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Study of a Peptic Degradation Product of Cytochrome c

I. Purification and Chemical Composition

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A ferriporphyrin c-peptide obtained from *beef* cytochrome c by digestion with pepsin and previously described by Tsou [1,2] has been purified by a partition chromatographic method.

The peptide component of this haemopeptide has been shown to be composed of eleven amino acid residues arranged in the following sequence: Val-Glu(NH_2)-Lys-CySH-Ala-Glu(NH_2)-CySH-His-Thr-Val-Glu. The two cysteine residues occurring in this chain are linked to the side-chains 2 and 4 of the ferriporphyrin prosthetic group *via* two thioether bridges.

The sequence of amino acid residues in the ferriporphyrin c-peptide prepared by peptic degradation of *salmon* cytochrome c is identical with the one found in the split product of beef cytochrome c. The corresponding peptide derived from *chicken* cytochrome c, however, differs from the others by the replacement of the alanine residue by a serine residue.

Through digestion of horse cytochrome c with *pepsin*, Tsou [1] has obtained a brown-red coloured split product of relatively low molecular weight. This "pepsin-modified cytochrome c" contained the prosthetic group of the enzyme linked to a peptide component which represented a part of the original protein moiety of cytochrome c. The haemopeptide exhibited interesting enzymatic properties [2] different from those of native cytochrome c. Tsou's degradation method was recently applied to cytochromes of three different animal species [3]; the peptic split products of beef, chicken and salmon cytochromes c were prepared and found to show a high degree of similarity.

In another recent investigation[4] a *tryptic* degradation product of cytochrome c was purified and its chemical structure determined. The peptide component was shown to contain nine amino acid residues arranged in the following sequence *:

CySH—Ala—Glu(NH_2)—CySH—His—Thr—Val—Glu—Lys.

* Throughout this paper the abbreviations for the amino acid residues suggested by Brand and Edsall [5] will be used. The symbol for cysteic acid will be $CySO_3H$. The arrangement of amino acid residues in peptides will be described as suggested by Sanger [6].

The two cysteine residues occurring in this chain are linked to the side chains 2 and 4 of haematohaemin c[7] *via* two thio-ether bridges[8, 9]. The same sequence of nine amino acid residues could be demonstrated to occur in the ferriporphyrin c-peptides obtained by tryptic degradation of three different mammalian cytochromes[10].

In the present investigation the *peptic* degradation product, which Tsou had thought to be a pure substance, has been prepared free from contaminating peptides, and the chemical structure of the purified product has been established. By comparing the sequence of amino acid residues in the haemopeptides isolated from peptic digests of beef, chicken and salmon cytochromes c, an unexpected instance of species specificity of protein structure has been found.

MATERIAL

Cytochrome c. Beef, chicken * and salmon cytochromes were prepared from minced hearts by the method of Keilin and Hartree[11]. In the case of salmon, however, the initial extraction of the enzyme from the mince was carried out using dilute sulphuric acid[3] instead of trichloroacetic acid. After fractionation of the extracts with ammonium sulphate and precipitation of the enzyme with trichloroacetic acid, the cytochromes were dissolved in dilute ammonia, dialyzed against 0.05 *N* ammonia and finally against distilled water.

Beef cytochrome c was further purified as described by Paléus and Neilands[12]. For chicken and salmon cytochromes the method of Margoliash[13] was followed. In all three cases Amberlite XE-64 was the ion exchange resin used. The preparations of cytochrome c thus obtained had iron contents of 0.41 % (beef), 0.40 % (chicken) and 0.31 % (salmon).

Proteolytic enzymes. The samples of pepsin and of trypsin used in this work were crystallized products of the "Bios Laboratories". Their proteolytic activities were tested by Anson's haemoglobin method[14] and found to be, respectively, 0.0266 units/mg and 0.0065 units/mg. The crystallised *Bacillus subtilis* proteinase "subtilisin"[15, 16] was kindly made available to us by Professor K. Linderstrøm-Lang and Ing. M. Ottesen.

METHODS

Preparation of the peptic split product of beef cytochrome c. For the splitting of cytochrome c with pepsin the procedure of Tsou[1] was adopted. However, in contrast to his experience, after digestion in acid solution and neutralisation of the digest, the degraded pigment could not be precipitated quantitatively by making the solution 85 % saturated with ammonium sulphate. This indicated incomplete splitting. The incompletely digested material had to be subjected to another incubation with pepsin **. In the following a representative two-stage digestion experiment is described in detail.

