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A Comparative Study of Cytochrome c from Beef, Chicken and Salmon

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In 1941 Theorell and Åkeson [1] published the first extensive chemical analysis of cytochrome c. The enzyme was in this case prepared from beef heart; horse heart cytochrome c was reported to be identical as regards molecular weight, nitrogen and sulphur content. Moreover, the titration curves of the two cytochromes were identical between pH 1.6 and 11. Three histidine groups were found in the molecule and it was suggested that two of them participated in the linkage of the iron-porphyrin to the protein. In 1951 Paul [2] showed that at least one histidine residue is linked to the iron atom; he could not exclude the possibility that a carboxyl group of some other amino acid residue as well as the imidazole group was linked to the iron atom.

The main purpose of our experiments to be reported was to correlate the histidine content of the cytochromes from beef, chicken and salmon with their titration curves. In addition, the pepsin degradation method of Tsou [3] was applied to these cytochrome studies and the peptides obtained analyzed for histidine.

Since the time when Theorell and Åkeson's investigation was reported new methods of preparation and analysis have been introduced, which make it possible to carry out amino acid analysis on only a few milligrams of protein. Thus paper chromatography as well as spectrophotometric measurements and acid-base titrations could be employed in the present work.

METHODS

Histidine determinations. Since Pauly's reaction is not specific for histidine in a mixture of amino acids (Kapeller-Adler [4]) and only small amounts of cytochrome were available (in some cases only a few milligrams) Henderson and Snell's microbiological method[5] which permits determination of as little as 3 micrograms of histidine was used *.

The cytochrome (either before or after pepsin treatment) was hydrolyzed under reflux with 20 % HCl, usually for 20 hrs. It was checked in some experiments that histidine

* The microbiological determinations were kindly performed by Dr. A. Wretlind at the Pharmacological Department, Karolinska Institutet, Stockholm.

was not destroyed during the hydrolysis by adding a known amount of L-histidine to the cytochrome c preparation to be hydrolyzed and comparing it with a hydrolysate without any addition. As long as the temperature never exceeds 108° there is no danger of racemization (Fränkel [6], Neuberger [7]) which would interfere with the microbiological determination. The hydrolysate was freed from insoluble humin-substances * by filtration through a sintered funnel and evaporated *in vacuo* to remove the hydrochloric acid. The pH was adjusted with NaOH to 6.8 and the solution diluted to 5 or 10 ml depending on the amount of hydrolyzed sample. A suitable concentration of histidine for the assay is 3—10 μg per ml. The experimental error of the method is given as ± 5 % by Henderson and Snell [5], but in control experiments the author has found that the error is usually smaller.

Tryptophane determination. Samples of 0.4—1.5 mg protein were hydrolyzed in 2 ml 5 *N* NaOH at 56° for 15.5 hrs in sealed tubes and tested for tryptophane according to the method of Bates [9], which permits determination of as little as 50 μg of tryptophane.

Paper chromatography. The amino acids of the hydrolysates were chromatographed according to the method of Consden, Gordon and Martin [10]. The same solvents and paper were used as by Maehly and Paléus [11]. Usually 300—400 μg of amino acids were applied to the paper in each experiment.

Paper electrophoresis. A method described by the author [12] was used. Paper "Munktell 20, 150 g" was found most useful for analysing samples of 1—2 mg protein. The electrophoresis was performed at pH 9.87 and 7.0 and developed with 0.2 % ninhydrin in 95 % water saturated butanol and 5 % 2 *N* acetic acid.

Titrations. The measurements were performed in a glass beaker of about 10 ml volume into which fitted a plexiglas chamber with compartments for: a) a glass electrode (Radiometer G 100A), b) a burette capable of adding 0.2 μl at a time **, c) a salt bridge (3 % agar in saturated potassium chloride solution) and d) gas inlet and outlet tubes. An electronic potentiometer *** was used for measuring the potentials. Magnetic stirring was employed. The glass-electrode was checked against three buffers: 0.05 potassium biphthalate, pH 4.00, Sörensen phosphate buffer, pH 6.8, and a Beckman standard buffer pH 9.22. The temperature was kept at 20°.

The titrations of ferricytochrome were performed in an atmosphere of nitrogen freed from carbon dioxide by passing through sodium hydroxide. Ferrocytochrome was prepared according to Theorell [1] by adding 10—15 mg platinum black to the sample at neutral reaction and letting first N_2 and then H_2 pass through until the potential was stable. The platinum black was prepared according to O. Loew [13] and its reducing capacity checked by reducing an aliquot of the cytochrome that was used during the titration and measuring its absorption at 550 mμ before and after the addition of $Na_2S_2O_4$. The result showed that the reduction by platinum black + H_2 was 90 % complete. The hydrogen used was washed free from O_2 by passing it through a vanadous sulphate solution (Meites and Meites [14]) before entering the titration vessel.

