residues of asparagine and 1 of aspartic acid. Hydrolysis with the aminopeptidase for 18 hours liberated only threonine quantitatively in accord with the initial yellow color of the peptide with ninhydrin. Hydrolysis with Carboxypeptidase A (2 μμ) yielded the following results: 1 hour, Asn, 0.1; 4 hours, Asn, 0.5; 21 hours, Asn, 1.0; Lys, 0.1; thus establishing the COOH-terminal sequence, -Lys-Asn, in agreement with chymotryptic specificity. Four steps of the Edman degradation yielded the sequence Thr-Asp-Ala-Asn: Step 1: PTH-Thr (Systems A, F); residue: Thr, 0.05; Asp, 3.15; Ala, 1.02; Lys, 0.97; Step 2: PTH-Asp (Systems C, F); residue: Thr, 0.05; Asp, 2.27; Ala, 1.03; Lys, 0.97; Step 3: PTH-Ala (Systems A, F); residue: Ile, 0.02; Ala, 1.00; Tyr, 0.67; Step 4: PTH-Leu (Systems A, F); residue: Lys, 0.97.
residue: Thr, 0.03; Asp, 2.15; Ala, 0.23; Lys, 0.87; Step 4: PTH-Glycine. This peptide has the composition of Peptide C-9 plus aspartic acid and alanine. Tryptic digestion yielded two peptides which were separated on a column of Dowex 50-X2 (60 × 0.9 cm) with a gradient of pyridine-acetate buffer. Peptide Ta gave Ala, 1.01; Asp, 1.02; Lys, 0.97. Peptide Tb gave Gly, 0.98; Glu, 1.02; Arg, 0.99. Tb was yellow with ninhydrin like Peptide C-9, indicating that glycine is amino-terminal in both. Edman degradation on Peptides Ta and Tb established the sequence: Peptide Ta: Step 1: residue: Ala, 0.04; Asp, 1.01; Leu, 0.99; Peptide Tb: Step 1: residue: Gly, 0.06; Glu, 1.00; Arg, 0.94.

**Peptide C-8 (Residues 85 through 92):** Thr(Gly, Glu, Arg, Ala, Asp)Leu—This peptide has the composition of Peptide C-9 plus threonine. Leucine is presumably carboxyl-terminal, as in Peptide C-9, on the basis of chymotryptic specificity; therefore, threonine must be aminoterminal.

**Peptide C-9 (Residues 27 through 31):** Lys, Thr, Gly, Pro—Asn—The composition of this peptide corresponds to the first 5 residues of Peptide C-13.

**Peptide C-9 (Residues 89 through 94):** Gly-Glu-Arg-Ala-Asp-Leu—This peptide is negatively charged at pH 5.6 and thus contains no amides. Hydrolysis for 18 hours with Carboxypeptidase A (20 μM) liberated 1 eq of leucine and small amounts of aspartic acid and alanine. Tryptic digestion yielded two peptides which were separated on a column of Dowex 50-X2 (60 × 0.9 cm) with a gradient of pyridine-acetate buffer. Peptide Ta gave Ala, 1.01; Asp, 1.02; Leu, 0.97. Peptide Tb gave Gly, 0.98; Glu, 1.02; Arg, 0.99. Tb was yellow with ninhydrin like Peptide C-9, indicating that glycine is amino-terminal in both. Edman degradation on Peptides Ta and Tb established the sequence: Peptide Ta: Step 1: residue: Ala, 0.04; Asp, 1.01; Leu, 0.99; Peptide Tb: Step 1: residue: Gly, 0.06; Glu, 1.00; Arg, 0.94.

**Peptide C-10 (Residues 75 through 80):** Ile-Pro-Gly-Thr-Lys—Hydrolysis of Peptide C-10 with Carboxypeptidase A (0.5 μM) at 25° gave in 1 hour: Lys, 0.23; Met, 1.00; and hydrolysis with the enzyme at 50 μM and 40° gave in 18 hours: Thr, 0.64; Lys, 0.83; Met, 0.99. This establishes the carboxyl-terminal sequence—Thr-Lys—Met. Two steps of the Edman degradation gave Ile-Pro: Step 1: residue: Gly, 0.06; Glu, 1.06; Thr, 0.83; Lys, 0.70; Met, 1.03. Step 2:
TABLE V

A. Composition of Peptides T-2 through T-17 from tryptic digest

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Lysine</th>
<th>Arginine</th>
<th>Aspartic acid</th>
<th>Threonine</th>
<th>Serine</th>
<th>Glutamic acid</th>
<th>Proline</th>
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<th>Alanine</th>
<th>Valine</th>
<th>Isoleucine</th>
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<td>T-12</td>
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<tr>
<td>Residues</td>
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<td>14</td>
<td>4</td>
<td>3</td>
<td>8</td>
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<td>2</td>
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<tr>
<td>Yield</td>
<td>65%</td>
<td>54%</td>
<td>48%</td>
<td>36%</td>
<td>10%</td>
<td>8.5%</td>
<td>43%</td>
<td>46%</td>
<td>20%</td>
<td>52%</td>
<td>52%</td>
<td>56%</td>
<td>24%</td>
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</table>

* Total hydrolysis by leucine aminopeptidase.

\[ T-lla T-llb \]

\[ II \]