A solution of 0.830 g beef cytochrome c in 83 ml distilled water was mixed with 20 ml 0.3 *N* HCl. To the acid solution (pH 1.55) was added 16.1 mg pepsin dissolved in a few ml of 0.01 *N* HCl. The mixture was incubated 28 h at 25° C and the digestion stopped by adding 20 ml 0.3 *N* NaOH. Solid ammonium sulphate was added to the slightly alkaline solution (pH 8.5) to make it 85 % saturated. Most of the pigment was precipitated, but on filtration the solution also proved to be somewhat coloured. The filtrate was discarded. The precipitate was dissolved from the filter using 0.01 *N* HCl and the pH of the

* We wish to thank AB. Findus, Hälsingborg, for kindly supplying frozen chicken hearts.

** The incomplete digestion of cytochrome samples appears to have been caused by the presence of some substance inhibiting the peptic proteolysis. The inhibitor is likely to have its origin in the ion exchange resin used for the purification of the cytochromes. The inhibitor can be removed from the cytochrome preparations by reprecipitating them with trichloroacetic acid from ammonium sulphate solution. Inhibitor-free cytochrome preparations do not require the two-step digestion with pepsin described above.

solution adjusted to pH 1.60 with 0.3 *N* HCl. To the acid solution (124 ml) 9.8 mg pepsin were added. After 20 h at 28° C the digestion mixture was neutralized by the addition of 0.3 *N* NaOH and made 85 % saturated with ammonium sulphate. Now the precipitation of the pigment was complete. The precipitate was collected by filtration and dissolved from the filter with 100 ml water containing a few drops of ammonia. The dark red solution was mixed with a solution of 280 g ammonium sulphate in 500 ml water and the pigment precipitated by the addition of 19 ml of 20 % aqueous trichloroacetic acid. The precipitate was centrifuged down and dissolved in the smallest possible amount of 0.05 *M* NH_3. The ammoniacal solution was dialyzed in the cold against 0.01 *M* phthalate buffer of pH 5.0 (three changes) and finally against distilled water (two changes). This resulted in an almost complete precipitation of the pigment. The brown-red powdery material was dissolved in 0.02 *M* NH_3 and the precipitations by ammonium sulphate and trichloroacetic acid repeated. Following dialysis against phthalate buffer and distilled water, the peptic degradation product of cytochrome c was collected by centrifugation and dried. Yield: 101 mg.

Purification of the ferriporphyrin c-peptide by partition chromatography. The final purification of the peptide was achieved using essentially the same method as that previously described for the tryptic split product of cytochrome c[4]. To 15 g of carefully washed and dried Hyflo Supercel was added 5.5 g of the lower layer of a freshly prepared mixture of *n*-butanol, glacial acetic acid and water (4 : 1 : 5). After thorough mixing, the Hyflo Supercel was suspended in a suitable volume of the upper phase of the solvent system so as to form a slurry and was poured into a chromatography tube 1.9 cm in diameter. After settling, the Hyflo Supercel formed a column 18.5 cm high. On the top of this column was placed 74.5 mg of the ferriporphyrin c-peptide dissolved in the smallest possible volume of the lighter phase. The chromatogram was developed with the help of larger amounts of the upper layer of the solvent mixture. In order to prevent the pigment from being partially destroyed, the ferriporphyrin c-peptide during chromatography had to be protected from direct illumination. The main component formed a dark brown band nicely moving down the column, the R_F value being approximately 0.9 in the beginning and somewhat decreasing in the course of the development. The main band was closely preceded by a lighter brown-coloured fraction and followed by a greenish band. Sometimes another brown-coloured band travelling slowly was observed. The effluent containing the main fraction was collected separately and evaporated to dryness *in vacuo* in a desiccator over conc. H_2SO_4 and solid KOH. The dried material was dissolved in as little 0.05 *M* ammonia as possible and dialysed in the cold against several changes of 0.01 *M* phthalate buffer pH 5 and subsequently against distilled water. The precipitated pure ferriporphyrin c-peptide was centrifuged down and dried. The final yield was 41 mg.

