differences between second-order and first-order substitutions which are shown to depend on the relative isolation of the main reaction of substitution. The differences, of course, depend on the relative reactivity of the organic halides with specific reagents and are closely connected with structural and solvent effects. For reasons which have been given previously,[1] in reactions which involve hydroxylic solvents, such as water and the alcohols, we favor the "polymolecular interpretation" for the solvolysis of secondary and tertiary halides. When the solvent concentration is large and constant, the rate expression reduces to the first-order form. In the above examples, it is unnecessary to include the solvent concentration in the calculations.

If, for a given reaction which involves optically active molecules, ionization of the organic halide is definitely known to occur, this method should prove useful to calculate the degree of such ionization. The chief usefulness is for testing reaction mechanisms of the above type.

Summary

Rate equations were derived for the calculation of the change in the optical rotatory power of solutions in which several reactions proceed simultaneously. They were applied to three sets of experimental results for which the necessary data are sufficiently complete to test the equations. The agreement of theory and experiment is satisfactory in all cases and suggests the usefulness for the determination of reaction mechanisms.

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[CONTRIBUTION FROM THE BIOCHEMICAL INSTITUTION OF THE MEDICAL NOBEL INSTITUTE, STOCKHOLM]

Studies on Cytochrome c. I. Electrophoretic Purification of Cytochrome c and its Amino Acid Composition*

BY HUGO THEORELL AND Å. ÅKESSON

It has been mentioned in a previous report[1] that cytochrome c, which was obtained in 1935 by H. Theorell[2] and in 1937 by Keilin and Hartree[3] with an iron content of 0.34%, could be further purified by electrophoresis to a hemin iron content of 0.43%. The preparation purified in this way, unlike the preparation with 0.34% iron, migrates uniformly on electrophoresis at different hydrogen ion concentrations. As the electrophoretic test for purity seems to be very delicate with such a low molecular substance as cytochrome (M = 13,000), which shows, moreover, an extremely unusual ion mobility curve,[4] there was good reason to suppose that cytochrome with 0.43% iron really represented a chemically homogeneous substance. Moreover, on account of the low molecular weight, it was possible by further analyses of the content of sulfur and various amino acids to obtain additional confirmation of the homogeneity of the cytochrome, since in a pure substance all the constituent parts occur in whole number proportions in the molecule. There was another reason why the amino acid analyses were of interest. Keilin and Hartree[3] reported that the content of basic amino acids in cytochrome with 0.34% iron differed little from that of hemoglobin. These values seemed to us to be incompatible not only with the ion mobility curve of cytochrome, which we had previously found to constitute an almost constant plateau between *p*H 6 and *p*H 9, indicating a very low histidine content, but also with the strongly basic character of cytochrome: its isoelectric point lies at *p*H 10, in contradistinction to that of hemoglobin (about *p*H 7). Our amino acid analyses showed that the pure cytochrome contains much less histidine (3.3%) and much more lysine (24.7%) than was shown by Keilin and Hartree's analyses (7.8 and 9.1% respectively). The low histidine content partly explains the flat course of the ion mobility and titration curves in the range within which the imidazole groups are titrated (*p*H 5.5–8.5, Wyman[5]). In connection with the titration curve it will be shown, moreover, that probably two of the three histi-

* Reprints of this and the three following papers may be obtained from the Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena, California.

(1) H. Theorell and Å. Åkesson, *Science*, **90**, 67 (1939).

(2) H. Theorell, *Biochem. Z.*, **279**, 463 (1935).

(3) D. Keilin and E. F. Hartree, *Proc. Roy. Soc.* (London), **B122**, 298 (1937).

(4) H. Theorell, *Biochem. Z.*, **285**, 207 (1936).

(5) J. Wyman, *J. Biol. Chem.*, **127**, 1 (1939).

dine-imidazole groups constitute the hemochromogen forming groups, so that they are not titrated within the usual *p*H range.

For an accurate determination of the equivalent weight, which was necessary for the calculation of the number of amino acids per molecule, there were two possibilities: determination of either iron or of sulfur. The iron content has hitherto generally been made the basis of the calculation of the equivalent weight of hemin proteins. We found, however, that cytochrome c, even our purest preparations, sometimes contained a small quantity of non-hemin iron, amounting to 1–4% of the total iron. This showed itself in the fact that the relation between the iron content and light absorption (in reduced cytochrome c at 549.5 mμ) was not absolutely constant. On calculating the absorption coefficient $\beta_{549.5\ m\mu\ Fe^{++}}$ the values varied between 6.3 and 6.5 × 10^7 sq. cm./mole. The higher the iron content in relation to light absorption, the lower is β. The variations thus indicated that non-hemin iron was present, and that the correct value of β should be 6.5 × 10^7 or somewhat higher.

On the other hand, the relation between light absorption and sulfur content was found to be constant. Even before electrophoretic purification the cytochrome preparations contain about 6 atoms of sulfur per atom of iron.[4] On the final purification the color strength rises proportionally to the sulfur content. The impurities removed during the final purification are thus sulfur-free. As regards sulfur, there is no risk of "false" sulfur attaching to the molecule. For these reasons it proved more suitable to make the sulfur content (6 atoms of sulfur per mole) the basis of the accurate determination of the absorption coefficient. When we did this we obtained, in several different preparations, the mean value $\beta_{549.5,\ Fe^{++}}$ = 6.53 × 10^7 sq. cm./mole. As the sulfur content in the best preparations was 1.47–1.48%, we get as the probable value of the molecular weight 13,000.

