3. The urine of rats and rabbits injected with 2-naphthylamine was examined by paper chromatography. 2-Naphthylamine, 2-acetamidonaphthalene, 2-acetamido-6-naphthol, 2-amino-1-naphthyl hydrogen sulphate, 2-acetamido-6-naphthyl hydrogen sulphate and a compound which is probably 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene were detected. Rat urine sometimes contained 2-amino-6-naphthol and its hydrogen sulphate. The injection of 2-acetamidonaphthalene gave the same products except that 2-naphthylamine was not detected in rabbit urine and 2-amino-6-naphthol and its hydrogen sulphate were not detected in rat urine. 2-Amino-1-naphthol, 2-acetamido-1-naphthol and 2-acetamido-1-naphthyl hydrogen sulphate were not detected as metabolites of 2-naphthylamine or 2-acetamidonaphthalene in the urine of rats or rabbits.

4. The oxidation of 2-acetamidonaphthalene by an ascorbic acid-ferrous sulphate-oxygen system yields a compound which is probably 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene.

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REFERENCES

- Asatoor, A. & Dalglish, C. E. (1956). *J. chem. Soc.* p. 2291.
 Booth, J. & Boyland, E. (1949). *Biochem. J.* **44**, 361.
 Booth, J., Boyland, E. & Manson, D. (1955). *Biochem. J.* **60**, 62.
 Boyland, E. & Manson, D. (1957). *Biochem. J.* **67**, 275.
 Boyland, E., Manson, D. & Orr, S. F. D. (1957). *Biochem. J.* **65**, 417.
 Boyland, E. & Sims, P. (1957). *Biochem. J.* **66**, 38.
 Brodie, B. T., Axelrod, J., Shore, P. A. & Udenfriend, S. (1954). *J. biol. Chem.* **208**, 741.
 Corner, E. D. S. & Young, L. (1954). *Biochem. J.* **58**, 647.
 Corner, E. D. S. & Young, L. (1955). *Biochem. J.* **61**, 132.
 Crawford, T. B. B. & Outschoorn, A. S. (1951). *Brit. J. Pharmacol.* **6**, 8.
 Dobriner, K., Hoffman, K. & Rhoads, C. P. (1941). *Science*, **93**, 600.
 Ekman, B. (1948). *Acta chem. scand.* **2**, 383.
 Fieser, L. F. & Fieser, M. (1939). *J. Amer. chem. Soc.* **61**, 896.
 Kehrman, F. & Matis, M. (1898). *Ber. dtsch. chem. Ges.* **31**, 2413.
 Liebermann, C. & Jacobson, P. (1882). *Liebigs. Ann.* **211**, 36.
 Manson, L. A. & Young, L. (1950). *Biochem. J.* **47**, 170.
 Peters, J. H. & Gutmann, H. R. (1955). *J. biol. Chem.* **216**, 713.
 Sato, T., Yamada, M., Suzuki, T., Fukuyama, T. & Yoshikawa, H. (1956). *J. Biochem., Tokyo*, **43**, 25.
 Saunders, C. R. & Hamilton, C. S. (1932). *J. Amer. chem. Soc.* **54**, 636.
 Solomon, J. B. (1954). Ph.D. thesis: University of London.
 Sudborough, J. J. (1901). *Trans. chem. Soc.* **79**, 533.
 Udenfriend, S., Clark, C. T., Axelrod, J. & Brodie, B. T. (1954). *J. biol. Chem.* **208**, 731.
 Vesely, V. & Jakes, M. (1923). *Bull. Soc. chim. Fr.* **4e**, **33**, 942.
 Wiley, F. H. (1938). *J. biol. chem.* **124**, 627.
 Young, L. (1947). *Biochem. J.* **41**, 417.