Table 1. Amino acid composition of the peptic degradation products of three different cytochromes.

Ferriporphyrin c-peptide preparation		Amino acids found in the hydrolysate					
Beef	A	Glu	Thr	Ala	His	Val	Lys
	B	2.99	1.01	1.00	1.11	2.09	1.12
Salmon	A	Glu	Thr	Ala	His	Val	Lys
Chicken	A	Glu	Thr	Ser	His	Val	Lys

A: Amino acids determined by qualitative paper chromatography.
B: Moles of amino acid found, using the method of Moore and Stein [17, 18].

Determination of the constituent amino acids. For a qualitative determination 0.4 mg of the purified preparation were hydrolyzed overnight in a sealed capillary with 5.7 *N* HCl at 105° C, and the hydrolysate subjected to two-dimensional chromatography on Whatman No. 4 filter paper using phenol/NH_3/HCN in the first and collidine/lutidine/water in the second direction. For a quantitative determination 1 mg of the ferriporphyrin c-peptide was hydrolysed and the resulting amino acid mixture analysed by a modification [17] of the method of Moore and Stein [18]. The results of these determinations are summarised in Table 1.

Determination of the amino-terminal amino acid residues. The method used was Sanger's DNP technique [19]. 2.25 mg of the ferriporphyrin c-peptide were reacted with fluorodinitrobenzene in aqueous ethanol containing sodium bicarbonate. After 10 hrs. standing at room temperature with occasional shaking, the reaction mixture was diluted with water and extracted three times with peroxide-free ether. On acidification with HCl, the DNP derivative of the ferriporphyrin c-peptide precipitated quantitatively in the form of a brown powder. It was centrifuged down, washed with water, dried, and finally washed with ether.

0.5 mg of the DNP-derivative was hydrolysed with 5.7 *N* HCl at 105° in a sealed capillary. After 7 hrs. heating the yellow solution, in which black grainy material derived from the porphyrin moiety was floating, was diluted with water and extracted with ether. Paper chromatography of the aqueous solution with the butanol-acetic acid solvent of Partridge [20] revealed the presence of ε-DNP-lysine and im-DNP-histidine, besides free amino acids. In the ethereal extract DNP-valine was identified by the paper chromatographic methods of Biserte and Osteux [21] and of Blackburn and Lowther [22]. This DNP amino acid was further characterized by eluting it from the paper after chromatographic separation and heating it with concentrated ammonia solution [23] in a sealed capillary; after 12 hrs. at 108° a considerable part of the DNP-derivative had been split and free valine could be identified by paper chromatography.

On the paper chromatograms of the ether-soluble fraction a slow-moving and considerably weaker yellow spot (R_F 0.13 in phenol-*iso*amyl alcohol-water solvent [21]) was also found, besides the strong spot of DNP-valine (R_F 0.75). This substance was obtained as the main ether-soluble product, when the DNP-derivative of the ferriporphyrin c-peptide was dissolved in a mixture of equal parts of glacial acetic acid and concentrated HCl and hydrolysed at 108° for 1/2 h only. On further hydrolysis with 5.7 *N* HCl (12 hrs. at 108°) this yellow product gave rise to DNP-valine and free glutamic acid and was thus shown to be DNP-Val-Glu.

Splitting with trypsin. 1.0 mg of the peptic split product of bovine cytochrome c was dissolved in 0.50 ml of a 0.005 *M* ammonia solution, mixed with 0.05 ml 0.2% aqueous trypsin solution and incubated 23 hrs. at 36° C. The digest was then heated 5 minutes in a boiling water bath to inactivate the trypsin and was evaporated to dryness. The dry brown material was dissolved in a small volume of dilute ammonia and the solution placed on a strip of Whatman No. 4 paper in the form of a line 9 cm long. The chromatogram was developed one-dimensionally using butanol-acetic acid [20] as the solvent. The pigment moved down the paper in the form of a broad diffuse brown band (R_F 0.22—0.45). After drying of the chromatogram, a narrow strip was cut off from its long edge and sprayed with ninhydrin; on heating a strong violet colour developed at R_F 0.13. The ninhydrin-positive substance was eluted from the untreated part of the chromatogram.