An amount of cytochrome containing 1.3—1.5 × 10^{-6} gram atoms of iron dissolved in a volume of 2.0 ml glass distilled water was used. Prior to titration with NaOH (1.43 *N*) the solutions were acidified to about pH 3.5 with HCl (2.7 *N*) in order to remove CO_2. During the titration the ionic strength never exceeded 0.02. According to Paul [15] cytochrome c is stable between pH 1.6 and 12.3 but in these experiments the titration range was limited to pH 3.5—9.2, as beyond these limits too many groups will interfere with the evaluation of the curves.

Spectrophotometry. The measurements were made at room temperature in a Beckman spectrophotometer Model DU using, as a rule, cells of 1 cm optical path length.

The spectrophotometric data were obtained in 0.1 *M* acetate buffer, pH 6.0 (Tint and Reiss [16]). It was verified that the spectrophotometric values did not vary within the pH-range 5.5—6.5. The reducing agent was solid sodium dithionite. The cuvettes were covered with glass plates.

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* It has been emphasized by Horn *et al.*[8] that this step is important for the subsequent microbiological assay.

** In this case an "Agla" micrometer syringe manufactured by Burroughs Welcome & Co., London.

*** Radiometer, Copenhagen, type PHM 3 f.

MATERIAL

1) Preparations. As the source of cytochrome c, hearts from beef, chicken* and salmon were used. Chicken and salmon hearts were frozen after killing and collected until about 10 kg had accumulated, when they were thawed and treated like fresh beef hearts. The first steps of the cytochrome c preparation followed Keilin and Hartree [17]. The mince was thus extracted with 2.5 % trichloroacetic acid, except in the case of salmon, where the yield was much better when the trichloroacetic acid was replaced by 5 % H_2SO_4 to a pH of 4.0, fractionated with $(NH_4)_2SO_4$ after neutralization and the cytochrome precipitated with 20 % trichloroacetic acid. This precipitate was centrifuged down, suspended in the smallest possible volume of 0.05 *N* ammonia and dialyzed against the same solution. After lyophilization the enzyme was purified according to a modification of the method of Paléus and Neilands [18]. The precipitation of the eluate from the ion exchanger by trichloroacetic acid was avoided by using lyophilization. This has also been recommended by Margoliash [19] and by Neilands [20]. The fraction with the highest iron content (Fraction II) was used.

The cytochromes used for titration were beef cytochrome with an iron content of 0.42 %, chicken cytochrome with 0.40 % iron and salmon cytochrome with 0.43 % iron.

2) Digestion with pepsin. The cytochrome preparations described above were degraded by Tsou's [3] method. Crystallized pepsin "Bios Laboratories" was used and its activity tested (Anson [21]). Currie and Bull [22] have shown the pH to be an important factor determining the size of the peptide produced. In all digestion experiments the pH was therefore carefully adjusted to 1.50 against a glass electrode. The same number of Hb-units of pepsin per milligrams of cytochrome were used in all experiments.

RESULTS

The results of the Fe and N determinations are summarized in Table 1.

From the above figures it is seen that the iron contents of the beef and salmon preparations are lower than those found by the author in 1950 (0.466 % and 0.461 %, resp.). The chicken preparations, however, contain a little more iron than is usually reported. The author found an iron content of 0.42 % (unpublished experiments) in agreement with Tint and Reiss [16].

Table 1. Nitrogen and iron determinations of the different cytochromes.

Preparation	N %	Fe-content %	Molecular weight based on Fe-content
Beef	15.01	0.449	12 400
Chicken			
Prep. 1	—	0.434	12 900
Prep. 2	15.33	0.436	12 800
Salmon			
Prep. 1	—	0.434	12 800
Prep. 2	15.57	0.452	12 350

* The chicken hearts were kindly supplied by courtesy of AB Findus, Hälsingborg.

Table 2. Results of the histidine determination of the different cytochromes.

Source	Histidine (%)	Histidine (moles) found	probable	Earlier results found	probable
Beef	3.39	2.70	3	2.91 (Theorell and Åkeson [1])	3
				2.97 (Paul [2])	3
Chicken	3.38	2.82	3		
Salmon	2.16	1.76	2		

The histidine analysis of the above preparations gave the results recorded in Table 2.

The experimentally determined histidine values lie somewhat below the theoretical numbers but this is to be expected. During the handling of the preparation losses occur especially during the filtration from humin substances. There is also some error inherent in the assay method which, however, is diminished by taking the mean value of several hydrolysates.