The Amino Acid Composition of Cytochrome c from Horse Heart

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Though the physiological properties of haemoproteins such as haemoglobin, catalase, peroxidase and cytochrome *c* ultimately depend on the structure of their common prosthetic group they differ so widely that the properties of the prosthetic group must be greatly affected by the nature of the protein to which it is attached and the way in which it is bound to the protein. Our understanding of this problem is limited largely by our ignorance of the structure of the proteins involved. A certain amount is known about the relation

between the protein and haem parts of the molecule of cytochrome *c*. Theorell (1938, 1939) discovered that the prosthetic group is firmly attached to the protein by thioether linkages with two cysteine residues. Tsou (1951) subsequently isolated from a peptic hydrolysate of cytochrome *c* a peptide carrying the prosthetic group, and the amino acid sequence of this peptide has been worked out (Tuppy & Paléus, 1955; Leaf & Gillies, 1955). Tuppy & Bodo (1954) also examined similar peptides obtained by tryptic and acid hydrolysis. As none of these peptides possesses the specific enzymic properties of cytochrome *c*, however, the remainder of the peptide chain must contribute significantly to the properties of the intact protein.

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It seems necessary therefore to know the structure of the whole protein moiety before the problem of its association with the prosthetic group can be solved.

The first step in the investigation of the structure of a protein is usually the determination of its amino acid composition. Though Keilin & Hartree (1937) analysed cytochrome *c* for basic amino acids and cystine, it subsequently transpired that their preparation was impure and the first extensive study of what would now be regarded as a pure sample of the protein was made by Theorell & Åkeson (1941). Their success was limited by the methods of amino acid analysis then available. Since then methods have greatly improved and it appeared desirable to investigate the problem with the elegant methods of amino acid analysis developed by Moore and Stein (Stein & Moore, 1948; Moore & Stein, 1949, 1951). Since this work was undertaken three other amino acid analyses of cytochrome *c* have been published, one of cytochrome *c* from cow heart by Theorell & Ehrenberg (1955) and two of horse-heart cytochrome *c* by de Barbieri & Zamboni (1951) and de Barbieri & Scevola (1955). These are discussed below.

EXPERIMENTAL

Materials

Cytochrome *c*. This was prepared from fresh horse heart by the method of Keilin & Hartree (1945). Care was taken to prevent frothing, particularly when the protein was in acid solution, as this appeared to promote denaturation. The final dialysis was carried out against distilled water rather than sodium chloride solution and the solution then lyophilized. The product varied greatly in purity, from run to run, containing from 0.30 to 0.42% of Fe. It was further purified by chromatography on the ion-exchange resin Amberlite IRC-50. This method was first described by Paléus & Neilands (1950) and was later studied by Margoliash (1954). The conditions used in this Laboratory differed from those used by these workers in that sodium phosphate buffers were used to develop the columns. The finely divided resin (100–240 mesh) was purified by washing with acid and alkali and stored dry in the hydrogen form. Columns (3 cm. × 20 cm.) were prepared by a method

similar to that described by Hirs, Moore & Stein (1953), being poured in phosphate buffer (pH 7.0, 0.162M-Na⁺). Phosphate buffer was allowed to pass through the column until the effluent and influent solutions had the same pH and optical density at 280 mμ. The cytochrome *c* (200–500 mg.) was dissolved in 2–5 ml. of phosphate buffer (0.1M, pH 7.0). This solution was run into the top of the column and the chromatogram developed with the same buffer. A light-brown band ran rapidly down the column. This material showed a weak metmyoglobin spectrum and was probably a mixture of metmyoglobin and its globin as suggested by Margoliash (1954). Two further bands moved more slowly down the column, a narrow pink band consisting of the ferrous form of cytochrome *c*, and following it the main band of the ferric cytochrome. Near the top of the column there remained a number of narrow reddish-brown bands which probably consisted of cytochrome *c* modified by the action of trichloroacetic acid. The elution with 0.1M-buffer was continued until the main band was approximately half way down the column, by which time the optical density of the effluent had returned to its initial value, and the concentration of the buffer was then increased to 0.25M. This accelerated the movement of the main band and yielded the purified cytochrome in more concentrated solution. This procedure gave a more efficient separation of the oxidized and reduced forms of the cytochrome than the method of Margoliash (1954), and eliminated the necessity of repeating the fractionation at pH 9. The main fraction of the cytochrome moved down the column as a symmetrical band which appeared to be homogeneous by spectrophotometric analysis of different fractions of the eluate at 550 and 280 mμ. The eluate containing the oxidized cytochrome was dialysed against distilled water until free of phosphate, and either lyophilized or stored at –20°.