One third of the eluted material was hydrolysed with 5.7 *N* HCl overnight at 105°. In the hydrolysate 3 amino acids were identified by paper chromatography: valine, glutamic acid and lysine. One third of the peptide was investigated using the DNP technique [6]; the dinitrophenylated peptide, on hydrolysis with 5.7 *N* HCl (10 hrs. at 105°), yielded DNP-valine, ε-DNP-lysine and glutamic acid. The remainder of the eluted peptide was subjected to electrophoresis using the method previously described by the senior author [24] ("Munktell 20, 150 g" filter paper; *M*/15 phosphate buffer pH 6.4; 75 volts; 2.5 mA; 5 hrs.) and it was found to migrate towards the cathode.

Preparation of the porphyrin-free peptide component of the peptic split product of cytochrome c. The thio-ether bridges were split by the method of Paul [25] following the procedure previously described for the removal of the pigment moiety from the tryptic degradation product of cytochrome c[4]. The thiol groups set free through the action of silver salts were oxidized to sulphonic acid groups by treatment with performic acid [26].

12 mg of the peptic split product of cytochrome c were dissolved in 5 ml of 60 % acetic acid, mixed with a warm solution of 80 mg silver sulphate in 10 ml water and heated at 50° for 9 hrs. Then the solution was evaporated to dryness *in vacuo* at low temperature. To the residue a mixture of 3 ml 87 % formic acid and 0.3 ml 30 % hydrogen peroxide was added. After 20 minutes standing at room temperature the reaction mixture, which had turned light yellow through oxidative degradation of the porphyrin, was diluted with water and evaporated to dryness *in vacuo* at a temperature not exceeding 30° C. Addition of water and evaporation to dryness was repeated twice in order to remove all the hydrogen peroxide. The residue was dissolved in 40 ml distilled water of 60°, 2 drops of 20 % H_2SO_4 added and H_2S passed into the warm solution. The precipitate of Ag_2S was removed by filtration and the clear filtrate reduced to a volume of about 10 ml by careful evaporation *in vacuo* at a temperature not higher than 25°. The sulphuric acid was removed by extracting three times with a solution of 0.25 ml tri-*n*-octylamine in 10 ml chloroform [27, 28], followed by chloroform and ether. The aqueous solution was evaporated to dryness leaving behind the porphyrin-free peptide.

A small amount of the peptide was heated 20 hrs. with 5.7 *N* HCl at 105°; in the hydrolysate the following amino acids were found by paper chromatography: $CySO_3H$, Glu, Thr, Ala, Val, His and Lys.

Partial acid hydrolysis of the porphyrin-free peptide. Separation and examination of the split products. The peptide prepared from 12 mg of bovine ferriporphyrin c-peptide as described above was hydrolysed 4 days at 37° C in concentrated HCl. After removal of excess HCl, the hydrolysate was adjusted to pH 5 and fractionated in the four-compartment ionophoresis cell described by Sanger and Tuppy [29]. The peptide and amino acid mixtures present in the acidic and neutral fractions were investigated by two-dimensional paper chromatography using essentially the same methods as those reported by Sanger and coworkers [29, 30].

Digestion of the porphyrin-free peptide with subtilisin. Porphyrin-free peptide obtained from 6 mg of ferriporphyrin c-peptide was dissolved in 0.5 ml water and adjusted to a pH of 7 to 8 by dropwise addition of 0.05 *M* ammonia. A solution of 0.2 mg subtilisin in 0.1 ml water was added and the mixture incubated at 37° for 23 hrs. After heating for 3 minutes in a boiling water-bath to inactivate the enzyme, the solution was evaporated to dryness *in vacuo*. No preliminary fractionation was necessary and the digest was investigated directly on paper chromatograms.

The peptic split products of salmon and chicken cytochromes. Salmon and *chicken* cytochromes c were digested with pepsin in the same way as has been described above for beef cytochrome. In the purification involving partition chromatography on Hyflo Supercel, the ferriporphyrin c-peptides obtained did not differ in their behaviour from the cor-

Table 2. Iron and nitrogen determinations.