The amino acid composition of the different cytochromes was studied by paper chromatography on samples of up to 400—500 μg protein in order to detect amino acids which occur only in small amounts (Table 3).

*Table 3. Amino acid composition of the different cytochromes according to paper chromatography. Weak spots are indicated by brackets *.*

	phe	ala.	gly.	ser.	thr.	pro.	opr	his.	arg.	lys.	cys.	tyr.	try.	glu.	asp.	leuc.	val.	met
Beef	(+)	+	(+)	+	+	+	—	(+)	+	+	+	+	+	+	+	+	+	(+)
Chicken	(+)	+	(+)	+	+	+	—	(+)	+	+	+	+	+	+	+	+	+	(+)
Salmon	(+)	+	(+)	+	+	+	—	(+)	+	+	+	+	+	+	+	+	+	(+)

* Abbreviations of the amino acids according to Brand and Edsall [40], cys. = cysteic acid.

Methionine gave only a very weak spot but the iodoplatinate test of Winegard and Toennies [23] (performed on the hydrolysate) gave a positive reaction in all samples.

Table 3 shows the amino acid distribution of the cytochrome of beef, chicken and salmon to be very similar. Only quantitative differences in the participating amino acids seem to exist. This is confirmed by the nitrogen content and, to a certain degree, by spectrophotometric and titrimetric data given below.

From Table 4 it is seen that the extinction coefficients ($E^{1\%}_{1cm}$) of the different species vary more than the molecular extinction coefficients (ε). The difference could mean that the protein part of the molecules have different compositions. This is also indicated by the ratios in Table 5, where, especially

Table 4.

Cyto-chrome (form)	Wave-length (mμ)	$E^{1\%}_{1cm}$ Beef	Salmon	Chicken	ε * Beef	Salmon	Chicken	M.W.
oxidized	280	19.15	20.19	18.46	22 940	26 250	23 630	Beef = 11 980
	520	12.91	11.56	11.62	15 466	15 028	14 870	Salmon = 13 000
reduced	535	5.87	5.40	5.67	7 035	7 020	7 260	Chicken = 12 800
	550	22.41	20.19	21.02	26 882	26 247	26 905	

* Molar extinctions per gram atom of iron.

Table 5. Some ratios between the molar extinction coefficients at different wavelengths.

$\frac{\varepsilon_{550}}{\varepsilon_{280}}$ Beef	Salmon	Chicken	$\frac{\varepsilon_{550}}{\varepsilon_{535}}$ Beef	Salmon	Chicken	$\frac{\varepsilon_{520}}{\varepsilon_{535}}$ Beef	Salmon	Chicken
1.17	1.00	1.13	3.82	3.70	3.70	2.20	2.13	2.05

in the case of salmon $\frac{\varepsilon_{550}}{\varepsilon_{280}}$ differs from the others, possibly due to a rather high content of the aromatic amino acids in this particular cytochrome. The alkaline hydrolysate of salmon cytochrome gives an especially strong tryptophane reaction.

Table 6. Results of the titrations. The first three columns show the titration values for the ferricytochromes. The last column shows the titration values of beef ferrocytochrome.

pH	Equivalents *			
	chicken [a]	salmon [a]	beef [a]	beef [b]
4.5	6.40	5.25	6.20	5.90
5.0	7.85	7.30	7.65	7.18
5.5	8.65	8.75	8.65	8.36
6.0	9.30	9.50	9.35	9.16
6.5	9.75	9.85	9.75	9.66
7.0	10.10	10.10	10.15	10.10
7.5	10.35	10.35	10.40	10.44
8.0	10.60	10.65	10.65	10.72
8.5	10.95	11.15	10.90	10.94
9.0	11.65	12.20	11.50	11.21

* The equivalent scale has an arbitrary origin and is arranged so that 10 equivalents are used at pH 6.8.

[a] oxidized.

[b] reduced.

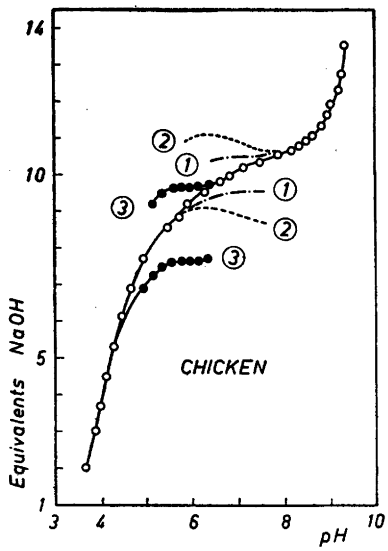

Fig. 1. The titration curve of chicken cytochrome c.