The data of Table I were obtained using this molecular weight and the analytically determined values for certain amino acids. The good agreement between the values actually found and the calculated ones, as regards histidine, arginine and tyrosine, speaks as much in favor of the purity of the cytochrome preparation as does the constant sulfur content. The lysine content is so high that it is not possible to decide with certainty whether the cytochrome molecule contains

TABLE I

Amino acid	Found, % Hydrolysis 1	Found, % Hydrol. 2	Calcd. for the number of amino acids given in column 5	Probable number of moles per mole of cytochrome
Histidine	3.22	3.27	3.57	3
Arginine	2.62	2.81	2.65	2
Lysine	22.1	24.7	24.5	22
Tyrosine	5.43		5.50	4
Tryptophan	1.21		1.55	1

21 or 22 moles of lysine. The high lysine content is, however, in itself very remarkable. As far as we are aware, no protein having a similarly high lysine content has hitherto been known. This shows, moreover, that the protein component of the cytochrome must be specially synthesized for its purpose in the cells. The analyses of Keilin and Hartree[5] might suggest that the protein component of the cytochrome was nearly related to globin. We see, however, that this is not the case.

It is of especially great interest that cytochrome appears to contain 31 or 32 free amino groups and only 22 lysine residues. From this we deduce that there are 9 or 10 free α-amino groups, which indicates that there are present in the molecule the same number of polypeptide chains. Evidence of this sort does not seem to have been reported for any other protein molecule.

Cystine,[6] lysine, tyrosine, glutamic and aspartic acids, and leucine have been isolated in the pure state from cytochrome c. A more detailed account will be found in the experimental section.

The total number of amino acids in the cytochrome molecule can be calculated as follows. The nitrogen content in the purified preparation was 15.37%, the molecular weight = 13050, whence 15.37 × 13050/14 × 100 = 143 atoms of nitrogen per mole.

From this is subtracted

	4 porphyrin-N
	8 amide-N
	6 histidine-N
	6 arginine-N
	22 lysine-N
	1 tryptophan-N
Total	47 N

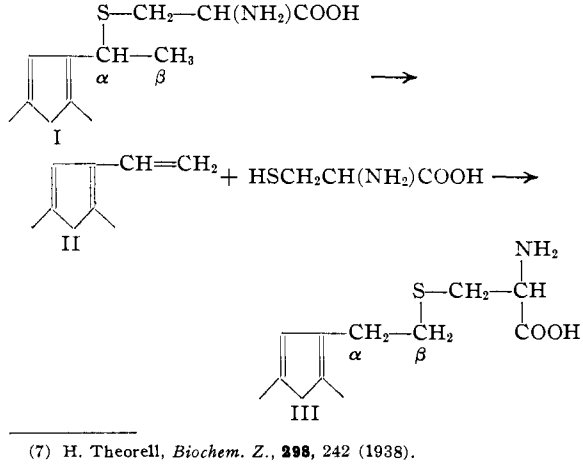
The difference gives 96 amino acids per molecule of cytochrome. It should not, however, be forgotten that two of these nitrogen atoms can also be regarded as belonging to the prosthetic group, "hemin c." As regards this question there is the following to be said:

The constitutional formula for "porphyrin c"

(6) H. Theorell, *Enzymologia*, **6**, 88 (1939).

as dicysteine adduct to the double bonds in protoporphyrin was first advanced by Theorell[6,7] on the basis of the production in the pure state of *l*-cystine from porphyrin c after splitting with hydrogen bromide in glacial acetic acid. It was, however, shown in a later work[8] that the sulfur content, after a short hydrolysis, was lower than 2 sulfur atoms per mole of porphyrin (lowest 0.9). On heating proto- or hemato-porphyrin with cysteine in hydrochloric acid solution it was, moreover, found that products resembling "porphyrin c" arose. It was therefore doubtful whether dicysteine-porphyrin existed preformed in the cytochrome, as it was conceivable that it was formed during the hydrolysis. Zeile and Meyer[9] have since carried out the hydrolysis under conditions that are supposed to exclude the possibility of an addition between the vinyl groups in the porphyrin and the SH-groups of the cysteine. Nevertheless, they obtained a dicysteine-porphyrin adduct, which is therefore assumed to be preformed in the cytochrome c. The constitutional formula that the authors advance is identical with that given earlier by Theorell.[6]

Theorell's observation that short hydrolysis with hydrochloric acid gives products with lower sulfur content than 2 S/1 mole porphyrin still remains unexplained, however. There is a possibility that the natural product might contain two cysteine residues in some sort of thio-ether linkages that are labile for hydrochloric acid hydrolysis; and these linkages might be split, and new, firmer thio-ether bindings be formed instead. It is possible that the cysteine residues go over from the α-position to the more stable β-position



In this way porphyrin mixtures with lower S content than two atoms per molecule might be obtained. It, is, however, difficult to understand why hydrochloric acid should split the α-configuration, while sulfuric acid left it intact. It may further be mentioned that Zeile and Meyer's synthetic dicysteine-porphyrin was not identical with the product obtained by the hydrolysis of cytochrome c.

