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On the Stereochemical Structure of Cytochrome c

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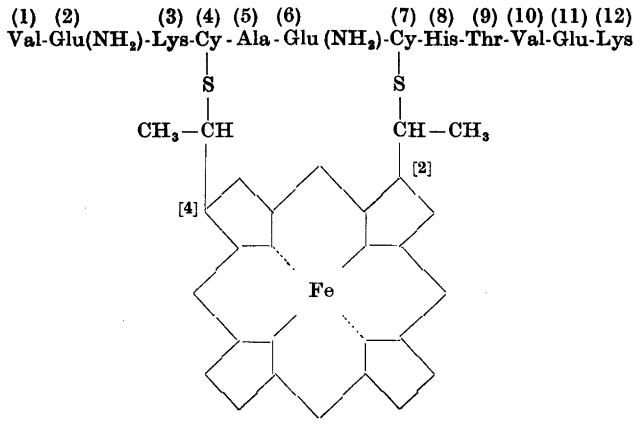
The haemopeptide from cytochrome c of Tuppy and Paléus contains 11 amino acid residues of known sequence, three of them linked to the haem moiety. This substance offered an unusual possibility of investigating the structure of the peptide chain by means of models. It was found that an α-helix structure is very probable. The histidine imidazole then forms a strainless covalent bond with the iron atom, and the axis of the α-helix is parallel with the plane of the haem. Further evidence suggested that the same is true for cytochrome c.

Sedimentation experiments on the haemopeptide showed that it is monomer in acid solution but polymer at alkaline reaction. The polymerisation is due to intermolecular haemochromogen bonds, and is reversed by histidine. The light absorption curve between 500 and 600 mμ of this di-histidine haemochromogen was indistinguishable from that of ferrocytochrome c. A "half" haemochromogen is supposed to exist at slightly acid reaction.

The partial specific volume of cytochrome c was calculated from determinations of its amino acid composition. Comparison with the experimental value indicated a compact structure in which the haem contributes very little to the volume. A "rhombic" packing of peptide chains, distorted to a "square" packing around the haem is suggested.

Earlier work in this institute has established that the haem of cytochrome c is linked to the protein moiety by two thioether bonds between cysteine residues and the α-carbon atoms of the vinyl side chains in the positions [2] and [4] of the haem disc [1–3]. Physico-chemical research made it likely that haemochromogen-forming groups are imidazoles of histidine residues [4,6], and Margoliash [6] has recently presented additional evidence in favour of at least one of the haemochromogen-forming groups being histidine.

The investigations of Tuppy and Bodo [7], and Tuppy and Paléus [8] on different haemopeptides obtained by partial hydrolysis of cytochrome c with proteolytic enzymes have established the following amino acid sequence in beef and salmon cytochrome c:



The sequence (4)—(12) was found to be exactly the same in horse and pig cytochrome c, whereas the chicken cytochrome c contained serine instead of alanine at position (5) — an interesting case of a chemically verified species difference in a protein [8].

The haemopeptide obtained by peptic degradation of beef cytochrome c contains the amino acids (1) to (11). Paléus, Ehrenberg and Tuppy [9] have studied this peptide in its ferric state by means of spectrophotometry, titrimetry, and magnetometry. They found one acid form with essentially ionic bonds to the iron and one alkaline form with covalent bonds to the iron and giving a haemochromogen spectrum upon reduction. The two groups linked to the ferric iron appeared to attach themselves stepwise with pK'-values of 3.4 and 5.8. The intermediate form, with only the first group titrated, had spectral and magnetic properties of intermediate character. At that time nothing could be said with certainty about the character of the bonds with the iron. It was assumed that the group titrated with pK' 3.4 is the imidazole residue of histidine (8) and that the other group with pK' 5.8 is either the α-amino group of valine (1) or the ε-amino group of lysine (3).

Experimental proof of the mode of hydrogen bonding in protein molecules has hitherto practically exclusively been furnished by X-ray crystallography. This method has not yet been applied in the case of cytochrome c *. However, this peptic degradation product of cytochrome c provides a compound of known amino acid sequence that is linked to a rigid structure (the haem-disc) by two chemically well-defined thioether bonds, and in addition, in neutral or alkaline solution, by covalent haemochromogen bonds. This offers a unique possibility of investigating which structures could be compatible with the restrictions introduced by the bonds between the peptide and haematin moieties. The feasibility of the different "spiral" or "pleated sheet" conformations recently suggested as alternatively present in protein molecules [10] could then be compared.

* However, see addendum.