The activity of the cytochrome *c* in the succinoxidase system (Potter & Dubois, 1942) bore a constant relation to its light absorption at 550 mμ during this stage of its purification, and the spectral properties of the final product were not affected by carbon monoxide (Tsou, 1951). It was assumed therefore that the protein had not undergone denaturation. The analytical values for the purified protein are shown in Table 1, where they are compared with those obtained by other workers. The homogeneity of the preparation was tested in the Tiselius apparatus and gave a single peak both in phosphate buffer (pH 7.0, *I* 0.2) and veronal buffer (pH 8.5, *I* 0.2). Analysis of samples from different parts of the U-tube after electrophoresis yielded the same analytical figures as the starting material.

Table 1. *Analytical values of cytochrome c preparations*

	Present work	Margoliash (1955) Horse heart	Tint & Reiss (1950)		Paléus & Neilands (1950) Cow heart	Theorell & Åkeson (1941)
Iron (%)	0.460	0.465	0.456	0.453	0.466	0.43
10 ⁻⁴ $\epsilon_{550 \text{ m}\mu}$ (reduced)	2.76	2.77	2.78	2.64	2.69	2.81
$\bar{E}_{550 \text{ m}\mu}$ (reduced)	1.20	1.19	—	—	1.17	—
$\bar{E}_{280 \text{ m}\mu}$ (oxidized)	—	—	—	—	—	—
Nitrogen (%)	15.98	—	—	—	—	15.10
Mol. wt.	12 100	12 000	12 270	12 350	11 980	13 000

Reagents. The reagents used were as far as possible of analytical-reagent quality. The amino acids used for recovery experiments were obtained from British Drug Houses Ltd. and their purity was checked by determination of carbon, hydrogen and nitrogen content and by paper chromatography. We are indebted to Mr J. L. Cameron of the Chemistry Department for the microanalyses.

Methods

Amino acid analysis. The protein was hydrolysed by heating in a sealed tube with 100 times its weight of glass-distilled 6*N*-HCl at 110° for 24 hr. The HCl was then removed by repeated evaporation *in vacuo* over NaOH and H₂SO₄. The residue was dissolved and made up to a known volume with the solvent which was to be used for chromatographic analysis and samples were taken for determination of total nitrogen. No attempt was made to determine 'humin nitrogen', though a small quantity of humin was present. The hydrolysate was qualitatively analysed by paper chromatography with butanol-acetic acid-water (4:1:5, by vol.; Partridge, 1948) and saturated aq. phenol soln. in the presence of ammonia (Dent, 1948). The dried chromatograms were sprayed with a 0.2% (w/v) solution of ninhydrin in aq. 50% (v/v) ethanol containing 1% (by vol.) of both acetic acid and collidine.

The quantitative analyses were at first carried out on starch columns as described by Stein & Moore (1948) and Moore & Stein (1949), with potato starch obtained from Gordon Slater Co. The results obtained were as described by these authors except that the separation of alanine and glutamic acid with 2-methylpropan-2-ol-butan-2-ol-0.1*N*-HCl (2:1:1) was unsatisfactory. This separation was later achieved by chromatography on ion-exchange resin (Moore & Stein, 1951). The resin used was Zeo-Karb 225 with a nominal degree of cross-linking of 8% and average particle size of 50 μ . In this case the resolution was entirely satisfactory. No attempt was made to determine the basic amino acids on the 100 cm. columns, which were stopped after the emergence of phenylalanine. The basic amino acids and ammonia were determined separately with 15 cm. columns, which also yielded a combined value for phenylalanine and tyrosine.

The effluent from the columns was collected in fractions of 1 ml. and the amino acid content of these was determined with the ninhydrin reagent of Moore & Stein (1948). The reagent (1 ml.) was used for each analysis with a heating time of 30 min. and the optical density was measured in the Unicam SP. 500 or SP. 600 spectrophotometer. Standard curves (optical density/concentration) were prepared for each amino acid and the relative colour values agreed with those reported by Moore & Stein (1948). In our experience the ninhydrin reagent did not always keep for more than a week and it was found desirable to analyse a standard amino acid solution with each batch of column fractions. The standard solution was prepared by dilution of a stock solution of glycine (AnalaR) to 0.4 mM with the same solvent as was present in the column fractions to be analysed.