B. Composition of Peptides T-18a through T-H from tryptic digest

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Lysine</th>
<th>Histidine</th>
<th>Aspartic acid</th>
<th>Threonine</th>
<th>Serine</th>
<th>Glutamic acid</th>
<th>Proline</th>
<th>Glycine</th>
<th>Alanine</th>
<th>Valine</th>
<th>Isoleucine</th>
<th>Leucine</th>
<th>Tyrosine</th>
<th>Phenylalanine</th>
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<tbody>
<tr>
<td>T-18a</td>
<td>1.94</td>
<td>0.89</td>
<td>1.67</td>
<td>1.90</td>
<td>0.90</td>
<td>1.33</td>
<td>0.04</td>
<td>1.06</td>
<td>2.02</td>
<td>1.08</td>
<td>0.98</td>
<td>1.01</td>
<td>1.02</td>
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<td>T-18b</td>
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<tr>
<td>T-19a</td>
<td>1.76</td>
<td>0.96</td>
<td>1.70</td>
<td>0.01</td>
<td>0.98</td>
<td>1.01</td>
<td>0.07</td>
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<td>0.78</td>
<td>0.99</td>
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<td>Residues</td>
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<td>5</td>
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<td>6%</td>
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<td>42%</td>
<td>12%</td>
<td>2%</td>
<td>69%</td>
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</tbody>
</table>

* Incomplete hydrolysis of Lys-Lys bond (20 hours).

\[ T-22b \]

\[ T-23a \]

\[ T-23b \]

\[ T-23c \]

\[ T-23d \]

\[ T-24 \]

\[ T-H \]

PTH-Pro (System D); residue: Ile, 0.06; Pro, 0.19; Gly, 0.99; Thr, 0.96; Lys, not determined; Met, 1.01.

**Peptide C-11 (Residues 83 through 88): (Ala, Gly, Ile)Lys—**This peptide has 1 less residue of lysine than Peptide C-18 and is derived from the same part of the sequence. Hydrolysis with Carboxypeptidase B liberated only lysine.

**Peptide C-12 (Residues 1 through 10): R—CO—(Gly, Asp, Val, Glu)Lys—Lys—Lys—Ile—Phe—**The partial sequence of this peptide was established by hydrolysis with Carboxypeptidase A and by the composition of the tryptic peptides as described in Table VI. The remaining portion of the sequence was obtained by study of Peptide T-2 described below.

**Peptide C-13 (Residues 27 through 33): Lys—Thr—**Hydrolysis of Peptide C-13 (Table VII) with Carboxypeptidase A showed that the carboxyl-terminal sequence is Lys—Thr—His—. Degradation by the aminopeptidase established the amino-terminal sequence Lys—Thr— and removed both residues quantitatively but did not release any other amino acids. Pep-
Table VI

Sequence of Peptide C-12

<table>
<thead>
<tr>
<th>Sequence</th>
<th>R—CO—(Gly, Asp, Val, Glu) Lys-Gly-Lys-Lys-Ile-Phe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxypeptidase A (0.5 μM); 10 min</td>
<td>Ta →</td>
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<tr>
<td>(0.5 μM); 30 min</td>
<td>Te →</td>
</tr>
<tr>
<td>(10 μM); 16 hrs</td>
<td>Tb →</td>
</tr>
<tr>
<td>Tryptic peptides</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Elution volume (ml)</th>
<th>pH</th>
<th>Ninhydrin color</th>
<th>Composition</th>
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<tbody>
<tr>
<td>Ta</td>
<td>38-63</td>
<td>3.1</td>
<td>Blue</td>
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<tr>
<td>Tb</td>
<td>48</td>
<td>3.7</td>
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<tr>
<td>Tc</td>
<td>41</td>
<td>4.0</td>
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</tr>
<tr>
<td>Td</td>
<td>28</td>
<td>4.4</td>
<td>Blue</td>
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<tr>
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</tr>
<tr>
<td>Te</td>
<td>39</td>
<td>4.4</td>
<td>Yellow</td>
</tr>
</tbody>
</table>

* Peptides Tb and Te were separated by paper electrophoresis at pH 5.6.

Initial ninhydrin color.

tide C-13-L2 was positively charged at pH 5.6 and gave a bright yellow ninhydrin color; these findings indicate that glycine is amino-terminal and asparagine is present. Three steps of the Edman degradation of Peptide C-13-L2 gave the sequence Gly-Pro-Asn.

Peptide C-14 (Residues 87 through 104): Lys-Thr-Gly(Glu, Arg, Ala, Asp)-Leu—This peptide has the same composition as Peptide C-9 with the addition of lysine and threonine and, therefore, arises from the same part of the sequence since there are only 2 residues of arginine in cytochrome c. Hydrolysis with the aminopeptidase for 16 hours yielded Lys, 0.31; Thr, 0.19; Gly, 0.07; showing the amino-terminal sequence to be Lys-Thr-Gly, in agreement with the sequence of Peptide C-9. It follows from chymotryptic specificity that leucine is carboxyl-terminal.

Peptide C-15a (Residues 100 through 104): Lys-Ala-Thr(Lys, Glu)-This peptide differs in composition from the carboxy-terminal Peptide C-25 of which it must be part, in having 1 less residue of lysine. Hydrolysis with the aminopeptidase yielded in 20 hours: Lys, 0.38; Ala, 0.14; Thr, 0.115; Glu, 0.042, indicating the sequence Lys-Ala-Thr— for the first 3 residues.

Peptide C-15b (Residues 85 through 88): (Ala, Gly, Ile, Lys)-Lys—This peptide is derived from the amino terminus of Peptide C-20 and has the composition of Peptide C-18 plus threonine which must be carboxyl-terminal since the peptide does not give an initial yellow color with ninhydrin. Hydrolysis with Carboxypeptidase A (0.05 mM) for 4 and 18 hours removed threonine quantitatively and also a small amount of lysine, which increased with time parallel with the appearance of a Peptide C-15b-CP2 of lower cathodic mobility than C-15b-CP1, establishing the carboxyl-terminal sequence, —Lys-Thr.