We have therefore studied the possible hydrogen bonded structures of the haemopeptide by means of steric models. The degree of polymerization of the haemopeptide at different pH was studied in the ultracentrifuge. The α- and β-bands of the ferrohaemopeptide were measured spectrophotometrically under various conditions. A complete amino acid analysis of cytochrome c was made, and the results used for calculating the partial specific volume for comparison with the unusually low experimental value which indicates a particularly compact structure. The results were also correlated with previous data on the haemopeptides and cytochrome c. Short summaries have already been published [11].

MATERIAL AND METHODS

About 1.5 mg of a haemopeptide was used in the ultracentrifugal and spectrophotometrical experiments. The sample was one of those used by Paléus, Ehrenberg and Tuppy [9]. It had been obtained by peptic degradation of beef cytochrome c and contained 2.76 % iron.

Beef cytochrome c was kindly put at our disposal by Dr. S. Paléus. It was prepared according to Paléus and Neilands [12] and contained 0.433 % iron.

In the spectrophotometric studies we have also used a sample of protohaemin with 8.26 % iron. The amino acids used were obtained commercially.

All the spectrophotometric experiments were made at 20° C in a Beckman Spectrophotometer, Model DU.

The sedimentation experiments were made in a Spinco Ultracentrifuge, Model E. Because of the low molecular weight of the haemopeptide we used the synthetic boundary cell manufactured by Spinco. All experiments were made at 59 780 r.p.m. which corresponds to about 250 000 g in the middle of the cell where the boundary was formed. Because of the intense colour of the substance (on a weight basis its absorption is more than six times stronger than that of cytochrome c) special precautions had to be taken. We used a red glass filter at the slit, Schott-Jena R G 1, 2 mm, allowing only light of wavelengths longer than 600 mμ. With dilute solutions of the haemopeptide the light absorption was then sufficiently low to give enough blackening of the plate where the light had passed through the peptide solution, without overexposing the other part of the plate. The high diffusion rate of the haemopeptide made it advisable to use the highest concentration possible. The experiments therefore had to be carried out within a narrow concentration range of about 1 mg peptide per ml solution. With a slit width of 0.3 mm, a bar angle of about 30° and exposure intervals of 4 min., a good series of pictures could be obtained with exposure times of 30 *s* in alkaline and 60 *s* in acid solutions. Ten to fifteen exposures were taken in every experiment.

In preliminary experiments on cytochrome c it was found difficult to produce good synthetic boundaries between the solvent and solutions with 1 mg or less protein per ml. As we had only about 1.5 mg of the haemopeptide at our disposal, which was sufficient for only five experiments, it was necessary to insure the formation of good boundaries in each experiment. A sufficient stabilizing effect during the formation period was obtained by adjusting the content of sodium chloride in the peptide solution to 10.5 mg/ml and in the solvent, to be layered above, to 9.5 mg/ml. The layering usually occurred at 6 000 r.p.m. and the first exposure was taken at full speed some ten minutes later. At that time the high diffusion rate of sodium chloride had already flattened out the gradient curve due to the salt, so that it did not influence the position of the superposed and still sharp peptide gradient curve.

As a consequence of the low concentration and sedimentation velocity, and the high diffusion rate and light absorption of the haemopeptide, special precautions had to be taken in evaluating the results. Two different methods were used. In the first procedure the plate was placed in a common photographic magnifying apparatus and projected in the scale 10/1 on a paper where the image was copied with a pencil. On these drawings the position of the peak in comparison with the meniscus and the index could be estimated. Three persons evaluated every plate independently. In the other method a Hilger micro-

photometer for spectrographic plates was used as a comparator. The surface with the entrance slit to the photocell was used as a projection screen on which the images of peak and reference lines were successively brought to coincide with the slit by adjustment of the micrometer on which the differences were read. In both methods the sedimentation rates were calculated from the peak positions according to the method of independent intervals. When the means were calculated the double weight was given to the values obtained by the comparator method, which is considered to be the more objective one, and to those obtained from the drawings of the person who was specially trained in drawing and evaluating the sedimentation diagrams.

Professor L. Pauling generously presented to us a new type of steric bond models for building peptide chains with coplanar peptide bonds, in the scale 1 Å = 1 inch. A haem model was made from a metal disc in the same scale. All C—C and C—N distances were made 1.34″, the pyrrol rings as regular pentagons, and the angle between the bonds to the methine carbon atom 116°. This resulted in a N—Fe distance of 1.9″ in accordance with the known data for covalent radii [13]. The imidazole ring was represented by a regular pentagon with sides 1.42″. Side chain models were manufactured from bent metal rods and suitable joints allowing free rotation around the axis of the bonds.