Determination of cyst(e)ine by oxidation to cysteic acid. Preliminary experiments suggested that bromine water was a milder reagent for this purpose than performic acid and yet gave a quantitative yield of cysteic acid when acting on standard solutions of cystine. The amino acid solution or protein hydrolysate was made *N* with respect to

HCl and a saturated solution of bromine in water was added dropwise until the solution had a persistent but faint yellow colour. The solution was then evaporated in a desiccator over NaOH and H₂SO₄, dissolved in citrate buffer (pH 3.42) and run through a 100 cm. column of Zeo-Karb 225 prepared as for the other amino acid analyses. The cysteic acid emerged after 25 ml. of the buffer had passed through the column and was therefore well separated from all other amino acids present.

RESULTS

The results of the analyses are presented in Table 2. The results of qualitative analysis are implicit in the list of amino acids given. Serine was absent from all pure preparations and indeed its presence in a hydrolysate was taken as a sign of impurity in the sample of cytochrome used. In addition to the amino acids listed in Table 2 tryptophan was present. This amino acid was not detected by paper chromatography as only acid hydrolysates were examined by this technique, but it was shown to be present by the method of Graham, Smith & Hier (1947) with the intact protein. In Table 2 the values obtained by the use of starch and ion-exchange columns are presented separately as these techniques provide a differential analysis insofar as the amino acids emerge from the respective columns in different orders. It might therefore be expected that fortuitous contamination of particular amino acid fractions would not be the same for both types of column. The close agreement between the two sets of results therefore lends them greater significance.

The numbers in parentheses after the analytical values in Table 2 represent the occasions on which the particular amino acid was sufficiently separated from others for individual determination. On starch chromatograms developed with butanol-propanol-0.1*N*-HCl, resolution is incomplete and the following groups of amino acids are found in mixed fractions: leucine and isoleucine; valine, tyrosine and methionine; alanine and glutamic acid. The first two groups were separated on chromatograms developed with butanol-benzyl alcohol-water and the means of results of two such analyses are given in Table 2. These results agree with and are therefore supported by combined values obtained on four other columns where resolution of these particular amino acids was incomplete. The same applies to the results for phenylalanine and tyrosine obtained from ion-exchange chromatograms. These amino acids were determined separately on 100 cm. columns on two occasions and as a combined fraction on the short columns used primarily for the separation of the basic amino acids on four other occasions. As mentioned in the Experimental section alanine and glutamic acid were not separated on starch chromatograms, but

for ease of presentation the combined value for these amino acids obtained from starch chromatograms has been divided in proportion to the individual values obtained from the ion-exchange chromatograms. This makes it possible to correct for the loss of glutamic acid due to esterification on the starch columns.

The mean total recoveries of nitrogen from the starch columns was 94.3% and from the ion-exchange columns 94.8%. If allowance is made for the prosthetic group and tryptophan (assuming one residue of tryptophan/molecule of cytochrome *c*) these values are raised to 101.35 and 101.65 respectively. The mean-weight recoveries (based on residue weights), for the three analyses for which it was determined, was 90.5%, which with allowance for one residue of tryptophan and the prosthetic group is raised to 97.6%. The weight recovery was determined only in some experiments as it was thought that the weighing of the small amounts of protein used for the analyses was less accurate than duplicate determinations of nitrogen.

Recoveries of individual amino acids were determined for both starch and ion-exchange resin chromatograms before analysis of cytochrome *c* hydrolysates, but when values for the composition of the cytochrome *c* hydrolysate became available an amino acid mixture made up to have the same composition was analysed by ion-exchange chromatography. The percentage recoveries of the amino

acids from this mixture are shown in Table 2. In general the recoveries were similar to those obtained by Moore & Stein (1951) but those of cystine, methionine, isoleucine and arginine were low.