Ferriporphyrin c-peptide preparation	% Fe		% N		Mol.wt		Molar ratio N : Fe	
	found	calcd.	found	calcd.	found (based on Fe content)	calcd.	found	calcd.
Beef prepn. 1	2.70	2.97 *			2 068	1 879 *		
Beef prepn. 2	2.85	2.97 *			1 959	1 879 *		
Beef prepn. 3	2.76	2.97 *	13.85	14.91 *	2 023	1 879 *	19.99	20
Salmon	2.76	2.97 *			2 023	1 879 *		
Chicken	2.77	2.95**			2 016	1 895**		

* Based on the formula $C_{50}H_{84}O_{17}N_{16}S_2 \cdot C_{34}H_{32}O_4N_4Fe+OH^-$.
** Based on the formula $C_{50}H_{84}O_{18}N_{16}S_2 \cdot C_{34}H_{32}O_4N_4Fe+OH^-$.

responding peptide of bovine origin. The amino acid composition and the iron contents of the three haemopeptides are compared in Tables 1 and 2. After splitting with silver salt in acetic acid, the porphyrin-free peptides were subjected to further digestion with subtilisin and the resulting mixtures of lower peptides investigated by paper chromatography.

RESULTS AND DISCUSSION

In the hydrolysate of the purified ferriporphyrin c-peptide of *bovine* origin, 6 amino acids were identified by paper chromatography: glutamic acid, threonine, alanine, histidine, valine and lysine (Table 1). For comparison, it may be noted that the peptic degradation product of cytochrome c as obtained by the isolation procedure of Tsou [1], which did not include a partition chromatographic purification, contained aspartic acid, glycine, leucine, phenylalanine, proline and serine [2,3] in addition to the six amino acids found in the purified preparation.

A quantitative analysis by the method of Moore and Stein [17] gave evidence that the peptide was composed of one residue of threonine, alanine, histidine and lysine, while valine occurred twice and glutamic acid three times (Table 1). Neither paper chromatography nor ion exchange chromatography revealed the presence of cysteine or cystine, but this is not inexplicable since it is known that the two sulphur containing amino acid residues linked to the prosthetic group of cytochrome c are not set free by mild acid hydrolysis, but remain combined with the pigment in the form of porphyrin c [31,9]. However, after fission of the thioether bridges by the silver salt method of Paul [25] and oxidation of the —SH groups to —SO_3H groups, the porphyrin-free peptide moiety, after hydrolysis with acid, yielded cysteic acid in addition to the other six amino acids. Since the cysteic acid found is derived from two porphyrin-bound cysteine residues, and since the six other amino acids account for nine amino acid residues, it can be concluded that the peptide component of the peptic degradation product of cytochrome c is composed of 11 amino acid residues.

In the ferriporphyrin c-peptide only one α-amino group was found free to react with fluorodinitrobenzene; after dinitrophenylation and hydrolysis, one of the two valine residues was recovered in the form of DNP-valine. After short hydrolysis, however, DNP-Val-Glu was found in high yield. This indicates that the 11 amino acid residues of the ferriporphyrin-c-peptide are arranged in one single peptide chain having the dipeptide sequence Val-Glu in the N-terminal position. In contrast, Tsou [2] had found that his "pepsin-modified cytochrome c" contained 3.4 α-amino nitrogen atoms per atom of iron.

On treatment of the ferriporphyrin c-peptide with trypsin, a small fragment was split off which, on acid hydrolysis, yielded valine, glutamic acid and lysine. DNP treatment showed valine to be the N-terminal residue. Lysine, on the other hand, could be assigned the C-terminal position since trypsin is known to split specifically peptide bonds involving the carboxyl groups of lysine and arginine residues [32-34,4]. A tripeptide containing one glutamic acid and one lysine residue would be expected to behave as a neutral substance at a pH of 6 to 7; the peptide under investigation, however, at a pH of 6.4, proved to be a basic substance. This suggests that the glutamic acid residue is not present as such, but is in the form of its amide, glutamine, and that the tripeptide is Val—Glu(NH_2)—Lys.

Table 3. Partial acid hydrolysis of bovine "porphyrin-free peptide". Peptides from the acidic fraction (see Fig. 1.).