RESULTS

Sedimentation experiments

The results of the sedimentation experiments are presented in Table 1, and some exposures are reproduced in Fig. 1. From the mean values of S_t the sedimentation constants referred to standard conditions S_{20}°, were obtained after proper correction for viscosity and density as described by Pedersen [14]. In these calculations we have used the mean concentration of sodium chloride, 10 mg/ml, and an estimated value of the partial specific volume of the haemopeptide of 0.7.

The sedimentation constant of beef cytochrome c obtained by the present technique is about 10 % in excess in comparison with earlier data determined with the same centrifuge [15]. This increase might be due to the salt gradient. We have not investigated the question further.

The diffusion coefficients were calculated as follows. From the drawn copies the maximal height, H_{max}, was read, and its inverse square was plotted *versus* the time, which gave an approximately straight line with slope K. The surface A under the curve was measured and an apparent diffusion coefficient

Table 1. Summarized results of the sedimentation experiments on beef cytochrome c and its peptic haemopeptide (Php). The standard errors of S_{20}° are given.

Expt. No.	Sample	Buffer	pH	S_t med $\times 10^{13}$	S_{20}° $\times 10^{13}$	D $\times 10^{7}$	M	f/f_0
1	Php	0.03 M citrate	2.3	0.53	0.57±0.08	26.5	2 000	1.08
2	Php	0.03 M citrate	2.3	0.57	0.62±0.06	21.5		
3	Php	0.05 M borax	8.7	1.54	1.57±0.04	11.8	10 800	1.25
4	Php	0.05 M borax + 0.145 M histidine	8.7	0.76	0.85±0.04	30	[2 300]	[0.83]
5	Php	0.05 M acetate	4.0	2.34	2.53±0.20	56	[3 800]	[0.38]
6	Cyt-c	0.05 M borax	8.7	1.70	1.83±0.06	(11.3)	13 600	1.21

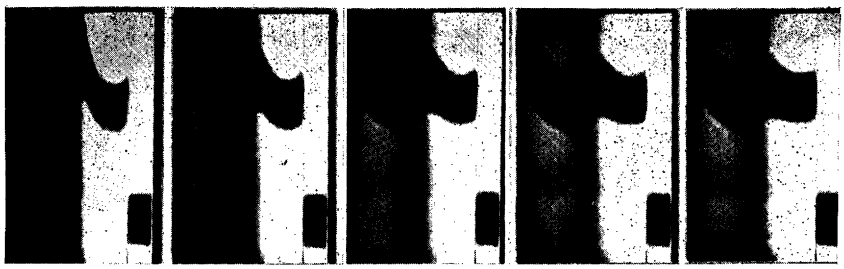


Fig. 1. Sedimentation experiment 2 of Table 1. Exposures taken 4, 12, 20, 28 and 36 min. after full speed had been attained. Exposure time 1 min. Full speed, 59 780 r.p.m. was reached 8 min. after layering.

could be obtained by the formula $D_{app} = \frac{A^2K}{4\pi}$. In case of cytochrome c, D_{app} was about twice as high as the value of 11.3×10^{-7} cm^2. s^{-1} determined by Pedersen [16]. Experiments on cytochrome c without a stabilizing salt gradient revealed that this difference was only partly an effect of the salt gradient. By means of the values of D_{app} the diffusion coefficients of Table 1 have been calculated in proportion to Pedersen's value for cytochrome c.

The molecular weights and the frictional ratios were calculated according to the well known formulae [14]

$$M = \frac{R\ T\ S}{D(1-V\varrho)} \text{ and } \frac{f}{f_0} = \left(\frac{1-V\varrho}{D^2SV}\right)^{1/3} \times 10^{-8}$$

The formula of the haemopeptide gives a molecular weight of 1 880 if one hydroxyl is assumed to be bound to the iron. The iron content of 2.76 % corresponds to the value of 2 020. Our results of experiments 1, 2 and 3 reported in Table 1 show that the haemopeptide is monomer at low pH and polymerized at alkaline reaction. The reasonable values of the frictional ratios and the molecular weight of the monomer indicate that the diffusion coefficients are of the right order and that the species are essentially monodisperse both at pH 2.3 and 8.7. The molecular weight of the polymer is estimated to be 10 800, indicating a pentamer or a hexamer.

In experiment 4 histidine was added to a sample at pH 8.7. The decrease of the sedimentation constant shows that histidine has a depolymerizing effect. The depolymerisation is not complete, which is reflected in a too high diffusion coefficient and a too low value of the frictional ratio.