The poor recovery of the sulphur-containing amino acids was presumably caused by their oxidation during passage through the column. The presence of freshly distilled thiodiglycol in the eluting buffer and the use of boiled buffer did not appreciably improve the situation. The value of 62% for the recovery of cystine was based on three determinations for which the individual values were 50, 67 and 69%. The degree of loss is therefore sufficiently consistent to allow of correction of the values obtained for the cyst(e)ine content of the hydrolysate if it is assumed that the loss is the same for the experimental as for the control chromatograms. A suitable correction has therefore been applied to the value given in Table 2. No value for cyst(e)ine was obtained from the starch chromatograms, on which this amino acid was completely destroyed. The figure quoted in Table 2 cannot therefore be regarded as very reliable. An attempt was made to overcome this difficulty by oxidizing the cystine to cysteic acid before chromatography of the hydrolysate. In an artificial mixture of amino acids cystine was oxidized quantitatively to cysteic acid and the recovery of this acid from ion-exchange columns was complete. When, however, a sample of the protein hydrolysate was treated in

Table 2. *Amino acid composition of cytochrome c*

The number of separate estimations on which each value is based is given in parentheses.

	Starch columns		Ion-exchange columns		
	Amino acid N as % of total N	Residues/mole of cytochrome <i>c</i>	Amino acid N as % of total N	Residues/mole of cytochrome <i>c</i>	Recovery from amino acid mixture (%)
Glycine	7.72 (4)	10.6	7.94 (2)	10.9	99
Alanine	3.92*	5.4	3.77 (2)	5.2	97
Valine	1.60 (2)	2.2	1.53 (2)	2.1	98
Leucine	3.94 (2)	5.4	3.81 (2)	5.2	97
Isoleucine	3.97 (2)	5.4	3.70 (2)	5.1	92
Proline	2.60 (4)	3.5	2.10 (1)	2.9	97
Phenylalanine	2.25 (5)	3.1	2.34 (2)	3.2	104
Tyrosine	2.61 (2)	3.6	2.75 (2)	3.8	97
Threonine	6.24 (4)†	8.6	6.37 (2)†	8.8	100
Cyst(e)ine	Lost	—	1.66 (2)†	2.2	62
Methionine	0.98 (2)	1.3	Lost	—	40
Arginine	5.51 (5)	1.9	5.19 (4)	1.8	91
Histidine	4.44 (4)	2.0	5.64 (5)	2.6	98
Lysine	26.75 (5)	18.3	25.75 (5)	17.8	95
Aspartic acid	5.18 (5)	7.1	5.15 (2)	7.1	98
Glutamic acid	8.00*	11.0	7.92 (2)†	10.8	94
Ammonia	5.08 (5)	7.0	6.24 (3)	8.5	100

* Alanine and glutamic acid were not separated on starch columns. The mean combined value from five determinations has been divided in proportion to the separate values obtained for these amino acids from ion-exchange chromatograms. Allowance has been made for 7% loss of glutamic acid on starch columns owing to esterification.

† Corrected for 8% loss on hydrolysis.

‡ Corrected for loss on column.

the same way the yield of cysteic acid corresponded to 1.38 residues of cysteine/molecule of cytochrome *c*, similar to the yield without oxidation. This suggests that some constituent of the hydrolysate, perhaps a breakdown product of the prosthetic group, interferes with the oxidation of cystine. Schram, Moore & Bigwood (1954) recommend the oxidation of the cyst(e)ine residues in the intact protein, but it is doubtful whether this method could be applied successfully to cytochrome *c* as the effect of the oxidizing agent on the thioether linkages between the protein and the prosthetic group cannot be predicted.

In contrast with cystine, methionine could be determined on starch but not on Zeo-Karb 225. When the artificial mixture of amino acids was analysed on an ion-exchange column some 60 % of the methionine was lost, but from the cytochrome hydrolysate the loss of methionine was practically complete. Fortunately the conditions used for the determination of this amino acid on starch columns were less destructive. From an amino acid mixture 98 % of the added methionine was recovered. The value obtained for the methionine content of the cytochrome hydrolysate is not, however, as precise as this would suggest. The amount of hydrolysate which can be separated on a starch column with butanol-benzyl alcohol-water as eluting solvent is severely limited by the disturbing effect of the hydrophilic amino acids, which are present in unusually high proportion in cytochrome *c*, on the equilibrium between the starch and the solvent. This meant that the methionine peak was low and the accuracy of the determination reduced. It is thought that the value obtained is low rather than high and therefore indicates the presence of two residues of methionine in the protein molecule.