Despite these obscurities there nevertheless seems at present to be fair reason for accepting Theorell's original view[6,7] that the "natural" c-porphyrin is a dicysteine-porphyrin.

If we count the two nitrogen atoms of the two cysteine residues as belonging to the protein component, there are thus 96 ± 1 amino acids in the protein component, cystine being reckoned as two amino acids. The error in the nitrogen content, corresponding to the given probable error in this figure, is 0.1 % N.

Cytochrome c fits very well in the multiple system that Bergmann has shown to be valid for a number of proteins, so far as the total number of residues is concerned, in fact so well that the analyses were not able to show any discrepancy here at all. According to Bergmann[10] it is possible to express the number of amino acid residues in a number of different protein molecules as $2^n \times 3^m$. With $n = 5$ and $m = 1$, we get 96 as the number of amino acid residues.

Moreover Bergmann and Niemann require a similar expression to hold for each individual amino acid. We do not verify this for lysine, for which we find 22 residues; the nearest Bergmann–Niemann number is 24.

The molecular weight of cytochrome lies quite near $^1/_3 \times 35000 = 11700$. For if the hemin is subtracted from the molecular weight of the cytochrome, we get the molecular weight of the protein component = 12350. *It thus constitutes the first known representative for a new weight-class in the system of proteins with multiple molecular weights first set up by Svedberg.*

The electrophoretically purified cytochrome is free from carbohydrate.

Finally, it may be mentioned here that cytochrome c from the cow's heart seems to be identical with the cytochrome from the horse's heart as regards molecular weight, nitrogen content and sulfur content. Both give the same titration

(7) H. Theorell, *Biochem. Z.*, **298**, 242 (1938).
(8) H. Theorell, *ibid.*, **301**, 201 (1939).
(9) K. Zeile and H. Meyer, *Naturwissenschaften*, **27**, 596 (1939); *Z. physiol. Chem.*, **262**, 178 (1939).
(10) M. Bergmann, *Chem. Rev.*, **22**, 423 (1938); M. Bergmann and C. Niemann, *J. Biol. Chem.*, **122**, 577 (1938), and other papers.

curve within the investigated range between *p*H 1.6 and 11, which proves a close similarity in amino acid composition, especially as regards histidine (*cf.* part III).

Some Remarks Concerning the Electrophoretic Purification.—The original material for the electrophoretic purification was cytochrome c, prepared by the method of Keilin and Hartree[3] or that of Theorell.[4] Regarding the first mentioned method it may be said that the shaking with chloroform in 1% sodium chloride solution did not, at least in our hands, lead to any further purification, so we omitted this stage. Further it has been our experience in connection with a large number of preparations, that Keilin and Hartree's method generally gives a preparation containing less than 0.34% iron. We have found percentages as low as 0.22%, and as a rule one gets a preparation with about 0.30 % iron. These variations, however, do not seem to affect the degree of purity of the preparation obtained by electrophoresis. On the other hand, the purer the original material, the better is the percentage yield of the final product.

This is due to an interesting circumstance. The cytochrome does not, on electrophoresis, migrate independently of the impurities, but seems to enter into dissociable salt formations with one or several of them. Consequently the cytochrome migrates in a different manner at the cathode end and at the anode end. Below the isoelectric point (*p*H 10.65 at 0°, see Fig. 1) pure cytochrome migrates upward at the cathode end with a definite boundary. The concentration above the original boundary is, however, lower than it is below it. This is obviously due to the fact that below the original boundary there is not only pure cytochrome in the same concentration as above this boundary, but also cytochrome that has been electrically neutralized through salt formation with acid impurities. At the anode end the same phenomenon is shown by the formation of an extended zone without definite boundary surfaces, in which the cytochrome is in part motionless and in part migrates more or less rapidly toward the cathode end. Furthermore, one observes boundaries migrating toward the anode, which derive from colorless or faintly brown-colored impurities. Toward the end, the concentration of the still pure cytochrome migrating toward the cathode diminishes more and more. This is due to the fact that after the originally free cytochrome, not neutralized by impurities, has separated, the originally neutralized cytochrome is dissociated from the impurities and begins to migrate toward the cathode. Thanks to this circumstance it is possible on prolonged electrophoresis to obtain high yields, *e. g.*, as much as 88% in example 1, although at the beginning only half was migrating toward the cathode. To get a yield of 100% however, is not practically possible.

The observed phenomenon has of course a general significance in connection with all electrophoretic investigations. It should be remembered that various kinds of combinations between impurities and the substance to be investigated may give rise to all sorts of disturbances. An undissociated or only slightly dissociated union of the substance and an impurity may show a homogeneous electrophoresis. In fact, we have in such a case a homogeneous substance—which may nevertheless be of slight interest, as the impurity may be of an adventitious and uninteresting nature.

Experimental Investigations

Examples of Electrophoretic Purification of Cytochrome c

1. **Electrophoresis in Sodium Phosphate *p*H 7.82.**—6.78 grams of cytochrome prepared according to Keilin and Hartree[3] with 0.285% iron (degree of purity, 0.67), dissolved in 164 ml. of sodium phosphate buffer solution (ionic strength, 0.1; *p*H 7.82) and previously dialyzed against the same buffer solution, was introduced into a large Tiselius apparatus.[11] The cytochrome solution filled the bottom cell, which contained glass globules to diminish its volume, and two vertical cells on each side. The third and topmost cell on each side contained buffer solution. When the apparatus had attained the temperature of the thermostat, 0°, the boundaries were compensated half a cell toward the anode end. The current was 35 milliamp.