Peptide C-15c (Residues 23 through 26): Gly-Gly-Lys—Hs—From the initial ninhydrin color, the 1st residue is glycine. Hydrolysis with Carboxypeptidase A (0.05 mM) at 40° for 16 hours gave: Lys, +++; His, ++ + +; indicating the above sequence for Peptide C-15c. There are only 3 residues of histidine in the protein, 1 in the tryptic Hemopeptide T-H, and another in the sequence Leu—His—Gly of Peptide T-24. It follows that Peptide T-22a, His-Lys, is an extension of Peptide C-15c and by tryptic specificity must be preceded by lysine in the protein sequence. This confirms that Peptide C-15c must be Gly-Gly-Lys-His, and Peptide T-10, Gly-Gly—Lys, then corresponds to the first 3 residues.
Peptide C-16 (Residues 69 through 74): Glu-Asn(Pro, Lys, Lys) Tyr—The Edman degradation established the amino-terminal sequence, Glu-Asn. Step 1: PTH-Glu (Solvents C, F); residue: Glu, 0.22; Asp, 0.97; Pro, 1.03; Lys, not determined; Tyr, 0.39(1). Step 2: PTH-Asn (Solvents C, F); residue: Glu, 0.14; Asp, 0.43; Pro, 1.00; Lys, 1.09(2); and Tyr, 0.33(1). The low values for Lys and Tyr indicate incomplete decomposition of the phenylthio carbamyl derivative.

Peptide C-18 (Residues 83 through 87): Ala-Gly(Ile, Lys)Lys—The first 2 residues, Ala-Gly, were established by the Edman degradation. Step 1: Ala, 0.12; Gly, 1.07; Ile, 0.95; Lys, 1.53(2). Step 2: Ala, 0.11; Gly, 0.43; Ile, 1.00; Lys, 1.47(2). Hydrolysis with Carboxypeptidase B (0.4 PM) at 25° for 21 hours gave only 1 eq of lysine and a residual basic peptide. The apparent resistance to enzymic digestion of the Ile-Lys bond is indicated by the evidence that the sequence Leu-Glu-Asn-Pro-Lys-Lys-Tyr of Peptide C-19 was not resolved by the Edman degradation. Together, the above evidence indicates the sequence Leu-Glu-Asn-Pro-Lys-Lys-Tyr for Peptide C-19. This is confirmed by the isolation of Peptide T 22b, Lys (Tyr, Ile, Pro, Gly, Thr, Lys) which can only have originated by tryptic cleavage of a Lys-Lys bond and not of a Pro-Lys bond.

Peptide C-20 (Residues 83 through 94): Ala-Gly-Ile-Lys-Lys-Thr-Gly-Glu-Ag-Ala-Asp-Leu—This peptide contains 1 of the 2 residues of arginine of dog cytochrome c. Its composition is identical to the sums of those of Peptides C-11 plus C-14, C-18 plus C-8, and C-15b plus C-9. The second peptide of each pair contains 1 residue of leucine and is thus presumably carboxy-terminal in Peptide C-20 from tryptic specificity. Three steps of the Edman degradation on Peptide C-20 gave the sequence Ala-Gly-Ile. Step 1: PTH-Ala (System F); residue: Ala, 1.13; Gly, 2.03; Ile, 1.03; Lys, 1.65; Thr, 0.96; Glu, 1.09; Arg, 0.92; Asp, 1.01; Leu, 1.03. Step 2: PTH-Glu (Systems A, F); residue: Ala, 1.09; Gly, 1.22; Ile, 0.58; Lys, 1.63; Thr, 0.98; Glu, 1.09; Arg, 1.97; Asp, 1.06; Leu, 0.99. Step 3: PTH-Ile (Systems A, F); residue: Ala, 1.09; Gly, 1.23; Ile, 0.18; Lys, 1.99; Thr, 0.98; Glu, 1.05; Arg, 0.99; Asp, 1.03; Leu, 1.00.


**Table VIII**

**Sequence of Peptide C-23**

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly-Arg-Lys-Thr-Gly-Gln-Ala-Pro-Gly-Phe</td>
<td>Residues 22 and 23</td>
</tr>
<tr>
<td>Gly-Phe</td>
<td>Residues 22 and 23</td>
</tr>
<tr>
<td>Phe (4+)</td>
<td>Residues 22 and 23</td>
</tr>
<tr>
<td>Carboxypeptidase A</td>
<td>Residues 22 and 23</td>
</tr>
<tr>
<td>Tryptic digestion, trypsin (10 μM), 30 min</td>
<td>Residues 22 and 23</td>
</tr>
<tr>
<td>Ta</td>
<td>Residues 22 and 23</td>
</tr>
</tbody>
</table>
| Composition: Gly, 0.98; Arg, 1.02; Lys, 0.98(1); Ala, 1.04(1); Tyr, 1.05(1); Gly, 0.07. The failure of the aminopeptidase to remove more than 2 residues from Peptide C-19 is again consistent with proline being the 4th residue. Since Peptide C-19 is basic (Table III), it contains at least one amide, which must be asparagine.

**Peptide C-23**

Peptide C-23 has the partial sequence Glu-Asn (Pro, Lys, Lys) Tyr and thus arises from Peptide C-19 by tryptic cleavage of the amino-terminal Leu-Glu bond. Together, the above evidence indicates the sequence Leu-Glu-Asn-Pro-Lys-Lys-Tyr for Peptide C-19. This is confirmed by the isolation of Peptide T 22b, Lys (Tyr, Ile, Pro, Gly, Thr, Lys) which can only have originated by tryptic cleavage of a Lys-Lys bond and not of a Pro-Lys bond.