The low recovery of isoleucine was at first puzzling, as the sample of the amino acid used in making up the amino acid mixture gave satisfactory analytical figures for carbon, hydrogen and nitrogen. The explanation may, however, be found in the observations of Piez (1954) and van Dam-Bakker (1958) that commercial samples of synthetic isoleucine usually contain some *alloisoleucine* which runs with methionine on the ion-exchange column. The low recovery of methionine makes it impossible to determine from our results whether this is the true explanation but it suggests that correction of the isoleucine values obtained by analysis of the hydrolysate is unnecessary.

The low value for the recovery of arginine from the ion-exchange resin column may be due to error in the ninhydrin determination rather than incomplete elution from the column. Arginine is eluted from the column as a rather broad band and as there is only a small quantity present this means

that the ninhydrin-colour values given by the tubes containing arginine are low. Any uncertainty in the blank value can therefore quite easily introduce an error of 10 % into the determination. In spite of this uncertainty the analyses indicate fairly clearly that there are two residues of arginine in the molecule of cytochrome *c*.

The recovery of other amino acids and ammonia lay between 95 and 100 % for both starch and ion-exchange chromatograms. The values obtained by the two methods agree well with one another except for those of ammonia, histidine and proline. The value for ammonia obtained from the ion-exchange chromatogram is appreciably greater than that obtained on starch chromatograms but it is thought that the lower value is more reliable. The ion-exchange resin may pick up traces of ammonia inadvertently present in the buffers used for the preparation of the column and this ammonia will subsequently be eluted from the column at about the same time as the ammonia added in the hydrolysate. This is not likely to happen with a starch column. Of the two values obtained for histidine, that obtained from ion-exchange columns is taken to be the more accurate. The precision of the determination of histidine on starch columns is limited by the same factors as were mentioned in connexion with the determination of arginine. The low colour value given by proline in the ninhydrin reaction similarly reduces the accuracy with which this amino acid can be determined. It is impossible on the basis of the present analyses to say whether there are three or four residues of proline in the molecule of cytochrome *c*.

Loss of amino acids during the process of hydrolysis. The process of hydrolysis itself causes loss of some amino acids. The values for threonine have been corrected for loss with the factor found by Hirs, Stein & Moore (1954) for similar conditions of hydrolysis. This correction is arbitrary, particularly in view of the findings of Smith & Stockell (1954) and Smith, Stockell & Kimmel (1954), but even Smith's method of determining the loss involves the assumption that the rate of loss is constant throughout the period of hydrolysis. Tryptophan, as was expected, appeared to be destroyed completely during hydrolysis. An attempt was made to determine the tryptophan content of the intact protein by the method of Graham *et al.* (1947). The result obtained corresponded to 0.58 residue/molecule of cytochrome, which suggests that the method does not work very well for this protein, but in the absence of better results may be taken to show the presence of one residue of tryptophan in the cytochrome molecule. Direct spectrophotometric methods such as that of Holiday & Ogston (1938) cannot be used owing to interference by the prosthetic group.

DISCUSSION

The results expressed as residues/mole of cytochrome *c* corrected to the nearest whole number are presented in Table 3. For comparison the values obtained by de Barbieri & Scevola (1955) for cytochrome *c* prepared from horse heart and cow heart and those of Theorell & Ehrenberg (1955) for cytochrome *c* prepared from cow heart are also included. The earlier results of de Barbieri & Zamboni (1951) differ so widely from those given in Table 3 that they have not been included. The values of de Barbieri & Scevola (1955), quoted in Table 3, show closer agreement with results of the present work on the one hand and those of Theorell & Ehrenberg (1955) on the other. There are, however, marked discrepancies, and it is thought that the results of de Barbieri & Scevola (1955), which were obtained by paper chromatography, are more likely to be in error than results obtained by column chromatography. The technique of quantitative paper chromatography requires further refinement before it is adequate for purposes of this sort. There is in general closer agreement between our results and those of Theorell & Ehrenberg (1955) for cow-heart cytochrome *c*. It is not clear from the report of the Swedish workers whether cow-heart cytochrome *c* contains serine. If it does not, the most significant difference between the two proteins lies in their content of glycine. Another notable difference is that most of the values for cow-heart cytochrome are rather higher than ours for the