The sample of experiment 5 was dissolved in dilute acetic acid and then brought to pH 4.0 by addition of a measured amount of sodium hydroxide. The peptide appeared to be polymer and polydisperse at this pH.

Spectrophotometric investigations

Tsou [17] determined the absorption spectrum of his peptic degradation product, which was somewhat less pure than our sample. He found that the spectrum of the ferrous form was "identical with that of ferrocytochrome c in

Table 2. Light absorption data of cytochrome c and some of its degradation products in the reduced state. $\varepsilon = \frac{1}{c} \cdot \frac{1}{d} \log \frac{I_o}{I}$, *where c in mM and d in cm.*

Sample and source	Peptic haemopeptide from beef cytochrome c			Cytochrome c from beef	Cytochrome c from horse	Tryptic haemopeptide from horse cytochrome c		
Author	Tsou[17]	Our data		Our data	Tuppy & Bodo[7]	Tuppy & Bodo [7]		
pH	7.3	8.9	8.9	8.9	7.3	7.3	7.3	7.3
In presence of	—	—	0.14 M histidine	—	—	—	1 000-fold excess of α-benzoyl-L-histidine	Extrapolated to infinite excess of α-benzoyl-L-histidine
ε_{550}	28.0	26.9	27.0	26.9	28.0	15.0	23.4	24
ε_{520}	14.5	13.6	15.8	15.9	16.4	9.4	14.6	15
$\varepsilon_{550}/\varepsilon_{520}$	1.93	1.98	1.71	1.69	1.71	1.60	1.60	1.60

the visible region and in neutral and alkaline solutions". However, a close examination of his absorption curve reveals that this conclusion is not entirely justified: the ratio between the heights of the α- and β-bands is a little higher than in ferrocytochrome c. Our preparation also showed the same property, see Table 2. It appeared to us that the difference might be due to the fact that the haemopeptide in contradistinction to cytochrome c contains only one imidazole residue per iron atom. We therefore investigated the light absorption upon addition of histidine. As seen from Fig. 2 and Table 2 the histidine

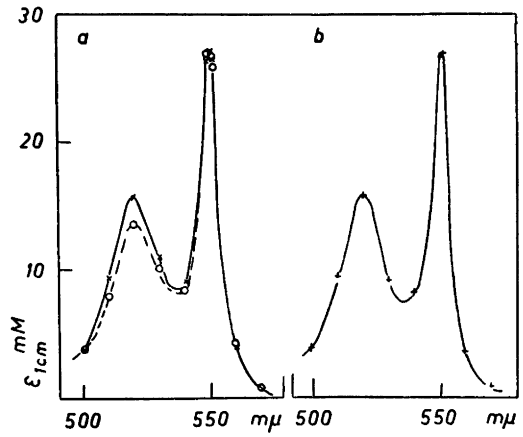


Fig. 2. a) Absorption spectrum of reduced peptic haemopeptide at pH 8.9 (O) and at the same pH with 0.14 M histidine added (×).
b) Absorption spectrum of reduced beef cytochrome c. The concentration of the haemopeptide was determined as pyridine haemochromogen in 0.1 N NaOH + 25 % pyridine + $Na_2S_2O_4$. The same extinction was assumed as for cytochrome c under corresponding conditions.

increased the height of the β-band, giving the same ratio $\varepsilon_\alpha/\varepsilon_\beta$ as cytochrome c itself. Thus the peptic haemopeptide gives α- and β-bands that are indistinguishable from those of cytochrome c only after the addition of histidine.

Tuppy and Bodo [7] examined a tryptic degradation product in the same spectral region at pH 7.3. They found that this haemopeptide alone gave rather low α- and β-bands which increased on the addition of histidine or α-benzoyl-L-histidine. We have extrapolated their data graphically to infinite concentration of α-benzoyl-L-histidine. It is seen from Table 2 that the band heights never reach those of cytochrome c, and that the ratio $\varepsilon_\alpha/\varepsilon_\beta$ remains constant at a somewhat lower level than for cytochrome c.