Abscissa: pH, ordinate: equivalents of NaOH. The origin of the ordinate is arranged so that ten equivalents are reached at pH 6.8. —O—O—O—O experimental curve.

(1) The curve obtained by subtracting the theoretical dissociation curve for one equivalent with pK 6.8.

(2) The curve obtained by subtracting the theoretical dissociation curve for two equivalents.

(3) The curve obtained by subtracting the theoretical dissociation curve for two equivalents with a pK of 5.5 from curve (1).

Titrations; Table 6 and Figs. 1—3 give the values from the titrations of beef, chicken and salmon cytochromes. There is a striking similarity between the titration values of ferricytochromes from beef and chicken. The difference in shape between the curves for ferri- and ferrocytochrome from beef is very small (Table 6), especially in the region pH 5.5—8.5. In the case of salmon more equivalents are titrated below pH 5.0 than in ferricytochromes of beef and chicken. Between pH 5 and 8.5 the difference between the three ferricytochromes is small, but becomes greater again at higher pH.

The titration curve of a complex protein molecule is composed of the dissociation curves of individual dissociating groups. The number of equivalents titrated and the positions of the dissociation curves of the individual groups along the pH axis can vary with the protein studied. Nevertheless it seems possible by a process of trial and error to obtain the pH values corresponding to the inflections of the individual curves, *i.e.* the pK's of the groups *.

It is known from earlier work of Greenstein [24] and Wyman [25] that histidine residues are usually titrated in the region of about 5 to 8.5. In cytochrome c from beef and chicken (Figs. 1 and 3) roughly three equivalents and in salmon

* Personal communication from Dr. A. C. Maehly (paper in preparation).

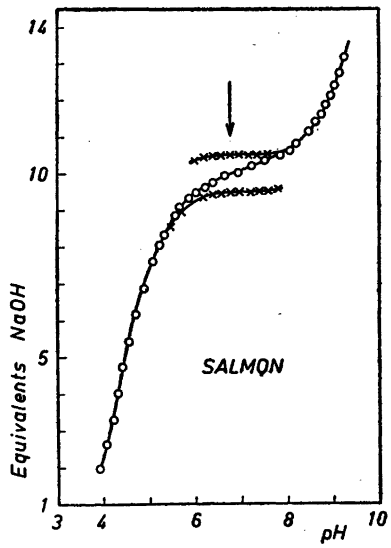

Fig. 2. The titration curve of salmon cytochrome.

The coordinates are the same as those in Fig. 1. The experimental curve (—O—O—O—O) is corrected by subtracting one equivalent with pK 6.5. (✱✱✱✱✱).

cytochrome four are titrated in this range, but it cannot be excluded that other groups than histidine are titrated within this range, as will be discussed later.

A comparison of the experimental titration curves with the theoretical dissociation curve of a single dissociating group immediately shows a great similarity in the range between 6 and 7. A subtraction from the titration curve of the theoretical curve for one acid group with a pK 6.8 (for beef and chicken), and a pK 6.5 (for salmon) gives a resultant curve with a horizontal plateau around pH 7. If other positions are tried, the resultant curve shows a maximum, which is absurd, or rises slowly, implying that other amino acid residues are still being titrated in that range (Fig. 4). Since the resulting curve has almost perfectly horizontal portions it is evident that the two pK-values (6.5 and 6.8) found represent the upper limits (Figs. 1—3).

In the relevant range (pH 5.0—8.5) there are now still two equivalents (for beef and chicken) and three (for salmon) to account for, but in the curve corrected for one equivalent, no position for a further single group can be located in the manner described above. If it is assumed that the two groups titrated in this range have equal affinity for hydrogen ions and do not interact, it is permissible to double the ordinates of the theoretical curve for one equivalent (von Muralt [26], Klotz [27]) and try whether this curve for 2 equivalents can be fitted into the corresponding experimental curve. In beef and chicken cytochrome a satisfactory plateau (Figs. 1 and 3) is obtained by subtracting two equivalents with pK 5.5 and 5.4 respectively. In salmon, no dissociating groups could be located (Fig. 2) with a pK between 4.75 and 6.5. The possibility that the tail of the titration curves of carboxyl groups in the pK region 3.0—4.0 might interfere with the plotting was excluded since a subtraction of 8 equivalents at pK 3.7 did not appreciably influence the shape of the curve around pH 5.4.