Peptide C-24 (Residues 83 through 94): Ala-Gly-Ile-Lys-Lys-Thr-Gly-Glu-Ag-Ala-Asp-Leu—This peptide contains 1 of the 2 residues of arginine of dog cytochrome c. Its composition is identical to the sums of those of Peptides C-11 plus C-14, C-18 plus C-8, and C-15b plus C-9. The second peptide of each pair contains 1 residue of leucine and is thus presumably carboxy-terminal in Peptide C-20 from tryptic specificity. Three steps of the Edman degradation on Peptide C-20 gave the sequence Ala-Gly-Ile. Step 1: PTH-Ala (System F); residue: Ala, 1.13; Gly, 2.03; Ile, 1.03; Lys, 1.65; Thr, 0.96; Glu, 1.09; Arg, 0.92; Asp, 1.01; Leu, 1.03. Step 2: PTH-Glu (Systems A, F); residue: Ala, 1.09; Gly, 1.22; Ile, 0.58; Lys, 1.63; Thr, 0.98; Glu, 1.09; Arg, 1.97; Asp, 1.06; Leu, 0.99. Step 3: PTH-Ile (Systems A, F); residue: Ala, 1.09; Gly, 1.23; Ile, 0.18; Lys, 1.99; Thr, 0.98; Glu, 1.05; Arg, 0.99; Asp, 1.03; Leu, 1.00.


**Peptide C-22 (Residues 55 through 60): Lys-Glu-Ile-Thr-Arg—**Hydrolysis with the aminopeptidase for 1 hour and 4 hours released predominantly lysine with smaller, approximately equal amounts of all the other residues, showing that lysine is the amino-terminal residue. Digestion with carboxypeptidase at 25° for 6 hours (Ile, 0.017; Thr, 0.057; Trp, 0.92) showed that the carboxy-terminal sequence is -Ile-Thr-Arg. By difference, glycine is the 2nd residue.

**Peptide C-23 (Residues 67 through 69): Gly-Lys-Ala-Thr-Glu-Arg-Ala-Pro-Gly-Phe—**Hydrolysis with Carboxypeptidase A (0.1 mM) at 40° for 18 hours yielded only phenylalanine, quantitatively. Tryptic digestion yielded two peptides (Table VIII). Peptide C-23-Ta (Gly, Arg, Lys) must be amino-terminal in Peptide C-23, possessing amino-terminal glycine, as seen from the initial yellow ninhydrin color. Carboxypeptidase B liberated lysine quantitatively. Peptide C-23-Ta contains phenylalanine and hence is carboxy-terminal in Peptide C-23. It is neutral and must therefore contain glutamine. The sequence was established by five steps of the Edman degradation.

**Peptide C-24 (Residues 99 through 104): Lys-Glu-Ala-Thr-Lys Glu—**Since this peptide was obtained in good yield (90%) and has no obvious point of chymotryptic cleavage, it is apparently the carboxy-terminal peptide of dog cytochrome c.

Tryptic digestion yielded five components (Table IX). C-25-Ta, free glutamic acid, must be the carboxy-terminal residue and is preceded by lysine. Peptides C-25-Ta (Ala, Thr, Lys, Glu) and C-25-Tb (Ala, Thr) Lys then establish the partial sequence (Ala, Thr) Lys-Glu for the COOH terminus. By difference and from tryptic specificity, the first 2 residues must both be lysine. This was confirmed by the isolation of C 25 Ta,
Limited Chymotryptic Digest—The following peptides were isolated from the limited chymotryptic digest of cytochrome c.

Peptide C-26 (Residues 60 through 67): (Gly, Glu, Glu, Thr, Leu, Met, Glu) Tyr—This peptide corresponds in composition to the methionine Peptide C-1, Gly-Glu-Glu-Thr-Leu-Met, plus Peptide C-3, Glu Tyr. Of the 3 residues, tyrosine, leucine, and methionine, tyrosine is the preferred point of cleavage by chymotrypsin. Accordingly, after digestion of Peptide C-26 with Carboxypeptidase A (10 μg) for 15 min, only tyrosine (4+) was detected, and it is therefore carboxyl-terminal. Thus Peptide C-26 established the extended sequence Peptide C-1 followed by Peptide C-3.

Peptides C-27a and C-27b (Residues 57 through 59): (Ser, Tyr, Thr) (Asp, Ala, Asn, Lys, Gly, Ile, Thr) Tyr—These two peptides have identical amino acid compositions and hence must differ only in the number of amide groups or in the mode of peptide linkage at an aspartyl residue (α or β). The unique serine and tryptophan residues of dog cytochrome c which occur in Peptides C-4b, Ser-Tyr, and C-22, Lys-Gly-Ile-Thr-Tyr-Trp, respectively, are present. The remaining residues represent the compositions of Peptides C-5a, Thr (Asp, Ala, Asp, Lys) Asn, and C-6a, Thr-Asp-Ala-Asn-Glu-Asn. Prolonged digestion of Peptide C-27a by the aminopeptidase (400 μg per ml) at 40° released stoichiometric amounts of serine, tyrosine, and threonine and small amounts of other residues. It follows that the sequences are in the order Peptide C-4b—Peptide C-6a—Peptide C-22, since the residues removed from Peptide C-27a correspond to those of Peptide C-4b and in the amino-terminal threonine peptide of Peptide C-6a. This limited action of the aminopeptidase resembles that found with Peptide C-6 from which the enzyme removed only threonine.