The peptic haemopeptide contains three potential haemochromogen-forming groups: α—NH_2 in Val(1), ε—NH_2 in Lys(3) and imidazole in His(8). We considered it of interest to compare the ability of such groups to form compounds with ferri- or ferro-protoporphyrin at pH 7, since we have not been able to find such data in the literature. It was found that no spectrophotometrically observable compounds were formed with lysine or glycyl-glycine. This was not unexpected since the pK' of primary amino groups is between 8 and 10.5. Thus at pH 7 they are practically entirely in the $-NH_3^+$ form which cannot combine with haematin iron. Histidine, on the other hand, with its pK' of the imidazole of 6.1, combined both with ferri- and ferro-protoporphyrin. Each iron atom coordinated simultaneously two histidine molecules, in accordance with the findings of Cowgill and Clark [18], who studied the combination of several imidazoles with ferri-mesoporphyrin. The dissociation constants of the two compounds were found to be $K_{ox} = 0.004$ M^2 and $K_{red} = 0.015$ M^2.

Partial specific volume of cytochrome c in relation to its amino acid composition

The partial specific volume of cytochrome c is unusually low, $V = 0.702$—0.707 [16,19]. It was considered to be of interest to determine whether this is due to an unusually high content of heavy amino acids, or whether the haem part is perhaps so intimately connected with the protein part that its contribution to the molecular volume is reduced.

The partial specific volume, V, has been calculated for many proteins from their amino acid composition and the empirical values for the partial specific volumes of the different amino acid residues [20,21]. The values were always in very satisfactory agreement with those obtained by the direct determination of V on the proteins.

In horse haemoglobin the haem part does not seem to have any great influence on V; the value calculated from the amino acid composition, disregarding the haem, was 0.741, the experimentally observed value 0.749 [21].

We now calculated V for horse myoglobin using both Tristram's [22] and our unpublished data on the amino acid composition. V was found to be 0.744 and 0.742, respectively, in close agreement with the experimental value 0.741 [23].

Beef cytochrome c with 0.433 % Fe was analyzed for the amino acid contents according to a modified Stein and Moore procedure. The results are given in Table 3.

Table 3. Amino acid composition of beef cytochrome c with 0.433 % Fe and M = 12 900.

Amino acid residue	Number per molecule	Amino acid residue	Number per molecule
Aspartic acid	9.4	Leucine	6.2
Serine / Threonine	8.5	Tyrosine	3.8
		Lysine	18.3
Glutamic acid	11.9	Histidine	3.1
Glycine	15.5	Arginine	3.4
α-alanine	6.6	Phenylalanine	3.3
Valine	3.3	Methionine	2 *
Proline	3.9	Cysteine	2 *
Isoleucine	6.2	Tryptophane	1 **

* According to Åkeson [24] and Paléus [25].
** According to Theorell and Åkeson [4].

V calculated from these values was = 0.737, thus considerably higher than the experimentally found V = 0.702—0.707. If we assume that the haem part does not increase the molecular volume, but only the weight, V would be = 0.700. The haem thus seems to occupy a space within the protein molecule, where it fits in without expanding the molecule to any large extent.

Structural Models

It was assumed that all the amino acids had the L-configuration. Both left and right hand screws of the α, π, and γ-helices [10] (3.7, 4.4 and 5.1 amino acids per turn, respectively) were constructed. "Pleated sheet" structures were also considered.

We first investigated the possibilities of attaching the haem part to the peptide chain by the two thioether bonds. As seen from the formula (p. 1194) there are two alternatives to be considered: A) Cy(4) to haem side chain [4], Cy(7) to [2], and B) Cy(4) to [2], Cy(7) to [4].

All of the four combinations between γ-helices and the haem were found to be very improbable because of strong steric hindrance. When the peptide chain was formed as an element of a "polar pleated sheet" the thioether bonds could not at all be attached; and when it was formed as required by the "parallel" or "antiparallel" pleated sheet configurations, the attachment of the thioether bonds resulted in distortion of the chain. These structures were therefore considered to be very unlikely.

Thioether bonds could readily be formed with either α- or π-helices.

We now investigated the possibilities of attaching haemochromogen-forming nitrogen atoms to the iron. The free amino group of valine (1) was always too far away from the iron; and it is of small interest, since it is very probably not free in cytochrome c. The histidine imidazole or the ε-amino group in lysine(3) could in many cases be attached to the iron, but never simultaneously from opposite sides of the haem. Because of the flexibility of the lysine side chain its ε-amino-group could easily be attached to the iron in all

Table 4. Comparison of the feasability of the different α- and π-helix structures for the haemopeptide.