DIGESTION OF CYTOCHROME C

Enzymatic breakdown of the cytochromes was performed with pepsin (Tsou [3]). In the case of chicken and salmon the available material was not sufficient for a complete analysis but was only used for the histidine determination and the spectrophotometric and chromatographic investigations. In Table 7 the iron content, molecular weights and histidine contents of the different cytochrome peptides are listed, and in Table 8 the chromatographic results are presented.

Table 7. Iron and histidine content of the different cytochromes degraded by the method of Tsou.

	Fe-content %	Molecular weight	Histidine %	Histidine (moles) found	probable
Beef	2.45	2 280	6.12	0.90	1
Chicken	2.27	2 460	5.35	0.85	1
Salmon	2.35	2 370	7.35	1.05	1
Beef (Tsou [41])	2.21	2 520			

Table 8.

Table. 8. Amino acid composition of the different degraded cytochromes according to paper chromatography.

	Ala.	Arg.	Asp.	Cys.	Glu.	Gly.	His.	Leu.	Lys.	Met.	Phe.	Pro.	Thr.	Try.	Tyr.	Val.	Ser.
Beef:	+	−	+	+	+	+	+	+	+	−	+	+	+	−	−	+	+
Chicken:	+	−	+	+	+	+	+	+	+	−	+	+	+	−	−	+	+
Salmon:	+	−	+	+	+	+	+	+	+	−	+	+	+	−	−	+	+

It follows from these tables that there is only one mole of histidine per mole of peptide. In the case of chicken the value is rather low which could indicate that the preparation is not quite homogeneous. During the course of the digestion different breakdown products are formed. The possibility exists that during the dialysis other peptides could remain inside the dialyzing bag. Therefore paper electrophoresis was used for checking the homogeneity of the pepsin-treated cytochromes. In all these cases only one spot moving towards the anode was found both at pH 7.0 and 9.9.

Table 7 shows that only one mole of histidine is present per mole of digested cytochrome. A striking similarity between the different species is shown as in the case of the intact cytochromes. In all the peptides the same amino acids are absent after peptic digestion, namely arginine, methionine, tryptophane and tyrosine.

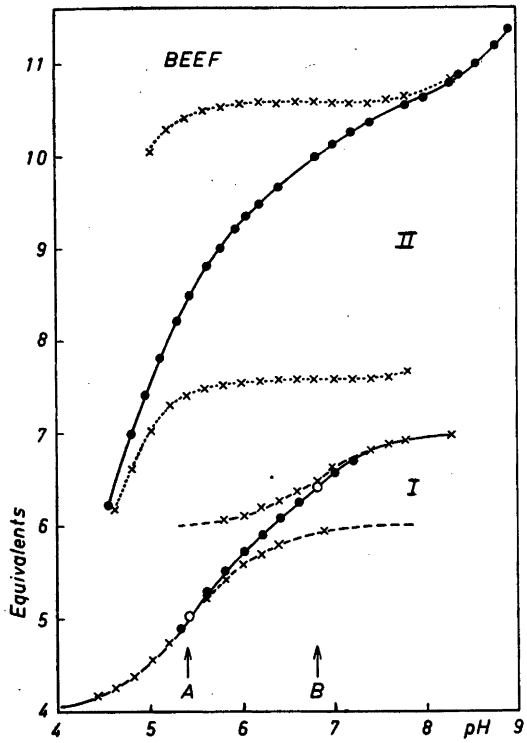

Fig. 3. The titration curve of beef cytochrome showing the theoretical dissociation curve (I) and the experimental curve (II).

I. The theoretical dissociation curve calculated from the theoretical curve for one equivalent with pK 6.8 (B) and two equivalents with pK 5.4 (A).
II. The experimental curve (—●—●—●—●—) and the curve obtained by subtracting curve (I) from the experimental values (···×···×—×—).
The coordinates are the same as in Figs. 1 and 2.

DISCUSSION

Chemical analysis. The work of Theorell [28,29] and Paul [30] has shown that two of the porphyrin-protein linkages are formed between two cysteine residues and the α-carbon atoms of the side chains 2 and 4 of the porphyrin. The nature of the iron protein linkages on the other hand, is not so certain and the information available about the protein structure of cytochrome c is still incomplete.

Using different methods of preparation it has been possible to obtain cytochrome c with different iron contents. Even cytochrome c prepared according to the same method can show different iron content in different batches. This could mean that the molecule could have been partly decomposed by the preparation procedure. The most common methods used for preparation of cytochrome c from animal tissues are listed in Table 9.

Table 9. Different preparation methods for cytochrome c from animal sources.