The cytochrome at the cathode end migrated with a uniform boundary with the ionic mobility $u = 3.1 \times 10^{-5}$ sq. cm./volt × sec.

The phenomena occurring on electrophoresis have been described above. Considerable quantities of brown impurities moved toward the anode. Electrophoresis was continued for nine days, during which time further compensation was made, and cytochrome which had migrated to the cathode was four times taken from the apparatus with the help of the device described by Tiselius for convection-free suction, which was applied immediately under the uppermost cathodic boundary. Each portion was separately analyzed, by measuring the light absorption at 550 mμ after reduction, and by dry weight determinations. The dry weight (110°) of a certain volume of cytochrome solution was corrected for the dry weight of the buffer present. The sample of buffer used

(11) A. Tiselius, *Kolloid-Z.*, **85**, 129 (1938).

for this estimation was taken each time from the buffer solution just above the boundary at the cathode end. Table II shows the result.

TABLE II

Portion	Removed after hr.	Vol., ml.	% Fe according to $\beta_{550} = 6.53 \times 10^7$ cm.²/mole	G. cytochrome c	Mg./cc.
1	48	41.5	0.426	0.578	13.9
2	96	77.5	.422	1.080	13.9
3	146	109	.420	1.408	13.0
4	223	104	.423	0.915	8.8
		332		3.981	

The total yield was thus 3.981/6.78 × 0.67 = 88%. The original solution contained 6.78 × 0.67/164 = 27.7 mg. of pure cytochrome c per ml. The cytochrome moving toward the cathode, however, is, even at the beginning, only half as concentrated as this; and the latter portions, 3 and 4, are progressively more dilute. That the total yield is as high as 88% is due to the fact that no less than 332 ml. of solution was sucked up from the cathode end, whereas the whole original volume was 164 ml.

The portions 1–4 were combined, and smaller parts of the solution were dialyzed against different buffer solutions, after which new electrophoretic investigations were carried out in a smaller apparatus (for 10 ml. of test solution), to determine whether the preparation obtained was electrophoretically uniform at different *p*H values. *This was found to be the case.* No foreign boundaries could be observed, so there is strong reason for assuming that the preparation was pure. The values obtained for ionic mobility may be found in Fig. 1.

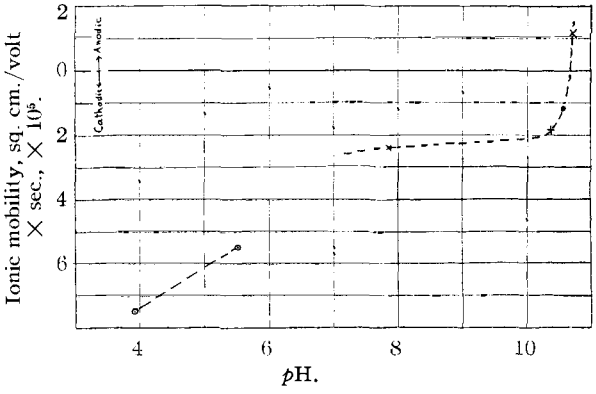


Fig. 1.—Ionic mobility of pure cytochrome c: ionic strength 0.1; temp., 0°; ⊙, acetate buffer; ×, phosphate buffer; +, borate buffer; ●, glycine buffer.

The isoelectric point (I. P.) for ferri-cytochrome at 0° lies at about *p*H 10.65, which corresponds to I. $P._{20°}$ = 10.05 according to the titrimetric determinations of the heat of dissociation (see part 3). This value agrees with the result of the titrations at 20°, and fairly well also with the value 9.86[4] previously obtained on less pure material.

The removal of the sodium phosphate from the solution by dialysis offered certain difficulties, as cytochrome c has a strong tendency to pass through cellophane or collodion. It is, however, generally possible with only slight loss to dialyze in a cellophane tube against very dilute ammonia of *p*H 10–10.2. In this case we first dialyzed in a parchment case against ammonia (concentrated solution diluted 3000 times) for sixty hours, after which the solution was evaporated to dryness. The ammonia is thus driven out on account of the basic properties of the cytochrome. The preparation was analyzed at this stage, although some phosphoric acid still remained.

Anal. N (Dumas), 15.12, 15.14; (Kjeldahl) 15.07, 15.00; S,[12] 1.435, 1.453; Fe,[14] 0.447, P,[13] 0.467 (= 1.48% H_3PO_4).

As the pure cytochrome is free of phosphorus, all the phosphorus is present as phosphate ion.

Light absorption, measured on reduced cytochrome at 550 mμ, gave $\beta = 6.54 \times 10^7$ cm.²/mol., if 1.444% S corresponds to 6.00 atoms per mol.

Calculation of the Molecular Weight.—If the S-content is corrected for the presence of 1.48% H_3PO_4, the sulfur content of the pure cytochrome is calculated to be 1.466% and M = 13,120.

The hemin iron content is then calculated as 55.84: 13,120 = 0.425%. The directly determined iron content was slightly higher, which indicates the presence of traces of iron that do not belong to the cytochrome hemin.