Alternative No.	Helix type	Screw type	Cysteine 4 linked to α-carbon	Imidazole imino group pointing towards amino acid	Angle between helix axes and haem plane	Strain for linking imidazole to haem iron
1	α	left	4	1	<5°	none
2	»	»	4	11	30°	some
3	»	»	2	1	15°	none
4	»	»	2	11	45°	some
5	»	right	4	1	<5°	none
6	»	»	2	1	<5°	none
7	π	left	4	—	70°	much
8	»	»	2	—	70°	much
9	»	right	4	—	60°	some
10	»	»	2	—	80°	very much

the α-helix alternatives, but less easily in the π-helices. However, since there are manifold reasons to believe [4–6] that imidazoles are the haemochromogen-forming groups in cytochrome c, and we have already shown in this paper that lysine is unable to form proto-haemochromogen at neutral reaction, we focussed our interest on the imidazole group of our haemopeptide. When the imidazole was attached to the iron by a bond, perpendicular to the plane of the haem, rigid structures were formed. In the case of left-handed α-helices we observed that two slightly different structures were possible, both with the imidazole disc essentially parallel with the axis of the helix, but with the free imino group pointing either towards amino acid (1) or (11).

The results with the α- and π-helices are summarized in Table 4. It is seen that the π-helices are all unfavourable for attaching imidazole to the iron. Not only is the bond formation possible only with considerable strain; but it further-

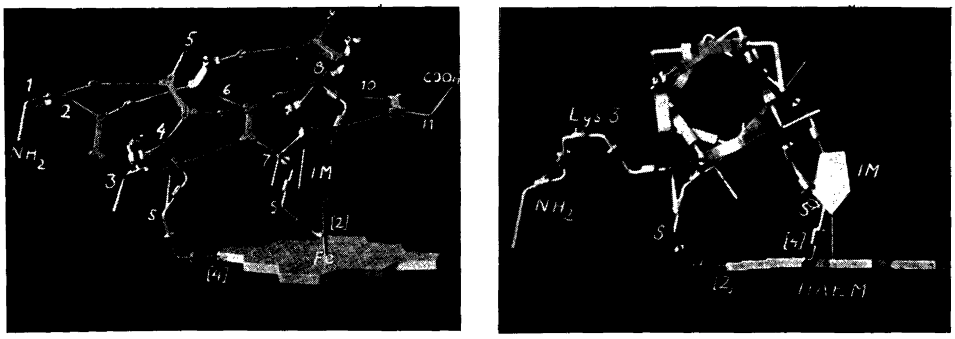


Fig. 3. Pictures showing the structure model of the haemopeptide with left handed α-helix corresponding to alternative 1 of Table 4. a) perpendicular to the helix axes and b) along the helix axes. Figures without brackets indicate the α-carbon atoms of the corresponding amino acid residues. Figures in brackets indicate the substitution positions of the haem.

more results in structures where the haem plane forms angles of 60—80° with the axis of the helix. For spatial reasons such structures seem less likely to occur in cytochrome c than such with the haem parallel to the helix axis. If this argument is applied to the α-helix structures, the most probable alternatives are numbers 1, 5 and 6 (Table 4). And finally, if the left-hand screw is preferred because it has been found to occur in a great number of proteins [26,27], whereas right hand helices do not seem yet to have been found, we are left with alternative 1 as the most likely one. Fig. 3 shows this structure from the side and along the helix axis.

DISCUSSION

When haemochromogens are formed in solution under usual conditions the attachment of one group to the iron on one side of the haem disc so greatly favours the coupling of another on the opposite side that the reaction appears to be trimolecular. Therefore, "half" haemochromogens have never been observed.

The haemopeptide from cytochrome c offers an interesting example of a molecule where the intramolecular formation of a half haemochromogen should be strongly favoured. It contains one histidine residue in an optimal position for coordination to the iron; after this bond has been formed no nitrogenous groups are left that can easily be used to complete the haemochromogen in slightly acidic solution. The spectrophotometrical transition with a pK' of 3.4 and $n = 1$ [9] is thus likely to be a reflection of the imidazole forming a half haemochromogen. A water molecule or an anion is likely to be bound to the iron on the opposite side. The paramagnetic susceptibility $\chi_m = 8\,800 \times 10^{-6}$ c.g.s. e.m.u.[9], indicates dsp^2 bonds with 3 odd electrons to be present.