	Fe-content	Molecular weight	Method	Author	Year
1.	0.34	16 400	A,B,C	Keilin and Hartree [17]	1937
2.	0.43	13 000	A,B,C,D	Theorell and Åkeson [31]	1939
3.	0.34, 0.43	16 400, 13 000	A,B,C	Keilin and Hartree [32]	1945
4.	0.466	11 980	A,B,C,F	Paléus and Neilands [18]	1950
5.	0.45	12 400	A,B,C,E	Paléus [12]	1952
6.	0.43	13 000	A,B,C,F	Margoliash [19]	1950

A = Extraction with 2.5 % trichloroacetic acid
B = $(NH_4)_2SO_4$ fractionation
C = Precipitation with 20 % trichloroacetic acid
D = Electrophoresis
E = Paperelectrophoresis
F = Ion-exchanger

This table shows a variation of 11 980 to 16 400 in the molecular weights of cytochrome c from beef, assuming one iron atom per molecule. Considering the variations of the iron content due to the preparation method used, it was felt that a comparative study of cytochromes from different sources would give valuable information about cytochrome c, especially the iron protein bonds, only if the same method of preparation was employed. The method of preparation will affect the iron protein bonds to a much less degree than the rest of the protein structure, as shown by the fact that the enzymatic activity is not lost in any of the methods of preparations listed above.

It can be seen from Table 1 that there is a slight variation in the iron content between the three cytochromes studied. The values for beef and chicken cytochrome agrees rather well with those of Tint and Reiss [16], 0.453 % and 0.421 %, respectively. Salmon cytochrome has been previously investigated only by Paléus and Neilands [18], who found an iron content of 0.461 %.

The present data as well as those of Theorell [1] and Paul [2] (Table 2) clearly show that beef cytochrome contains three residues of histidine per mole. The same number of histidine groups is found in chicken cytochrome, while salmon cytochrome was found to contain only two. The analytical results for horse cytochrome c published by de Barbieri and Zamboni [33] are hard to explain in the light of these findings. These authors found less than one mole of histidine per mole of cytochrome but do not give any of the very important details of the microbiological and chemical methods used for determining the histidine.

Table 4 shows that the molecular absorption-coefficients of beef and chicken cytochrome at 280 mμ are very similar but that the corresponding coefficient of salmon cytochrome shows a marked deviation. This result, together with the data from paper chromatography indicates that the different cytochromes contain the same varieties of amino acids but not the same number of each.

Titrations. From the titration curves (Figs. 1—3, Table 6) it is seen that three equivalents are titrated in the range pH 5.0—8.5 for beef and chicken cytochrome. One equivalent in each is titrated with pK 6.8 and the other two

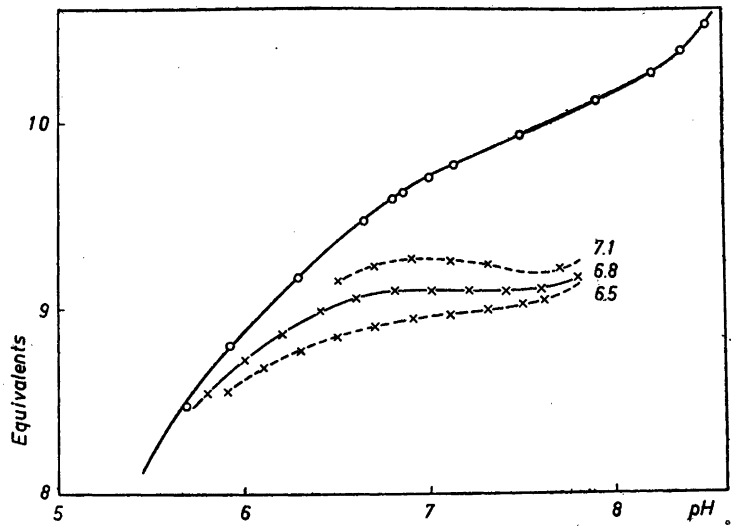

Fig. 4. The sensitivity of the plotting method.

The theoretical dissociation curves for one equivalent with the pK-values 6.5, 6.8 and 7.1 respectively are subtracted from the experimental acid-base titration curve of beef cytochrome and the resulting curves are plotted. It can be seen that the position of the appropriate pK value can be determined to within 0.3 units with certainty. The correct value is obtained when the resulting curve shows a horisontal plateau (6.8).

equivalents with pK 5.4 for beef and 5.5 for chicken. In the case of salmon cytochrome one equivalent with a pK 6.5 was titrated, but it was impossible with the method used above to find another pK above pH 4.75. The three remaining equivalents titrated in this range must belong to groups which dissociate below pH 4.75.