The number of N atoms per molecule is: 15.08 × 13,120/(100 × 0.985 × 14) = 143.

This preparation was used for the determination of certain titration curves after the phosphoric acid had been quantitatively removed by a week's dialysis, first against *n*/1000 hydrochloric acid and then against water. In this process the phosphoric acid is replaced by hydrochloric acid, but the cytochrome is so strongly basic that it cannot be dialyzed free from negative ions against water (but only against a base such as ammonia). In this case the chloride content in the preparation was determined after the dialysis and the chloride content obtained was included in the calculation of the titration curve.

Example 2.—Cytochrome c (300 mg.) of a degree of purity of 0.72 was subjected to electrophoresis first in a Tiselius apparatus with a volume of 10 ml. at *p*H 10.68 in phosphate buffer. The cytochrome here moved slowly in the direction of the anode. Various impurities moved more rapidly in the same direction, so that it was possible by suitable compensation to remove them. The degree of purity then rose to 0.95. The final purification was carried out in phosphate buffer at *p*H 7.3 in an electrophoretic apparatus for preparative purposes, according to the principle described by Theorell.[15] This apparatus was constructed of glass and vulcanite. Each cell held 35 ml. The membranes consisted of hardened filter paper. In this way pure cytochrome c was obtained.

Anal.: S, 1.440 (cor. 1.465); Fe, 0.422 (cor. 0.429); C, 52.56, 52.48; H, 7.63, 7.89. The corrected Fe and S values were based on the finding that 1.7% phosphate was present.

The molecular weight, assuming six atoms of sulfur per molecule = 13,120. *The determination of the light absorption coefficient* for reduced cytochrome at 550 mμ gave $\beta = 6.51 \times 10^7$ sq. cm./mole.

(12) B. Josephson, *Analyst*, **64**, 181 (1939).
(13) C. H. Fiske and Y. Subbarow, *J. Biol. Chem.*, **66**, 375 (1925).
(14) L. Lorber, *Biochem. Z.*, **181**, 391 (1927).
(15) H. Theorell, "Abderhaldens Handbuch der biol. Arbeits methoden," Abt. V., Teil 10, p. 1097, 1936.

Example 3.—It is from some points of view advantageous to use ammonium phosphate instead of sodium phosphate as the buffer substance in electrophoresis, in which case the silver–silver chloride electrodes may suitably be surrounded with saturated ammonium chloride. The advantage is that most of the ammonium phosphate can easily be removed by the addition of barium hydroxide, the greater part of the phosphoric acid being precipitated as barium phosphate, whereupon the ammonia is removed by evaporation in vacuum. Finally, the excess of barium hydroxide is removed with sulfuric acid.

The electrophoresis was carried out in the larger Tiselius apparatus: 6.3 g. of cytochrome preparation of a degree of purity of 0.6 was dissolved in 165 ml. of ammonium phosphate buffer, *p*H 7.99, H = 3.67×10^{-3}, and was dialyzed against the same buffer. The current used during the experiment was 50–60 milliamp.; temp., 0°.

The phenomena previously described as taking place at the cathode and anode ends were also in evidence here. The boundary of pure cytochrome moving toward the cathode showed an ionic mobility of 2.67×10^{-5} sq. cm./volt × sec.

The electrophoresis was continued for 245 hours. Altogether 240 ml. of cytochrome solution was drawn off in three portions at different intervals from the cathodic limb. To the sum of the three portions saturated barium hydroxide solution was added until there was no more precipitation (70 ml.), after which a further 10 ml. of barium hydroxide was added. The barium phosphate was removed and washed, and the solution freed of ammonia by evaporation *in vacuo*. The barium was removed quantitatively with sulfuric acid. The solution then contained 2.2 g. of pure cytochrome, yield 58%. The solution still contained, however, some Cl^-, which had entered from the ammonium chloride solution at the negative electrode during the prolonged electrophoresis. For this reason dialysis was carried out for two days in a cellophane tube against dilute ammonia (*p*H 10.2), followed by evaporation *in vacuo* over sulfuric acid to dryness.

Anal. N (Dumas): 15.05, 15.06; Cl^-,[16] 0.29.

Measurement of the hemin iron content, spectrophotometrically at 550 mμ in reduced cytochrome, $\beta = 6.53 \times 10^7$ sq. cm./mole: Fe, 0.418%.

It was probable that the chloride ions retained an equivalent quantity of ammonia during the evaporation to dryness. This was confirmed in two ways: (1) after the addition first of hydrochloric acid to a solution of the cytochrome, and expulsion of all carbonic acid, and subsequent addition of an exactly equivalent amount of sodium hydroxide, we obtained *p*H 10.03 at 20°, which is the correct isoelectric point in the ion milieu employed. (2) On hydrolysis for amino acid determination we obtained a correspondingly higher value for the amide nitrogen than on the occasion of two previous hydrolyses of ammonium-free cytochrome.

It is thus most reasonable to assume that the preparation contained 0.44% ammonium chloride. If the nitrogen and the hemin-iron contents are corrected accordingly one gets nitrogen = 15.06% and iron = 0.420%. This again gives 143 atoms nitrogen per atom iron.