Spot No. (Fig. 1)	Colour with ninhydrin	Amino acids present after hydrolysis	After DNP treatment and hydrolysis		Structure *
			DNP amino acids	amino acids	
1 **	purple	$CySO_3H$ (× × × ×)			cysteic acid
2 **	purple	$CySO_3H$ (× × ×) Glu (× × ×)	DNP-Glu (× ×)	$CySO_3H$ (×)	Glu-$CySO_3H$
3	purple	$CySO_3H$ (×) Glu (×) His (×)	DNP-Glu (×) im-DNP-His (×)	$CySO_3H$ (×)	Glu-[$CySO_3H$, His]
4	yellow → purple	$CySO_3H$ (×) Glu (× ×) Ala (×)			[$CySO_3H$, Ala, Glu]
5	yellow → purple	$CySO_3H$ (× ×) Ala (× × ×)	DNP-$CySO_3H$(× ×)	Ala (× ×)	$CySO_3H$-Ala
6	purple	Glu (× × ×)			glutamic acid
7	purple	Glu (×) Ala (×)	DNP-Ala (?)	Glu (?)	Ala-Glu
8	purple	Glu (×) Val (×)	DNP-Val (?)	Glu (?)	Val-Glu

* Square brackets indicate that the sequence of amino acids inside the brackets has not been established.

** Spots 1 and 2 can be separated by prolonged chromatography in butanol-acetic acid.

Further information about the amino acid sequence in the ferriporphyrin c-peptide was obtained by hydrolysis of its porphyrin-free peptide moiety and investigation of the split products. The results obtained with the acidic and neutral fractions of a partial acid hydrolysate are recorded in Figs. 1 and 2 and Tables 3 and 4. Fig. 3 and Table 5 give results obtained through the use of the proteolytic enzyme subtilisin.

Special attention must be given to peptides 3 and 4 in Fig. 3 and Table 5. These two dipeptides would seem to be identical with the dipeptides 7 and 8 (Ala—Glu and Val—Glu) found in the acid hydrolysate (Fig. 1 and Table 3), but they differ markedly in their R_F values. Whereas the peptides 7 and 8 (Fig. 1) show chromatographic properties previously found to be exhibited by Ala—Glu [4] and Val—Glu [29], the dipeptides 3 and 4 of the enzymic digest (Fig. 3) are travelling faster in phenol and slower in butanol-acetic acid. This behaviour appears to be characteristic of peptides containing glutamine rather than glutamic acid, and it may be mentioned that free glutamine shows the same difference in chromatographic properties with respect to free glutamic acid. The conclusion that peptides 3 and 4 of the enzymic digest are Ala—Glu(NH_2) and Val—Glu(NH_2) is strongly supported by the known

Table 4. Partial acid hydrolysis of bovine "porphyrin-free peptide". Peptides from the neutral fraction (see Fig. 2).

Spot No. (Fig. 2)	Colour with ninhydrin	Amino acids present after hydrolysis	After DNP treatment and hydrolysis		Structure
			DNP amino acids	amino acids	
1 *	purple	$CySO_3H$ (× ×) Lys (× × ×)	di-DNP-Lys (×)	$CySO_3H$ (× ×)	Lys-$CySO_3H$
2 *	yellow → grey	$CySO_3H$ (× × ×) His (× × × ×)	DNP—$CySO_3H$ (× × ×) im-DNP-His (× × × ×)		$CySO_3H$—His
3	purple	Glu (×) Ala (×)	DNP-Ala (×)	Glu (×)	Ala-Glu
4	purple	Ala (× ×)			alanine
5	purple	Glu (× ×) Val (× ×) Lys (× ×)	DNP-Val (× ×) ε-DNP-Lys (× ×)	Glu (× ×)	Val-[Glu, Lys]
6	purple	Glu (× ×) Val (× ×)	DNP-Val (×)	Glu (×)	Val-Glu
7	grey-brown	Glu (× × ×) Thr (× ×) Val (× × ×)	DNP-Thr (×)	Glu (× ×) Val (×)	Thr-[Val, Glu]

* Spots 1 and 2 were separated well when collidine-lutidine was used as the second solvent instead of butanol-acetic acid.

proteolytic specificity of subtilisin; this bacterial enzyme has previously been reported to attack peptide bonds involving the carboxyl groups of glutamine and asparagine residues [35, 36], whereas the amide groups are left intact. Mineral acid, on the contrary, readily breaks amide bonds.