A second, spectrophotometrically operable transition occurs at higher pH ($pK = 5.8$, $n = 1$), resulting in a complete covalent structure with 1 odd electron [9]. Upon reduction haemochromogen bands appear which are very similar, but not identical with those in ferrocytochrome c (See Fig. 2). Primary amino groups are here coordinated opposite to the imidazole, because no other groups likely to form covalent bonds with the iron are present. The pK' of the free amino group in a valylpeptide is likely to be about 8. A shift of this value to 5.8 in the haemopeptide is plausible as a result of the much higher stability of the covalent d^2sp^3 structure than that of the "half" haemochromogen structure. The ε-amino group of lysine with its pK' about 10.5 is less likely to take part in this reaction. A d^2sp^3 structure cannot be formed with the aid of the imidazole and an amino group belonging to the same molecule, unless the peptide chain is unfolded. If the peptide chain is an α-helix, as proposed, the iron atom must therefore be bound to the imidazole of the same molecule and a primary amino group of another; in neutral or alkaline solution polymerization would be expected. This was found to be the case; for some reason a monodisperse penta- or hexamer is formed. The aggregates were depolymerized upon addition of histidine. This indicates that the free histidine competitively replaces the amino group, thus forming a di-histidine haemochromogen with α- and β-bands that are spectrophotometrically indistinguishable from those of cytochrome c.

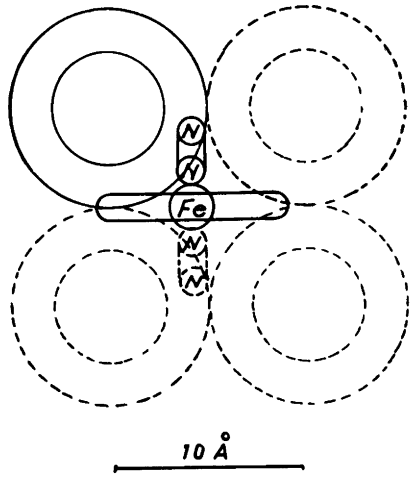


Fig. 4. Schematic axial view of proposed structure with four parallel α-helices in "square" packing around the haem. Full-drawn portion corresponds to haemopeptide. Space between inner and outer circles is occupied by side chains.

The peptic haemopeptide precipitates from solutions of low ionic strength between pH 4.3 and 7.4 [9]. It contains seven groups that are titratable from pH 2 to pH 11. Four of these are carboxyl groups, one imidazole and two primary amino groups. Furthermore, for calculating the isoelectric point the positive charge of the iron atom has to be considered. The haemopeptide should therefore be isoelectric when four equivalents are titrated starting from pH 2. According to Paléus *et al.*[9] this occurs at pH 5.4. Their data indicate that precipitation takes place when the molecule bears less than 1 ½ plus or minus charges. We believe that this precipitation is essentially isoelectric in character and not necessarily connected with the arrangement of the haemochromogen-forming groups.

There are good reasons to believe that the steric structure of the peptide fragment studied here really forms a part of native cytochrome c. The α-helix is in itself the most probable of the peptide helices, because it gives the most compact structure with minimal van der Waals energy [10]. The parallelity of the helix axis and the haem disc makes it possible to arrange peptide chains in the close packing that is necessary in order to explain the low partial specific volume of cytochrome c. If the side chains of the α-helix structure presented in Fig. 3 are considered, a cylinder with an average diameter of 10.5 Å results [10]. This occupies just one quadrant of a plane perpendicular to the plane of the haem disc. It is plausible to imagine that other helices in cytochrome are arranged parallel to each other as indicated in Fig. 4. Two haem-linked imidazoles are shown, one belonging to the haemopeptide part, the other to another part of the protein.

The proposed structure certainly avoids any steric hindrance, but the "square" packing of the helices is not the closest possible arrangement, and would result in a partial specific volume as high as 0.73 if it is assumed to extend over the whole length of the molecule. In an α-helix the axial projection per amino acid residue is about 1.5 Å [10]. Since there are about 100 resi-

dues in cytochrome c, the length of the molecule would be 35—38 Å, if four parallel helices of equal length are assumed. If the helices are packed in the closest possible manner, "rhomboic" packing, and the "square" packing is only a local distortion effected by the presence of the haem, which has an axial projection of 10—12 Å, then the theoretical partial specific volume comes out as 0.71 in satisfactory agreement with the experimental data.

The structure shown in Fig. 4 would shield the haem iron from direct contact with, for example, oxygen, carbon monoxide or cyanide, in accordance with the known properties of cytochrome c. On the other hand, the electron exchange with suitable donors and acceptors must in some way be facilitated. On both sides of the haem the non iron-bound imino nitrogen of the haem-linked imidazole has a rather exposed position in the crevice between two α-helices. An active group of another protein, *e. g.* the iron of DPNH cytochrome c reductase [28] could perhaps coordinate such a nitrogen atom. If the iron is present as a non-haem chelate compound this coordination would be sterically possible. The electron transfer could then be imagined to take place through the resonating bonds of the imidazole ring. A similar reaction mechanism was previously suggested for the reduction of cytochrome c by means of leucoflavins or hydrogen [29].