Peptide C-28 (Residues 58 through 64): (Leu, Lys, Lys, Ala, Thr, Lys, Glu)—This peptide has the composition of Peptide C-25, Lys-Lys-Ala-Thr-Lys-Glu, plus leucine and of Peptide T-23a, (Leu, Lys, Lys), plus Peptide T-8, Ala-Thr-Lys (Glu, Lys). Thus this serves to establish the sequence of Peptide 98 through 104.

Tryptic Peptides

Fraction T-1 (Residue 104): Glu—This fraction contained only one component which had the electrophoretic mobility and ninhydrin color of glutamic acid (Table III). After acid hydrolysis, glutamic acid was identified by chromatography in Solvent 1 and on the analyzer. Free glutamic acid can arise only from the carboxyl terminus of the protein.

Peptide T-2 (Residues 1 through 5): R—CO—Gly-Asp—Val—Glu-Lys—This peptide has the composition and properties of Peptide C-12-Ta. Digestion of Peptide T-2 (5 μl) with pepsin (20 μg) for 24 hours yielded two peptides which were separated on paper electrophoresis at pH 5.6. One is an acidic, ninhydrin-negative Peptide T-2-Pa, R—CO—[Gly, Asp], [Gly, 1.01; Asp, 0.99]; this peptide was detected by the method of Rydon and Smith (16). The neutral Peptide T-2-Pb, (Val, Glu) Lys, [Val, 0.96; Glu, 1.04; Lys, 1.00] must be carboxyl-terminal in Peptide T-2. Hydrolysis of Peptide T-2-Pb with leucine aminopeptidase for 2 hours gave: Val, 0.31; Glu, 0.035; Lys, 0.039, showing that the sequence is Val—Glu—Lys.

The peptide “T-20” (of the same composition as Peptide T-2 described here), isolated by Kreil and Tuppy (17) from the tryptic digest of horse heart cytochrome c, behaved in identical fashion to T-2 on digestion with pepsin. For the ninhydrin-negative peptide they found the sequence Acetyl—Gly—Asp. This was confirmed by repetition of the experiments of Kreil and Tuppy (17) on Peptide T-2-Pa. Hydrolysis in 0.01 M HCI for 2 hours at 105° gave free aspartic acid as the only ninhydrin-positive product. Hydrolysis of Peptide T-2-Pa in 2 M HCI for 30 min at 100° gave some free aspartic acid and a good yield of a negatively charged peptide, which gave a yellow color with ninhydrin and therefore could only be the dipeptide Gly—Asp. The identity of the acyl residue as acetyl was not established in the present case.

Peptide T-3 (Residues 92 through 97): Ala—Asp—Leu—Ile—Ala—Tyr—The complete sequence of this peptide was deduced by enzymic hydrolysis. After 20 min, Carboxypeptidase A (10 μg) released tyrosine quantitatively and smaller, equal amounts of alanine and isoleucine. This supports the sequence —Ile—Ala—Tyr rather than —Ala—Ile—Tyr, because the enzyme removes isoleucine more rapidly than alanine. The 4th residue from the carboxyl-terminal end, leucine, was removed only 50% in 8 hours. Leucine aminopeptidase released alanine predominately in 1 hour.

Peptide T-6 (Residues 40 through 53): Thr—Glu—Gln (Ala, Pro, Gly, Phe, Ser, Tyr, Thr, Asp, Ala) Asn—Lys—Peptide T-6 is neutral and hence must contain two amides and 1 acidic residue. The single serine residue of dog cytochrome c which is present in the sequence Ser—Tyr of Peptide C-4b is present. The presence of both proline and phenylalanine indicates an overlap with the carboxyl-terminal part of Peptide C-23, Gly—Arg—Lys—Thr—Gly—Glu—Ala—Pro—Gly—Phe, since arginine is absent from Peptide T-6. Subtraction of the residues of Peptide C-23-Ta leaves the composition of Peptide C-6a less 1 residue of aspartic acid (or asparagine). The carboxyl-terminal residue, lysine, is also the 5th residue in Peptide C-6. Thus the amino acid composition of this peptide establishes the extended sequence in the order Peptide C-23—Peptide C-4b—Peptide C-6a. This was confirmed by enzymic hydrolysis of Peptide T-6. Hydrolysis with the aminopeptidase (21 μg per ml) at 40° in pH 8.5 am-
monium acetate buffer, 2.5 mm in MtrCl gave in 3 hours: Thr, 0.32; Gly, 0.16; GluNH₂, 0.11; Glu, 0.02; and in 8 hours: Thr, 0.02; Gly, 0.35; GluNH₂, 0.29; Glu, absent. Thus the aminonic terminal sequence is Thr-Gly-Gln—. Hydrolysis with Carboxypeptidase B (0.08 μM) for 3 hours yielded only lysine; addition of Carboxypeptidase A (to 1.25 μM) gave 1 residue each of lysine and asparagine, thus establishing the carboxy-terminal sequence—Asn—Lys.

Peptide T-8 (Residues 101 through 104): Ala-Thr (Lys, Glu); and Peptide T-9a (Residues 101 through 108): (Ala, Thr) Lys—Peptide T-8 has the same composition as Peptide T-25-Tb from the carboxy terminus and the same as the sum of T-9a and T-1 (Glu). Hydrolysis of T-8 with the aminoprotease gave in 2 hours: Ala, 0.143; Thr, 0.093; Glu, 0.012; Lys, not determined, establishing the sequence Ala-Thr— for the first 2 residues. The order of the 2 remaining residues follows from the compositions of T-9a and T-1, placing Lys as the 3rd residue, in agreement with the specificity of trypsin.