(16) K. Linderstrøm-Lang, A. H. Palmer and H. Holter, *Z. physiol. Chem.*, **231**, 226 (1935).

Amino Acid Analyses

Hydrolysis 1.—Cytochrome c (0.677 g.) of a degree of purity of 0.96, employed for the determination of titration curves, contained, according to the nitrogen determination (micro-Kjeldahl), 102.9 mg. nitrogen. By saturation with hydrogen chloride gas at 0° porphyrin c was split off (compare[7]), the solution was afterward hydrolyzed in 70 ml. of 38% hydrochloric acid by boiling on a sand-bath under a reflux condenser for eight hours. The product of the hydrolysis was evaporated in vacuum to dryness and taken up in water, after which insoluble humin substances were centrifuged off and washed. The humin nitrogen was 1.80 mg. (micro-Kjeldahl).

The supernatant liquid and washings (volume 6 ml.) were saturated with hydrogen chloride at 0°. A certain amount of sodium chloride crystallized out and was removed. No glutamic acid appeared. The solution was evaporated to dryness over phosphorus pentoxide and solid sodium hydroxide, dissolved in water, to which saturated calcium hydroxide solution was then added to *p*H 4.3, and left overnight in a refrigerator. Porphyrin c had then precipitated almost quantitatively. It was centrifuged off and washed with water. Only after ten washings did the ninhydrin test become negative on the wash water.

The porphyrin was dissolved in *N* hydrochloric acid. A black, insoluble residue contained 0.17 mg. nitrogen. The porphyrin contained 3.02 mg. nitrogen and 2.66 mg. sulfur, nitrogen 9.12, sulfur 8.03, or 5.2 atoms of nitrogen per 2 atoms of sulfur. Thus, as was expected, a certain amount of nitrogen (0.8 atom per mole) had been split off in the way previously described,[8] probably partly in the form of ammonia, which is thus added to the amide nitrogen fraction.

To the amino acid solution (30 ml.), freed from porphyrin c, was added 1 ml. of 10% calcium hydroxide suspension and 10 ml. alcohol. The mixture was distilled for thirty minutes in vacuum at 45° (bath temperature). A suitable amount of sulfuric acid was present in the receiver. The amide nitrogen was determined by titration as 6.20 mg., 6.03% of the nitrogen.

The lime was separated off and washed eleven times, until it was free from nitrogen. The wash waters were added to the main solution, and the whole diluted to 100 ml. Cystine was then determined colorimetrically according to Kassell and Brand.[17] The whole solution contained 5.03 mg. cystine = 0.57% of the nitrogen. Cytochrome c thus contains cystine in excess of that which is included as cystine residues in the hemin. This excess cystine presumably does not occur in the cytochrome molecule as cysteine, the negative nitroprusside test indicating that free sulfhydryl groups are absent.

One molecule of cystine per mole of cytochrome would, however, correspond to 1.4% of the nitrogen instead of the 0.57% found. It is well known that cystine is largely destroyed during the hydrolysis with hydrochloric acid. It is naturally impossible on this basis to say how many of the 6 sulfur atoms arise from cystine. Two belong to the cysteine residues of the porphyrin. Of the other four it may be said that probably two, perhaps all four, derive from cystine. This question is being investigated.

(17) B. Kassell and E. Brand, *J. Biol. Chem.*, **125**, 115 (1938).

The whole solution contained (micro-Kjeldahl) 94.4 mg. of nitrogen, which agrees with the total nitrogen minus the sum of the amide-, humin- and porphyrin-nitrogen. 91.2 ml. of the solution was evaporated *in vacuo* to 15 ml.; 2.5 ml. of concentrated hydrochloric acid was added, and afterward 5 ml. of phosphotungstic acid solution (1 g. phosphotungstic acid per ml.). The suspension was diluted with water to 30 ml. and heated on a water-bath until no more solid dissolved. A certain amount remained undissolved even at 100°. The solution was then left to stand for sixty hours at room temperature, after which the precipitate was centrifuged down and washed seven times with 26 ml. of 2.5% phosphotungstic acid in *N* hydrochloric acid. The filtrate fraction ("F") was diluted to 100 ml. for analysis. The basic nitrogen fraction was freed from phosphotungstic acid with amyl alcohol + ether. After evaporation of the amyl alcohol and ether the residue was dissolved in 200 ml. of water = fraction "B."

Analyses of Fraction B.—Colorimetric determination of **arginine** according to Jorpes and Thorén[18] gave 15.96 mg., recalculated for the whole hydrolyzate. To this is added the correction for the solubility of arginine phosphotungstate in the filtrate, 1.81 mg. or together 17.77 mg. = 2.62% arginine.

Determination of **histidine** according to Jorpes[19] gave 19.25 mg., recalculated for the whole hydrolyzate, plus 2.56 mg. correction for the solubility of histidine phosphotungstate in the filtrate, together 21.81 mg. = 3.22% histidine.

Determination of **cystine** according to Kassell and Brand[17] gave 1.65 mg. of cystine in the whole base fraction.

Lysine was calculated from the difference between total-N and arginine + histidine + cystine-N in the base fraction, which gave 28.53 mg. of lysine-N plus correction for the solubility of lysine phosphotungstate in the filtrate, 0.18 mg. = 28.71 mg. lysine-N = 27.9% of total-N = 149.8 mg. lysine, or 22.1% of the dry substance. The value corresponds to 20 moles of lysine per mole of cytochrome c, if the molecule contains 143 atoms of N.