In Table 6 all the peptides are listed which have been obtained from the peptic degradation product of bovine cytochrome c. From their structure the following sequence of amino acids can be deduced:

$$Val—Glu(NH_2)—Lys—CySO_3H—Ala—Glu(NH_2)—CySO_3H—His—Thr—[Val\ Glu].$$

In this sequence the order of the last two residues has not yet been established in a straightforward manner. It can be assumed, however, that the order is not different from that occurring in the corresponding porphyrin-free peptide derived from the *tryptic* degradation product of cytochrome c [4, 10],

$$CySO_3H—Ala—Glu(NH_2)—CySO_3H—His—Thr—Val—Glu—Lys,$$

since both peptides have their origin in the same part of the protein moiety of cytochrome c. The perfect agreement between the two enzymic degradation products as regards amino acid sequence is clear evidence that no rearrange-

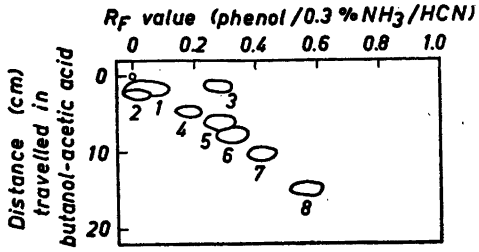

Fig. 1. Partial acid hydrolysate of bovine "porphyrin-free peptide". Chromatogram of the acidic fraction (see Table 3).

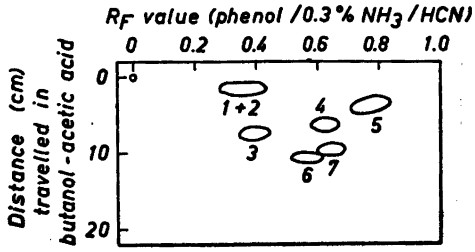

Fig. 2. Partial acid hydrolysate of bovine "porphyrin-free peptide". Chromatogram of the neutral fraction (see Table 4).

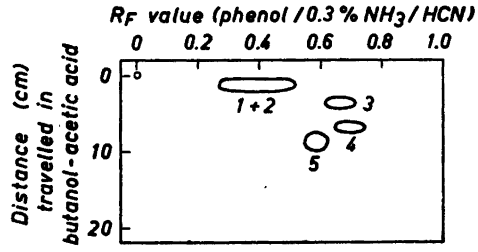

Fig. 3. Chromatogram of subtilisin hydrolysate of bovine "porphyrin-free peptide" (see Table 5).

ment has taken place within the peptide chain during enzymic digestion. Taking this for granted and remembering that the two cysteic acid residues of the porphyrin-free peptide are derived from two porphyrin-bound cysteine residues, the sequence of amino acids in the peptic degradation product of bovine cytochrome is concluded to be

$$\text{Val—Glu}(NH_2)\text{—Lys—CySH—Ala—Glu}(NH_2)\text{—}$$
$$\text{CySH—His—Thr—Val—Glu.}$$

On the basis of this peptide formula and of the known nature of the porphyrin component of cytochrome c[7], it is possible to calculate the iron and nitrogen contents of the peptic degradation product. In Table 2 the calculated figures are compared with the values actually found by analysis. The latter values prove to be considerably lower than expected although the iron content (2.76 %) is obviously far superior to that reported by Tsou (2.21 %) [2]. However, the molar ratio between the values found for nitrogen and iron is in excellent agreement with the theoretical figure of 20. This gives definite indication

Table 5. Subtilisin hydrolysis of bovine "porphyrin-free peptide" (see Fig. 3).

Spot No. (Fig. 3)	Colour with ninhydrin	Amino acids present after hydrolysis	After DNP treatment and hydrolysis		Structure
			DNP amino acids	amino acids	
1 *	purple	$CySO_3H$ (× × ×) Lys (× × ×)			[Lys, $CySO_3H$]
2 *	yellow → grey	$CySO_3H$ (× ×) His (× ×)			[$CySO_3H$, His]
3	purple	Glu (× × ×) Ala (× ×)	DNP-Ala (× ×)	Glu (× × ×)	Ala-Glu
4	purple	Glu (× × ×) Val (× × ×)	DNP-Val (× × ×)	Glu (× × ×)	Val-Glu
5	brown-yellow	Glu (× × ×) Thr (× ×) Val (× × ×)	DNP-Thr (× × ×)	Glu (× × ×) Val (× × ×)	Thr-[Val, Glu]

* Spots 1 and 2 were eluted from the 2-dimensional chromatogram (Fig. 3) and separated by 1-dimensional chromatography using collidine-lutidine as the solvent.

that the haemopeptide preparation is free from contaminating nitrogenous substances such as peptide impurities, though not free from other substances which may increase the molecular weight and lower iron and nitrogen values, such, for instance, as inorganic or organic (*e.g.*, phthalate) ions.