Peptide T-9b (Residues 92 through 99): (Ala, Asp, Leu, Ile, Ala) (Tyr, Leu, Lys)—This peptide has the composition of Peptide T-3, Ala-Asp-Leu-Ile-Ala-Tyr, plus that of Peptide T-17, Leu-Lys, (or the first 2 residues of the tripeptide T-23a, Leu-Lys-Lys). Hydrolysis of Peptide T-9b with Carboxypeptidases A and B released quantitatively the 3 carboxyl-terminal residues Tyr, Leu, and Lys. Peptide T-9b was obtained in low yield because most of the peptide was further degraded by hydrolysis of the Tyr-Leu bond to give mainly Peptides T-3 and T-17.

Peptide T-10 (Residues 25 through 28): Gly-Gly—Lys—The unique sequence of this tryptic peptide is confirmed by the yellow ninhydrin color.

Peptide T-11a (Residues 88 through 91): (Thr, Gly, Glu, Arg)—This peptide has 1 of the 2 arginine residues of cytochrome c and must arise from the same part of the sequence as Peptide C-9, Gly-Glu-Arg-Ala-Asp-Leu, and Peptide C-14, Lys-Thr-Gly (Glu, Arg, Ala, Asp) Leu. Peptide T-11b (Residues 6 and 7): Gly-Lys.

Peptide T-12 (Residues 54 and 55): Asn—Lys—This basic peptide contains asparagine.

Peptide T-13 (Residues 74 through 79): Tyr (Ile, Pro, Gly, Thr) Lys—The composition of this peptide, containing 1 of the 4 proline residues, resembles that of Peptide C-10, Ile-Pro-Gly-Thr-Lys, with tyrosine in place of methionine. Since lysine must be carboxyl-terminal in Peptide T-13, it follows that tyrosine is amino-terminal. This was confirmed by digestion for 17 hours with the aminopeptidase which released only tyrosine.

Peptide T-15 (Residues 9 through 13): Ile-Phe-Val-Gln-Lys—This peptide is positively charged at pH 5.6 and hence contains glutamine. The order of the first 3 residues was established by treatment with the aminopeptidase for 1 hour at 25°: Ile, 0.197; Phe, 0.146; Val, 0.042; Glu, 0.018; Lys, not analyzed. By difference, glutamine is the 4th residue.

Peptide T-17 (Residues 98 and 99): Leu—Lys.

Peptide T-18a (Residues 39 through 53): Lys (Thr, Gly, Gin, Ala, Pro, Gly, Phe, Ser, Tyr, Thr, Asp, Ala, Asn) Lys—This peptide has the composition of Peptide T-6 with an additional residue of lysine, which is presumably NH₂-terminal because of the overlap with Peptide C-23.

Peptide T-18b (Residues 80 through 86): Met-Ile-Phe-Ala (Ile, Gly) Lys—Aminopeptidase digestion for 140 min revealed the sequence of the first 3 residues: Met, 0.30; Ile, 0.19; Phe, 0.14. After digestion for 8 hours, some alanine was also detected. This peptide contains 1 of the 2 methionine residues of the protein.

Peptide T-19 (Residues 100 through 104): (Lys, Ala, Thr, Lys, Glu)—This peptide has the same composition as Peptide C-15a and was not investigated.

Peptide T-20 (Residues 56 through 73): (Gly, Ile, Thr) Thr (Gly, Glu, Thr, Leu, Met, Glu) Tyr (Leu, Gin, Asn, Pro, Lys) Lys—The composition of this peptide containing the only tryptophan residue and 1 of the 2 methionine residues of the protein is obviously related to Peptide C-22, Lys-Gly-Ile-Thr—Tyr, to Peptide C-1, Gly-Glu-Glu—Thr-Leu-Met, and to Peptide C-19, Leu—Glu—Asn—Pro—Lys—Lys—Tyr.

Hydrolysis of Peptide T-20 (1 mm) with chymotrypsin (10 μM) at 25° for 2 hours gave three peptides (T-20-Ca,Cb, and Ce) which were separated by electrophoresis at pH 5.6. Peptide T-20-Ca: (Gly, Gin, Glu, Thr, Leu, Met, Glu) Tyr; yield, 65%; composition: Thr, 1.06(1); Glu, 3.10(3); Gly, 0.92(1); Met, 0.00(1); Leu, 1.01(1); Tyr, 0.79(1). Digestion with Carboxypeptidase A (10 μM) at 25° for 2 hours gave three peptides (T-20-Ca, Cb, and Cc) which were separated by electrophoresis at pH 5.6. Peptide T-20-Ca, containing 1 of the 2 methionine residues, is presumably identical to Peptide C-1 plus Glu—Tyr at the COOH terminus. Peptide T-20-Cb: (Leu, Gin, Asn, Pro, Lys) Lys; yield, 94%; composition: Lys, 1.97(2); Asp, 1.04(1); Gin, 1.07(1); Pro, 1.04(1); Leu, 0.88(1). This peptide corresponds in composition to Peptide C-19 less 1 residue of tyrosine. Since it contains both of the lysine residues of Peptide T-20, it must be carboxyl-terminal in T-20. Peptide T-20-Cc: (Gly, Ile, Thr) Thr; yield, 61%; ninhydrin; yellow; composition (after hydrolysis with the aminopeptidase): Thr, 0.90(1); Glu, 3.10(3); Gly, 0.92(1); Met, 0.00(1); Leu, 1.01(1); Thr, 0.79(1). This peptide, containing the unique tryptophan residue, differs from Peptide C-22 in lacking (by tryptic cleavage) the amino-terminal lysine residue. Accordingly it must represent the amino-terminal tetrapeptide sequence in Peptide T-20.