Lysine was isolated from the remaining 166.5 ml. of the base fraction in the following way.

The solution was dried *in vacuo* and freed from most of the sodium chloride present by treatment of the dry residue with concentrated hydrochloric acid. This was followed by drying in vacuum over sodium hydroxide and treatment according to Kossel and Kutscher's silver–baryta method. The dry residue was dissolved in 25 ml. of warm water and solid silver sulfate was added until, on being tested with barium hydroxide solution, a yellow precipitate was obtained. This was followed by warm centrifuging and the precipitate was washed five times with water. The solution was saturated with baryta at 40°, the precipitate was centrifuged off and washed with saturated baryta. Ba^{++} was removed with an equivalent amount of sulfuric acid, the precipitate was washed, and the combined solutions were evaporated to about 4 ml. A little precipitate of barium sulfate was centrifuged off and washed with 4 ml. of water. To the main quantity was added 0.35 g. of picric acid in 6 ml. of alcohol. The resulting precipitate was dissolved again, but after the addition of the 4 ml. of washing fluid a large, beautiful crystalline precipitate of lysine picrate appeared. This was washed twice with 3 ml. of alcohol. The dry crystals weighed 191 mg. The mother liquor was freed from excess picric acid by shaking with ether and dried, when 56 mg. of crystals, resembling the foregoing, was obtained. The two portions were combined and recrystallized from 5 ml. of water. In this way 167 mg. of almost pure lysine picrate was obtained. The melting point was 267°. The picric acid was removed and the lysine analyzed: $\alpha^{20}D$ 100 × 0.12/0.008726 = +14° ± 1°. For pure l(+)-lysine there is given $\alpha^{20}D$ +14.6°.

Anal. Calcd. for $C_6H_{14}O_4N_2$: N, 19.2; carboxyl C,[20] 8.21. Found: N, 18.6; carboxyl C,[20] 8.65.

The whole amount of lysine picrate obtained according to the Kossel–Kutscher procedure was 167 mg. + 27 mg. (correction for the solubility in the amount of water used for the re-crystallization) = 194 mg.; re-calculated for the whole hydrolyzate: 194 × (1/0.912) × (200/166.5) = 256 mg. of lysine picrate, or 100 mg. of lysine = 14.8% of the dry substance.

The Filtrate Fraction "F."—The total nitrogen, determined according to micro-Kjeldahl, was 47.43 mg.: 0.912 = 51.95 mg. nitrogen for the whole hydrolyzate. This gives, if 102.9 mg. nitrogen is equal to 143 atoms per mole, 72 molecules of mono-amino acids per mole of cytochrome. If we add to this the already separated 25 basic amino acid molecules we get 97 amino acids per mole of cytochrome. Mono-amino nitrogen, determined according to Van Slyke, was 44:0.912 = 48.2 mg. for the whole hydrolyzate. The difference between 51.95 and 48.2 mg. = 3.8 mg. nitrogen may be due either to the presence of small quantities of proline or, more probably, to the fact that a certain amount of glutamic acid has gone over to pyrrolidone-carboxylic acid, in which case little or no proline is present in cytochrome (*cf.* hydrolysis 2).

Carboxyl carbon, according to Van Slyke and Dillon,[20] gave 47.6 mg. of carbon, recalculated to the original amount. This corresponds to 77 atoms of carbon, or 5 more than the total nitrogen, which would indicate the presence of about five molecules of aspartic acid, both of whose carboxyl groups react with ninhydrin, unlike glutamic acid, which reacts rapidly only with the α-COOH group.

The remaining part of the solution was freed from phosphotungstic acid by shaking with amyl alcohol and ether (1:1); the solution was 0.66 *N* in respect to hydrochloric acid. Only 0.9 mg. of nitrogen went into the amyl alcohol–ether layer. The solution was evaporated to dryness *in vacuo* over sodium hydroxide and the residue was dissolved in a little water. Calcium was afterward removed by means of ammonium oxalate, after which the solution was treated with baryta and alcohol according to Jones and Moeller.[21] No glutamic acid was obtained, probably owing to the small quantities and possibly to transformation to pyrrolidone carboxylic acid. From 58.5% of the originally hydrolyzed amount, on the other hand, we obtained by means of the copper salt 6.4 mg. of pure aspartic acid,

(18) E. Jorpes and S. Thorén, *Biochem. J.*, **26**, 1504 (1932).

(19) E. Jorpes, *ibid.*, **26**, 1507 (1932).

(20) D. Van Slyke and R. Dillon, *Compt. rend. trav. lab. Carlsberg, sér. chim.*, **22**, 480 (1938).

(21) D. B. Jones and O. Moeller, *J. Biol. Chem.*, **79**, 429 (1928).

crystallized out of water solution—thus 10.9 mg. of aspartic acid from 6.77 mg. of cytochrome = 1.6%, or 1.6 mole of aspartic acid per mole of cytochrome. This value is certainly too low, owing to unavoidable losses, which are proportionally much greater in the preparation of small quantities.

Anal. Calcd. for $C_4H_7O_4N$: amino N (Van Slyke), 10.53; carboxyl C,[20] 18.0. Found: amino N, 10.58; carboxyl C, 18.1.