In Tables 1 and 2 results are included which have been obtained with the peptic degradation products of *salmon* and *chicken* cytochromes. It can be seen

Table 6. Peptides obtained from the peptic degradation product of bovine cytochrome c.

DNP-technique	DNP-Val-Glu
Digestion with trypsin	Val-Glu(NH_2)-Lys
Hydrolysis with HCl	Val-Glu $CySO_3H$-Ala $CySO_3H$-His Val-[Glu, Lys] Ala-Glu Thr-[Val, Glu] Lys-$CySO_3H$ Glu — $CySO_3H$ [$CySO_3H$, Ala, Glu] Glu — [$CySO_3H$-His]
Digestion with subtilisin	Val-Glu(NH_2) Ala, Glu(NH_2) Thr-[Val, Glu] [Lys, $CySO_3H$] [$CySO_3H$, His]
Structure of the "porphyrin-free peptide"	Val-Glu(NH_2)-Lys-$CySO_3H$-Ala-Glu(NH_2)-$CySO_3H$-His-Thr-[Val, Glu]

that the iron contents of these two ferriporphyrin c-peptide preparations were found to be virtually the same as those obtained for the bovine material. In the amino acid composition, the *salmon* haemopeptide did not differ at all from the bovine one, whereas *chicken* ferriporphyrin c-peptide did not contain alanine, and serine was found instead. In all three haemopeptide preparations, the same N-terminal dipeptide sequence, Val—Glu, was found to be present.

To get further information, the peptic degradation products of salmon and chicken cytochromes c were split with silver salt and oxidized with performic acid, using the same methods as described above for bovine preparations. The porphyrin-free peptides thus obtained were subjected to the proteolytic action of subtilisin. In the enzymic digest of the *salmon* peptide the following 5 split products were found to be present preeminently:

Val—Glu(NH_2), Lys—$CySO_3H$, Ala—Glu(NH_2), $CySO_3H$—His and Thr—[Val, Glu].

These peptides being identical with the ones identified in the subtilisin hydrolysate of the corresponding bovine peptide preparation (*cf.* Table 6), the sequence of amino acid residues in the peptic degradation product of salmon cytochrome c appears to be the same as in bovine cytochrome,

Val—Glu(NH_2)—Lys—CySH—Ala—Glu(NH_2)—CySH—His—Thr—Val—Glu.

Subtilisin treatment of the *chicken* peptide also yielded

Val—Glu(NH_2), Lys—$CySO_3H$ and Thr—[Val, Glu];

instead of Ala—Glu(NH_2), however, Ser—Glu(NH_2) was found. This is taken to indicate that the amino acid sequence in the peptic degradation product of chicken cytochrome c will be

Val – Glu(NH_2) – Lys – CySH – Ser – Glu(NH_2) – CySH – His – Thr – Val – Glu *.

The fact that an alanine residue occurring in beef and salmon cytochromes c and previously shown to be also present in horse and pig cytochromes [4], is replaced by a serine residue in a bird cytochrome, is rather surprising. It is the more interesting, since an amino acid residue with a side-chain devoid of a polar group appears to be substituted by another residue possessing a polar group in its side chain, and since the amino acid residue found to be replaceable is situated in the very neighbourhood of the prosthetic group. Certainly, this is a remarkable example of how the species specificity of protein structure may be explicable in terms of differences in amino acid sequence, and the question arises what the genetic implication of such differences may be.

* Further evidence for this sequence was adduced by subjecting the porphyrin-free peptide (obtained from the peptic degradation product of chicken cytochrome c) to partial hydrolysis with hydrochloric acid and investigating the split products present in the hydrolysate. The following peptides were found and characterised: Val-Glu, Val-[Glu,Lys], Val-[Glu, Lys, $CySO_3H$], Lys-$CySO_3H$, Ser-Glu, Ser-[Glu, $CySO_3H$], Ser-[Glu, $CySO_3H$, His], $CySO_3H$-His, Thr-[Val, Glu].

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