The oxidation of ferrocytochrome c does not seem to be possible by direct contact between cytochrome oxidase haem iron and the haem-linked imidazole in cytochrome c, because of steric hindrance. But there is a possibility that an oxygen molecule, activated by being linked to cytochrome oxidase iron, could come close enough to be able to accept electrons from cytochrome imidazole.

The proposed structure and the reaction mechanisms discussed of course remain hypothetical and need further experimental confirmation. It is especially desirable to obtain crystalline cytochrome c in order to carry out detailed X-ray investigations. The type of helix, however, could perhaps be determined on amorphous material according to the X-ray technique used by Riley and Arndt [26,27].

Addendum May 6, 1955: Our results have been summarized in a forthcoming chapter of "*Currents in Biochemical Research*" (manuscript sent 17 February, 1955) and in a letter to the *Nature* of 30 March, 1955. When the present manuscript was ready a paper by Arndt and Riley (*Phil. Trans.* 247 A, p. 409, 24 March, 1955) came to our knowledge. These authors have classified cytochrome c as a closely packed "α"-protein with left hand α-helices, as revealed by their X-ray scattering method. The orientation of the haem *versus* the peptide chains was not discussed. On the basis of our independent results we can now say that our proposed structure must be essentially correct.

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REFERENCES

1. Theorell, H. *Biochem. Z.* **298** (1938) 242.
2. Theorell, H. *Enzymologia* **6** (1939) 88.
3. Paul, K. G. *Acta Chem. Scand.* **5** (1951) 389.
4. Theorell, H. and Åkeson, Å. *J. Am. Chem. Soc.* **63** (1941) 1804.
5. Boeri, E., Ehrenberg, A., Paul, K. G. and Theorell, H. *Biochim. et Biophys. Acta* **12** (1953) 273.

6. Margoliash, E. *Nature* **175** (1955) 293.
7. Tuppy, H. and Bodo, G. *Monatsh.* **85** (1954) 1024.
8. Tuppy, H. and Paléus, S. *Acta Chem. Scand.* **9** (1955) 353.
9. Paléus, S., Ehrenberg, A. and Tuppy, H. *Acta Chem. Scand.* **9** (1955) 365.
10. Pauling, L. in *Les Protéines*, Neuvième Conseil de Chimie Solvay, Bruxelles 1953, p. 63.
11a. Ehrenberg, A. and Theorell, H. *Nature.* **176** (1955) 158.
11b. Theorell, H. in *Currents in Biochemical Research*, Vol. II, New York 1955.
12. Paléus, S. and Neilands, J. B. *Acta Chem. Scand.* **4** (1950) 1024.
13. Pauling, L. *Nature of the Chemical bond*, Ithaca, New York 1945.
14. Pedersen, K. O. in Svedberg, T. and Pedersen, K. O. *The Ultracentrifuge*, Oxford 1940.
15. Ehrenberg, A. and Paléus, S. *Acta Chem. Scand.* **9** (1955) 538.
16. Pedersen, K. O. quoted by Paul, K. G. in Sumner, J. B. and Myrbäck, K. *The Enzymes*, Vol. II, Part I, Academic Press Inc., New York 1951, p. 375.
17. Tsou, C. L. *Biochem. J. (London)* **49** (1951) 367.
18. Cowgill, R. W. and Clark, W. M. *J. Biol. Chem.* **198** (1952) 33.
19. Theorell, H. *Biochem. Z.* **285** (1936) 207.
20. Cohn, E. J. and Edsall, J. T. *Proteins, Amino Acids and Peptides*, Reinhold Publish. Corp., New York 1943, p. 370.
21. Edsall, J. T. in Neurath, H. and Bailey, K. *The Proteins*, Academic Press Inc., New York 1953, p. 568.
22. Tristram, G. R. *Advances in Protein Chem.* **5** (1949) 83.
23. Theorell, H. *Biochem. Z.* **268** (1934) 46.
24. Åkeson, Å. *Acta Physiol. Scand.* **4** (1942) 362.
25. Paléus, S. *Acta Chem. Scand.* **9** (1955) 335.
26. Riley, D. P. and Arndt, U. W. *Nature* **169** (1952) 138.
27. Arndt, U. W. and Riley, D. P. *Proc. Roy. Soc. London* **B 141** (1953) 93.
28. Mahler, H. R. and Elowe, D. G. *J. Biol. Chem.* **210** (1954) 165.
29. Theorell, H. *Arkiv Kemi, Mineral. Geol.* **16 A** (1943) No. 14.

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