protein from horse heart. It must be noted, however, that calculation of the molecular weight from the figures of Theorell & Ehrenberg (1955) gives a value of some 15 600. This is much higher than their value of 13 000, determined in the ultracentrifuge, and the value of approximately 12 000 calculated from the iron content of the purest preparations (Paléus & Neilands, 1950). This may have a bearing on the calculation of specific volume of the protein made by Theorell & Ehrenberg. They obtained a value of 0.737 without considering the prosthetic group. As the observed value for the specific volume of cytochrome *c* is 0.702–0.707 they concluded that the prosthetic group must take up very little space. The value calculated from our results (Table 3) is 0.708, which makes any such assumption about the packing of the prosthetic group unnecessary.

In view of the observations of Theorell & Åkeson (1941) not much difference would be expected between the two proteins in respect of the balance of acidic and basic groups, and this is borne out by the figures in Table 3. The number of histidine residues present is of particular interest, as Theorell has suggested that two of these residues are bound to the iron atom of the prosthetic group. Margoliash (1955), by means of paper chromatography, obtained a value of four residues of histidine/mole of horse-heart cytochrome *c*. From our own results it seems very unlikely that there are more than three. If the value for ammonia in Table 3 is taken as a measure of the number of

Table 3. *Comparison of the amino acid compositions of cytochrome c from heart of horse and cow*

Values are given in terms of residues of amino acid/molecule of cytochrome *c*. The values from the present work are corrected to the nearest whole number (cf. Table 2), those of Theorell & Ehrenberg (1955) are quoted as they were published and those of de Barbieri & Scevola (1955) are calculated from the percentages given by these authors, assuming a molecular weight of 12 000.

	Present work	de Barbieri & Scevola (1955)		Theorell & Ehrenberg (1955)
		Horse heart	Cow heart	
Glycine	11	8.6	8.8	15.5
Alanine	5	5.3	7.0	6.6
Valine	2	3.5	2.8	3.3
Leucine	5	7.5	7.9	6.2
Isoleucine	5			6.2
Proline	3	4.3	6.0	3.9
Phenylalanine	3	2.8	3.3	3.3
Tyrosine	4	2.1	2.3	3.8
Tryptophan	1	—	—	1.0
Serine	0	0	1.2	8.5
Threonine	9	7.8	5.7	
Cyst(e)ine	2	1.0	2.7	2.0
Methionine	2	1.1	1.1	2.0
Arginine	2	3.2	3.0	3.4
Histidine	3	3.1	5.2	3.1
Lysine	18	12.9	12.2	18.3
Aspartic acid	7	7.8	9.6	9.4
Glutamic acid	11	8.6	8.3	11.9
Ammonia	7	—	—	—

amide groups in the molecule there is an excess of some 12 basic groups over acidic groups, and this is in keeping with the basic properties of cytochrome *c*.

The values quoted for the sulphur-containing amino acids in Table 3 require comment. Our experience suggests that the methods used in the present work are not suitable for accurate determination of these amino acids, but in the absence of other values we have put forward those that appear most likely, that is, two methionine and two cysteine residues/molecule of cytochrome *c*. It is not known whether the conditions of hydrolysis used here were sufficiently vigorous to split the thioether bonds linking the prosthetic group to cysteine residues in the protein. If so, these may be the only cysteine residues in the protein. Åkeson (1942) and Paléus (1955) concluded that this was true of cytochrome *c* from cow heart. Carruthers (1947), on the other hand, obtained evidence that even in this protein there are cysteine residues other than those involved in the binding of the prosthetic group. It may be that Carruthers's polarographic data are open to other interpretations but further examination of this problem seems desirable.

SUMMARY

1. Cytochrome *c* was prepared from horse heart and purified by chromatography on Amberlite IRC-50. The product contained 0.46 % of iron and 15.98 % of nitrogen.