From the foregoing, the alignment of the parts of Peptide T-20 is seen to be in the order Cc—Ca—Cb, and the extended sequence, Peptide C-22—Peptide C-1—Peptide C-3—Peptide C-19.

Peptide T-21a (Residues 87 through 91): (Lys, Thr, Gly, Glu) Arg—The composition of this peptide corresponds to the first 5 residues of Peptide C-14.

Peptide T-21b (Residues 100 through 103): (Lys, Ala, Thr, Lys) Lys—This peptide evidently arises from Peptide T-19 by tryptic cleavage of the carboxyl-terminal Lys—Glu bond.

Peptide T-22a (Residues 26 and 27): His—Lys.

Peptide T-22b (Residues 6 through 8): Gly—Lys—Lys—This peptide gave an initial yellow color with ninhydrin and hence must have amino-terminal glycine.

Peptide T-23a (Residues 98 through 100): (Leu, Gin, Lys—Lys—Aminopeptidase digestion for 1 hour showed that leucine was amino-terminal: Leu, 0.30; Lys, 0.18 (2 × 0.09). This important peptide, which is an extension of Peptide T-17, Leu—Lys, establishes the overlap via a lysine bridge of Peptide T-9b, (Ala, Asp, Leu, Ile, Ala) Tyr—Leu—Lys—Lys—Leu—Ala—Thr—Lys—Glu.

Peptide T-23b (Residues 73 through 79): Lys (Tyr, Ile, Pro, Gly, Thr) Lys—The composition of this peptide is the same as that of Peptide T-13, Tyr—Ile—Pro—Gly—Thr—Lys, plus a 2nd residue of lysine. Since Peptide C-10, Ile—Pro—Gly—Thr—Lys—
Met, is obviously related to this sequence, the extra lysine residue must be amino-terminal in Peptide T-23.

Peptide T-24 (Residues 28 through 38): Thr-Gly-Pro-Asn (Leu, His, Gly, Leu, Phe, Gly) Arg—Peptide T-24 must overlap by 2 residues with the amino-terminal portion of Peptide C 23, Gly-Arg-Lys-Thr-Gly-Gln-Ala-Pro-Gly-Phe. Peptide T-24 contains the unique composition of Peptide C-13, Lys-Thr-Gly-Pro-Asn-Leu—His less the amino-terminal lysine by tryptic cleavage of the Lys-Thr bond. This was confirmed by four steps of the Edman degradation: Step 1: PTH-Thr (System F); residue: Thr, 0.18; Gly, 3.24; Pro, 0.82; Asp, 1.02; Leu, 2.10; His, 0.95; Phe, 1.02; Arg, 0.88, Step 2: PTH Gly (System A, F); residue: Thr, 0.11; Gly, 2.28; Pro, 1.00; Asp, 1.08; Leu, 1.93; Phe, 0.96; His and Arg, not determined, Step 3: residue: Thr, 0.13; Gly, 2.20; Pro, 0.37; Asp, 0.98; Leu, 2.02; Phe, 1.00; His and Arg, not determined, Step 4: PTH-Asn (Systems C, F); residue: Thr, 0.13; Gly, 2.21; Pro, 0.36; Asp, 0.76; Leu, 2.00; His, 0.97; Phe, 1.02; Arg, 1.00. The remaining residues of Peptide T 24 correspond to Peptide C 4c, Gly-Leu-Phe. Hence, Peptide T-24 establishes by overlap the extended sequence Peptide C-13—Peptide C-4c—Peptide C-23.

Peptide T-H (Residues 14 through 22): (CySH-Ala, Gln-CySH, His, Thr, Val, Glu) Lys—This is the performic acid-oxidized tryptic hemopeptide. Lysine is presumably carboxyl-terminal. This peptide is identical in composition and other properties to the oxidized tryptic hemopeptide from horse heart cytochrome c. As in the case of the horse (18) and human (5) proteins, chymotrypsin produced extensive hydrolysis of dog cytochromes between consecutive lysine residues at 7 and 8, 86 and 87, and 99.

**Complete Sequence**—From the evidence given above for the compositions and sequences of the chymotryptic and tryptic peptides from dog heart cytochrome c, it is possible to place within this unique sequence of 104 residues shown in Fig. 3 all peptides obtained. Parts of these extended sequences have already been deduced in connection with the major overlapping peptides. Since it is apparent that the sequence of dog heart cytochrome c is homologous with the sequences of other mammalian cytochromes, it is unnecessary to discuss the derivation of the sequence in detail. Only the longer peptides useful in deriving the overlaps are noted in the figure, although many of the smaller peptides were important in establishing the detailed sequence. The sequence of the tryptic heme peptide has been assumed to be identical with that of horse cytochrome c.

**Sites of Proteolytic Action: Chymotrypsin**—In addition to the typical hydrolysis at the carboxyl bonds of all the residues of Tyr, Phe, Trp, Leu, and Met, strong action was found at other bonds, normally less susceptible, whenever these were followed by lysine, notably at the His-Lys (Residues 26, 27) and the Asn-Lys (Residues 55, 56) bonds. Good cleavage occurred at the His-Gly (Residues 33, 34) bond but poor cleavage at the Asn-Leu (Residues 31, 32) bond.