An attempt was made to relate the histidine groups obtained by microbiological assay with the groups titrated in the region pH 5.0—8.5. The pK 6.8 (beef and chicken) lies in the region where the imidazole group is usually titrated and it is therefore believed to belong to histidine. Wyman [34] has shown by potentiometry that oxyhemoglobin has a heme-linked group with a pK at 6.8, which he attributes to an imidazole group, since the apparent heat of dissociation of oxyhemoglobin in this pH range was found to be +6 500 calories per equivalent. Wyman's data on horse oxyhemoglobin gave very good agreement between the number of histidine groups calculated from the apparent heat of dissociation and from chemical analysis respectively.

Theorell and Åkeson [1] found two pK's at 9.35 and $\sim$ 9.85 respectively. Since these pK's shift upon reduction they probably correspond to heme-linked groups. The authors propose that it is the imino groups of the imidazole rings linked to the iron which are titrated there. Boeri *et al.*[35] recently studied the magnetic and spectrophotometric properties of different compounds of ferricytochrome of beef appearing in acid solution and concluded that the

heme was linked to two nitrogen atoms in the protein moiety. These findings fit well into the hypothesis that the hemin is linked to two imidazole groups in cytochrome c.

The fact that the reduction of cytochrome c does not shift the pK 6.8 (beef and chicken) can be regarded as a further proof that the third imidazole group is not heme-linked. The two other residues of histidine in beef and chicken found by microbiological assay are presumably bound to iron, as suggested by Theorell and collaborators (see above).

Amino acid analysis of salmon cytochrome reveals only two molecules of histidine. One of them is titrated with a pK 6.5 and by analogy with beef cytochrome it may be supposed that this group is not linked to the iron. The other histidine group is available for the linkage to the iron. The second heme-linked group in this cytochrome would thus not be an imidazole group.

In measurements of the oxidation-reduction potentials of beef cytochrome Paul [36] found a dissociation constant in ferricytochrome c with a pK of 6.86, while Rodkey and Ball [37] found one with a pK of 7.8. The discrepancy between these two values, as well as their incompatibility with our present results, which indicate a non heme-linked group at pK 6.8, (not shifting on reduction) still remains to be explained.

Let us now consider the nature of the groups titrated with pK 5.4 and 5.5 respectively. Theorell and Åkeson [1] concluded from their experiments on the apparent heat of dissociation of cytochrome c that groups other than histidine were titrated in the range pH 5.5—8.5. They could not observe any shift in the pK's within this range upon reduction, as is confirmed by the present experiments. This means that no heme-linked groups were titrated in the pH range 5.5—8.5. Of the carboxyl groups known to occur in proteins those belonging to aspartic acid show the highest values, 3.0—4.7 (Edsall [38]). Thus it is hard to see how carboxyl groups of the protein moiety could give rise to a pK of 5.4 and 5.5. On the other hand it may be expected that the two carboxyl groups of the propionic acid residues in the porphyrin are titrated in the neighbourhood of pH 5. Theorell [29] has titrated "porphyrin c" and found a pK of 5.63 for its two carboxyl groups.

The digestion experiments. Arginine, methionine, tryptophane and tyrosine could not be detected on the paper chromatograms, which might be due to the small amounts of hydrolysates available. The experiments of Tsou [3] (see his Fig. 2) indicated a rapid initial drop and a slow subsequent rise in the total cytochrome concentration as measured spechophotometrically during the digestion with pepsin. This could indicate a rearrangement of the molecule in one way or the other. Only one histidine group per mole of peptide is found by chemical analysis and hence the peptide must contain at least one heme linked group other than histidine. Noteworthy is that the peptide is autoxidizable and gives a CO compound Tsou [39]).

In the case of the peptide from beef cytochrome the total nitrogen content was 12.07 % corresponding to 20 N-atoms per mole. This is the number of N-atoms corresponding to one mole of each of the amino acids found in the peptide plus the four N-atoms belonging to the porphyrin nucleus. When all amino acids and the molecular weight of hemin (652) is added up, a minimum molecular weight of 2 170 is arrived at. The minimum molecular

weight according to the iron content is 2 280. The peptide molecule thus remarkably enough seems to contain scarcely more than one mole of each of the amino acids found to be present.

SUMMARY

1. Three histidine groups per mole were found by microbiological assay in beef and chicken cytochrome, two in salmon cytochrome.

2. Titration experiments showed one equivalent with a pK of 6.8 and two equivalents with a pK of 5.4 and 5.5 respectively for beef and chicken cytochrome. These pK-values were not shifted upon reduction of the cytochromes. Between pH 5—8.5 one equivalent with a pK of 6.5 was titrated in salmon cytochrome. The single equivalent with a pK of 6.8 and 6.5 is attributed to an imidazole group, not linked to the iron, the two equivalents with pK at 5.4 and 5.5 are attributed to the carboxyl groups from the porphyrin.