The filtrate from the copper aspartate gave, after removal of copper and evaporation in vacuum to a small volume, 8 mg. of tyrosine (7.67% N, calcd. 7.74%).

The filtrate from the tyrosine crystals contained 10.2 mg. of nitrogen, recalculated for the whole original amount. Since in hydrolysis 2 the presence of no other dicarboxylic acids besides aspartic and glutamic acid could be shown, we are justified in assuming that these 10.2 mg. of nitrogen derived from a mixture of glutamic and pyrrolidone carboxylic acid; 10.2 mg. of nitrogen corresponds to 107 mg. of glutamic acid = 15.8% of the dry weight of the cytochrome. The value is obviously rather uncertain.

Hydrolysis 2.—1.725 g. of cytochrome, of degree of purity 1 (Fe 0.428%, N 15.38%), was subjected to a similar hydrolysis. The results obtained are summarized briefly here.[22] The **humin nitrogen** was 1.78 mg. (2 nitrogen per molecule of cytochrome); **amide nitrogen** was 6.06% of total nitrogen = 8.67 moles per mole of cytochrome; since a little of this arises from cystine breakdown, the true figure is taken to be 8 moles.

In phosphotungstic acid precipitate, **arginine** found, 2.82% (calcd. for two moles per mole of cytochrome 2.67%). **Histidine** found, 3.27% (calcd. for 3 moles per mole of cytochrome, 3.57%). **Lysine** (estimated by difference) 24.7% = 22.1 moles per mole of cytochrome. Cystine (colorimetric): 4.83 mg. Filtrate from phosphotungstic acid precipitate contained 114.9 mg. nitrogen, and 117 mg. of amino nitrogen (Van Slyke). Hence the absence of proline was inferred.

***l*(+)-Glutamic acid** was isolated in pure form, by a complex procedure which inevitably involved loss, to the extent of 102.8 mg. (6.0% of the cytochrome). The actual content of glutamic acid is certainly considerably greater. Concerning the procedures used, see Jones and Moeller[21] and Jukes.[23] ***l*-Aspartic acid** was again isolated as the copper salt in pure form, corresponding to 1.02% aspartic acid (lower yield than in hydrolysis 1).

Following Dakin[24] an attempt was made to separate hydroxyglutamic acid, but no evidence of its presence was obtained.

Some **leucine** was isolated in pure form, although in small yield.

Amino nitrogen determinations on the unhydrolyzed cytochrome (reaction time thirty minutes) gave 31–32 atoms of amine nitrogen per mole of cytochrome. Since the figure of 22 atoms of ϵ-amino nitrogen from lysine appears to be maximal, this indicates that a number of free α-amino groups are present in cytochrome c, suggesting the presence of several peptide chains in the molecule of native cytochrome.

Determinations of tyrosine and tryptophan were carried out according to Brand and Kassell[25] after alkaline hydrolysis of 47–73 mg. of pure cytochrome in 2 ml. of 5 *N* sodium hydroxide, in sealed evacuated tubes on a water-bath. In the colorimetric procedure, analyzed preparations of the two amino acids were used as a standard. The results are given in Table III.

TABLE III

Period for hydrolysis in hours	Tyrosine, %	Tryptophan, %
22	5.18	0.98
25	5.40	1.07
30	5.43	1.17
72	(6.07)	1.21

In the last hydrolysis the tyrosine fraction, but not the tryptophan fraction, was discolored, so the colorimetric value for tyrosine is clearly too high, and is to be ignored.

The results of the analyses of amino acids and nitrogen are given in Table IV, which shows the distribution of the 143 nitrogen atoms in the cytochrome molecule, as far as this has been ascertained in the course of this investigation. Besides the composition of the monoamino-monocarboxylic acid fractions it remains to investigate the sulfur-containing amino acids. Whether methionine is present is not yet known.

TABLE IV

Fraction	No. of molecules	No. of N atoms	% of total N
Porphyrin c	1	6	4.2
Histidine	3	9	6.3
Arginine	2	8	5.6
Lysine	22	44	30.8
Cystine	1 ?	2	1.4
Tyrosine	5	5	3.5
Tryptophan	1	2	1.4
Glutamic + aspartic acid	19	19	13.3
Amide-N	8	8	5.6
Leucine (+ isoleucine + phenylalanine ?) — fraction	9	9	6.3
(Alanine + glycine + valine + hydroxyvaline) fraction	33	33	23.1
		Total 143	Total 100

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(22) For reasons of space, the editor has found it necessary to curtail drastically the detailed description of hydrolysis 2 presented in the original manuscript. Owing to the difficulty of communicating with the author in Sweden, the responsibility for preparing the condensed version given here has been assumed by Professors Carl Niemann and John T. Edsall.

For additional data order ADI Document 1510 from the American Documentation Institute, 2101 Constitution Ave., Washington, D. C., remitting 30¢ for microfilm, or $1.10 for photocopies readable without optical aid. This document gives a full account of Hydrolysis 2.

(23) T. H. Jukes, *J. Biol. Chem.*, **103**, 425 (1933).

(24) H. D. Dakin, *Biochem. J.*, **12**, 290 (1918).

(25) E. Brand and B. Kassell, *J. Biol. Chem.*, **131**, 489 (1939).