**Discussion**

Fig. 3. Amino acid sequence of dog heart cytochrome c. Only the major peptides from the tryptic (T) and chymotryptic (C) hydrolysates which were most useful in providing overlapping sequences are indicated.
and 100. In addition, several atypical bonds were hydrolyzed: Lys-Gly, (Residues 22, 23) 8%; Lys-Thr, (Residues 87, 88) 5%; and Thr-Gly, (Residues 88, 89) 20%. Hydrolysis at a carboxyl peptide bond of threonine was observed by Anderer et al. (19) in the chymotryptic digest of tobacco mosaic virus protein. In view of the extensive hydrolysis produced by chymotrypsin at unusual peptide bonds, it is probable that digestion was continued longer than necessary and that better yields of authentic chymotryptic peptides would have been obtained with a shorter period of digestion. Nevertheless, the highest yields found were little better than 40%, even when formation of the peptide involved the cleavage of normally susceptible peptide bonds and when sites of possible secondary cleavage were absent. It is thus possible that, despite exposure to 80% ethanol, a considerable proportion of the protein was not unfolded at the time of digestion and that it resisted chymotryptic hydrolysis (7, 18).

Trypsin—Since trypptic hydrolysis was performed for a short time (2 hours) anomalous cleavage was observed only at the Tyr-Leu (97, 98) bond as observed with a much longer period of trypptic digestion of human heart cytochrome c by Matsubara and Smith (5). Thus Peptide T-3, Ala-Asp-Leu-Ile-Ala-Tyr, was found in 54% yield whereas the authentic trypptic Peptide T-9b was found in 8.5% yield. It is likely that most, if not all, of this cleavage was caused by low levels of chymotrypsin in the chemical crystalline trypsin used. The preferential action of trypsin on Peptide C-23 (Table VIII) to yield mainly the tripeptide Gly-Lys-Arg of interest, and in contrast to that on Peptide C-12 (Table VI) which produced hydrolysis of the two lysyl bonds of the sequence −Gly−Lys−Lys−Be−Phe to the same extent.

Specificity of Exopeptidases—In this work, maximal use was made of exopeptidases in conjunction with quantitative amino acid analysis to determine the terminal sequences of peptides. This was greatly facilitated by knowledge of the sequence of the homologous horse protein (4). In favorable cases, the sequences of 3 or 4 consecutive residues were established on the basis of a single experiment, e.g., Peptides C-1, C-10, C-14, C-15, T-3, T-13, T-15, and T-18b. The sequences of Peptides T-3, Ala−Asp−Leu−Ile−Ala−Tyr, and T-15, Ile-Phe-Val-Gln-Lys, were determined entirely by enzymic hydrolysis. The position of proline residues in Peptide C-10, C-13, C-19, and C-23 could be deduced from the failure to remove the adjacent residues after prolonged exposure to both aminopeptidase and carboxypeptidase.

In most experiments a single concentration of the aminopeptidase sufficed. However, the presence of glycine, proline, and aspartic acid residues severely limited the extent of hydrolysis, e.g., in Peptides C-0 and C-22, and in Peptides C-15b, C-18, and C-20, in which the amino-terminal Ala−Gly−Ile−sequence was highly resistant. In Peptides C-6a and C-27a the aspartic acid residue, 50, was completely resistant to the prolonged action of high levels of the aminopeptidase at 40°, possible because of isomerization at the aspartyl peptide bond (20).

It was necessary to employ a wide range of concentrations of carboxypeptidase A, the usefulness of the higher levels of enzymes being partly, but not entirely, limited by the low solubility of the enzyme. Here, as in the work on the chymotryptic peptides of horse heart cytochrome c (7, 18), partial liberation of lysine residues was found, e.g., with Peptides C-6a, Thr−Asp−Ala−Asn−Lys−Asn; C-10, Ile−Pro−Gly−Thr−Lys−Met; and also with Peptides C-12, C-15b, C-15c and C-23. The sluggish action of the enzyme against threonine residues in Peptides C-1, C-2, C-10, and C-99 is noteworthy; however, the terminal residue of Peptide C-15b, Ala−Gly−Ile−Lys−Lys-Thr, was hydrolyzed more readily. The rapid liberation of the terminal histidine residue of Peptide C-13, Lys−Thr−Gly−Pro−Gln−Leu−His, permitted determination of the Leu−His sequence (18), histidine being predominantly a neutral aromatic residue at pH 8.

From the action of carboxypeptidase B on Peptide C-18, Ala−Gly−Ile−Lys−Lys, in removing 1 residue of lysine quantitatively, it would seem that the Ile−Lys bond is relatively resistant to the action of carboxypeptidase B. In contrast, carboxypeptidase A hydrolyzes the Ile−Phe bond of Peptide C-12 (Table VI) readily.

Comparative Structure—The numbers of amino acid sequence differences between the cytochromes c of dog and other mammals are, respectively, pig, sheep and ox, 4 (2); horse, 6 (4); and man, 11 (5). The significance of these and other differences among the cytochromes c of various species has been presented elsewhere, both from the viewpoint of gross evolutionary relations and structure-function considerations (2, 3, 21).

SUMMARY

Cytochrome c from dog heart muscle has the following amino acid composition: Trp, Lys4, His1, Arg2, Asp1, Thr, Ser, Glu6, Pro6, Gly16, Ala19, Val2, Cys2, Met3, Ile13, Leu9, Tyr4, Phe4, or 104 residues as is the case for other vertebrate cytochromes c. Dog heart cytochrome c was digested with chymotrypsin and with trypsin, and the digests were resolved by ion exchange chromatography on Dowex 50 X2 with volatile buffers. The sequences of the isolated peptides account for the complete composition and the sequence of the protein. Dog cytochrome c differs from the human protein in 11 residues and from the horse protein in 6 residues.

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Amino Acid Sequence of Dog Heart Cytochrome c
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