3. The amino acid composition of the three different cytochromes is rather similar but the number of each amino acid varies with the species.

4. Pepsin digestion of the three cytochromes yielded peptides containing only one histidine group. The amino acid composition was different from the original material but very similar in all three cytochromes studied. The heme linkages in the peptides must be different from that of ordinary cytochrome.

5. The results obtained here were correlated to those of Theorell and coworkers and have clarified to some degree our knowledge of the structure of cytochrome c.

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REFERENCES

1. Theorell, H. and Åkeson, Å. *J. Am. Chem. Soc.* **63** (1941) 1804.
2. Paul, K. G. *Acta Chem. Scand.* **5** (1951) 379.
3. Tsou, C. L. *Biochem. J. (London)* **49** (1951) 362.
4. Kapeller-Adler, R. *Biochem. Z.* **264** (1933) 131.
5. Henderson, L. M. and Snell, E. E. *J. Biol. Chem.* **172** (1948) 15.
6. Fränkel, S. *Beiträge zur Chemischen Physiologie und Pathologie* **8** (1906) 156.
7. Neuberger, A. *Advances in Protein Chem.* **4** (1948) 297.
8. Horn, M. J., Blum, A. E., Gerdsdorff, C. E. F. and Warren, H. W. *J. Biol. Chem.* **203** (1953) 907.
9. Bates, R. W. *J. Biol. Chem.* **119** (1937) VII.
10. Consden, R., Gordon, A. H. and Martin, A. J. P. *Biochem. J. (London)* **38** (1944) 224.
11. Maehly, A. C. and Paléus, S. *Acta Chem. Scand.* **4** (1950) 508.
12. Paléus, S. *Acta Chem. Scand.* **6** (1952) 969.
13. Loew, O. *Ber.* **23** (1890) 289.
14. Meites, L. and Meites, T. *Anal. Chem.* **20** (1948) 984.

15. Paul, K. G. *Acta Chem. Scand.* **2** (1948) 430.
16. Tint, H. and Reiss, W. *J. Biol. Chem.* **182** (1950) 397.
17. Keilin, D. and Hartree, E. F. *Proc. Roy. Soc. (London)* **B 122** (1937) 298.
18. Paléus, S. and Neilands, J. B. *Acta Chem. Scand.* **4** (1950) 1024.
19. Margoliash, E. *Nature* **170** (1952) 1014.
20. Neilands, J. B. *J. Biol. Chem.* **197** (1952) 701.
21. Anson, M. L. *J. gen. Physiol.* **22** (1938) 79.
22. Currie, B. T. and Bull, H. B. *J. Biol. Chem.* **193** (1951) 29.
23. Winegard, H. M., Toennies, G. and Block, R. J. *Science* **108** (1948) 506.
24. Greenstein, J. P. *J. Biol. Chem.* **93** (1931) 479.
25. Wyman, J. *J. Biol. Chem.* **127** (1939) 1.
26. von Muralt, A. L. *J. Am. Chem. Soc.* **52** (1930) 3518.
27. Klotz, I. M. in Neurath, H. and Bailey, K. *The Proteins I*, Academic Press Inc. New York 1953, p. 752.
28. Theorell, H. *Enzymologia* **4** (1937) 192.
29. Theorell, H. *Biochem. Z.* **298** (1938) 242.
30. Paul, K. G. *Acta Chem. Scand.* **5** (1951) 389.
31. Theorell, H. and Åkeson, Å. *Science* **90** (1939) 67.
32. Keilin, D. and Hartree, E. F. *Biochem. J. (London)* **39** (1945) 289.
33. de Barbieri, A., and Zamboni, A. *Boll. soc. ital. biol. sper.* **27** (1951) 343.
34. Wyman, J. *J. Biol. Chem.* **127** (1939) 581.
35. Boeri, E., Ehrenberg, A., Paul, K. G. and Theorell, H. *Biochim. et Biophys. Acta* **12** (1953) 273.
36. Paul, K. G. *Arch. Biochem.* **12** (1947) 441.
37. Rodkey, F. L. and Ball, E. G. *J. Biol. Chem.* **182** (1950) 17.
38. Edsall, J. T. in Cohn, E. J. and Edsall, J. T. *Proteins, Amino Acids and Peptides*, Reinhold Publ. Corp. New York 1943, p. 445.
39. Tsou, C. L. *Biochem. J. (London)* **49** (1951) 367.
40. Brand, E. and Edsall, J. T. *Ann. Rev. Biochem.* **16** (1947) 223.

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