2. The amino acid composition of the protein was determined by means of starch and ion-exchange chromatography.

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REFERENCES

- Åkeson, A. (1942). *Acta physiol. scand.* **4**, 362.
 Carruthers, C. (1947). *J. biol. Chem.* **171**, 641.
 de Barbieri, A. & Scevola, M. E. (1955). *Bull. Soc. ital. Biol. sper.* **31**, 688.
 de Barbieri, A. & Zamboni, A. (1951). *Bull. Soc. ital. Biol. sper.* **27**, 343.
 Dent, C. E. (1948). *Biochem. J.* **43**, 168.
 Graham, C. E., Smith, E. P. & Hier, S. W. (1947). *J. biol. Chem.* **168**, 711.
 Hirs, C. H. W., Moore, S. & Stein, W. H. (1953). *J. biol. Chem.* **200**, 493.
 Hirs, C. H. W., Stein, W. H. & Moore, S. (1954). *J. biol. Chem.* **211**, 941.
 Holiday, E. R. & Ogston, A. G. (1938). *Biochem. J.* **32**, 1166.
 Keilin, D. & Hartree, E. F. (1937). *Proc. Roy. Soc. B*, **122**, 298.
 Keilin, D. & Hartree, E. F. (1945). *Biochem. J.* **39**, 289.
 Leaf, G. & Gillies, N. E. (1955). *Biochem. J.* **61**, vii.
 Margoliash, E. (1954). *Biochem. J.* **56**, 529, 535.
 Margoliash, E. (1955). *Nature, Lond.*, **175**, 293.
 Moore, S. & Stein, W. H. (1948). *J. biol. Chem.* **176**, 367.
 Moore, S. & Stein, W. H. (1949). *J. biol. Chem.* **178**, 53.
 Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.
 Paléus, S. (1955). *Acta chem. scand.* **9**, 335.
 Paléus, S. & Neilands, J. B. (1950). *Acta chem. scand.* **4**, 1024.
 Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
 Piez, K. A. (1954). *J. biol. Chem.* **207**, 77.
 Potter, V. R. & Dubois, K. P. (1942). *J. biol. Chem.* **142**, 417.
 Schram, E., Moore, S. & Bigwood, E. J. (1954). *Biochem. J.* **57**, 33.
 Smith, E. L. & Stockell, A. (1954). *J. biol. Chem.* **207**, 501.
 Smith, E. L., Stockell, A. & Kimmel, J. R. (1954). *J. biol. Chem.* **207**, 551.
 Stein, W. H. & Moore, S. (1948). *J. biol. Chem.* **176**, 337.
 Theorell, H. (1938). *Biochem. Z.* **298**, 242.
 Theorell, H. (1939). *Enzymologia*, **6**, 88.
 Theorell, H. & Åkeson, A. (1941). *J. Amer. chem. Soc.* **63**, 1804.
 Theorell, H. & Ehrenberg, A. (1955). *Acta chem. scand.* **9**, 1193.
 Tint, H. & Reiss, W. (1950). *J. biol. Chem.* **182**, 397.
 Tsou, C. L. (1951). *Biochem. J.* **49**, 362, 367.
 Tuppy, H. & Bodo, G. (1954). *Mh. Chem.* **85**, 808, 1024.
 Tuppy, H. & Paléus, S. (1955). *Acta chem. scand.* **9**, 353.
 van Dam-Bakker, A. W. I. (1958). *Nature, Lond.*, **181**, 116.

The Assimilation of Amino Acids by Bacteria

25. THE PREPARATION AND ACTIVITIES OF A FACTOR INVOLVED IN THE INCORPORATION OF AMINO ACIDS IN DISRUPTED STAPHYLOCOCCAL CELLS*

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In previous papers of this series (Gale & Folk, 1955*a*, *b*) a description has been given of the preparation of disrupted cells of *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) which retain the ability to synthesize proteins and to incorporate

labelled amino acids into the protein of the preparation. Incubation of the preparation with ribonuclease or deoxyribonuclease, or extraction with *m*-sodium chloride, results in a loss of these abilities. The synthesis of proteins can be restored by addition to the incubation mixture of suitable combinations of nucleic acids or purines and

* Part 24: Gale & Folk (1957).