AMINO ACID COMPOSITION AND SOME OTHER PROPERTIES OF YEAST CYTOCHROME *C* IN COMPARISON WITH HORSE-HEART CYTOCHROME *C*

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From a detailed chemical and spectroscopic study of cytochrome *c*, isolated from beef heart, THEORELL AND ÅKESON[1] suggested in 1941 that both haemochromogen-forming groups were the imidazole groups of two histidine residues in the protein. Earlier, THEORELL had brought forward evidence that the prosthetic group was also linked to the protein by means of two thioether bridges, formed by attachment of –SH groups of cysteine residues in the protein to the vinyl groups of the proto-porphyrin[2]. These views have recently received support by determinations of the structure of the haem-containing fragments of the residue of the cytochrome *c* molecule remaining after proteolytic digestion[3,4,5]. However, definite proof exists for only one of the histidine groups presumed to be attached to the iron atom[6].

Little is known about the structure of the protein, apart from the immediate vicinity of the haem. As a first step in this direction and in the hope of obtaining some information concerning which amino acids were essential for the catalytic function of cytochrome *c*, a comparison has been made between the amino acid compositions of cytochrome *c*'s obtained from two very different sources, horse heart and yeast.

Although cytochrome *c* was first isolated from yeast[7], and shown to react with mammalian cytochrome oxidase, little further study had been made of the cytochrome from this source when this investigation was commenced. During its course, papers by LI AND TSOU[8] and by OKUNUKI and co-workers appeared[9,10].

Horse-heart cytochrome *c* has been used in most of the studies by the KEILIN school, but the detailed chemical studies, including amino acid analyses, by the THEORELL school were made with beef-heart cytochrome *c*. In view of the importance attached to the histidine groups, it appeared most desirable to see whether the difference between the number of histidine groups reported by THEORELL AND ÅKESON[1] for beef cytochrome *c* (three) and that reported by MARGOLIASH[11] for horse cytochrome *c* (four) could be confirmed.

METHODS

Determination of purity and concentration of cytochrome c

The purity of the cytochrome *c* was measured by the ratio of the optical density at 550 mμ of the reduced form to the optical density at 280 mμ of the oxidized form ($E_{550}:E_{280}$). Measurements were made first at 280 mμ in 0.1 *M* phosphate buffer, pH 7.3, after the addition of excess $K_3Fe(CN)_6$ to oxidize the cytochrome. Dithionite ($Na_2S_2O_4$) was then added to reduce the cyto-

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chrome and the optical density at 550 mμ determined. The concentration of the cytochrome *c* was calculated from the optical density at 550 mμ using a value of 27.6 for the millimolar extinction coefficient. The purity was also determined by measurement of the % Fe content, calculated from the cytochrome *c* content and the dry weight (determined after drying to constant weight at 110°).

The nitrogen content was determined by the Kjeldahl method, using red HgO as catalyst.

Free electrophoresis was carried out at 2° in the 2 ml cells of the Perkin-Elmer apparatus, model 38 A.

Catalytic activity

The catalytic activity in the cytochrome *c* oxidase reaction was measured manometrically at 25° with the following reaction mixture[12]: phosphate pH 7.4, 0.068 *M*; ethylenediaminetetraacetate (EDTA), 10^{-3} *M*; ascorbate, 0.025 *M*; Keilin and Hartree heart-muscle preparation, about 0.25 mg protein/ml; cytochrome *c* concentration, $5.8 \cdot 10^{-5}$ *M*. The rate of O_2 uptake in a blank measurement, without heart-muscle preparation, was subtracted from the rate in the test. This blank is a measure of the rate of auto-oxidation of the cytochrome *c* preparation.

Isolation and purification of cytochrome c

Horse-heart cytochrome c was isolated according to KEILIN AND HARTREE[13] and purified on a column of Amberlite IRC-50, according to the procedure of MARGOLIASH[14]. The $E_{550}:E_{280}$ was 1.24, and the iron content 0.454 %.

Yeast cytochrome c. Preliminary experiments were carried out with various types of yeast (*Pichia*, *Candida* and *Torula*) in addition to the usual baker's yeast (*Saccharomyces cerevisiae*). For the extraction of the cytochrome *c*, various methods were tried, including autolysis, extraction of fresh or dried yeast with trichloroacetic acid, organic solvents (butanol, acetone, ethyl acetate), or various salt solutions and bases. The procedure finally adopted was largely based on KEILIN's original procedure[7], since this was found the most suitable for extracting large amounts of yeast.

Stage 1. *S. cerevisiae* was specially grown under strong aeration in order to obtain a high yield of cytochrome *c*, and was washed in a centrifuge*. The washed and compressed yeast was treated in 6 kg batches. Each batch was plasmolysed with 300 g NaCl and added to 15 l boiling tap water in a stainless-steel container provided with a mechanical stirrer. The rapidly stirred suspension was quickly brought again to boiling, held at this temperature for 15 sec, and then rapidly cooled by the addition of 20 kg crushed ice. The stirring was continued until all the ice had melted and the temperature was below 20°. After standing 3 h, the greenish upper layer was removed by suction and enough tapwater was added to the remainder to bring the volume to 40 l. The mixture was then centrifuged in a Sharples separator and the residue (about 3 kg wet weight) suspended in about 1.75 l water containing 150 g $Na_2S_2O_5$. Then 150 g $Na_2S_2O_4$ were added and the suspension kept overnight in a well-stoppered amber bottle. The following day, a crude extract was obtained by filtration with suction through a layer of Hyflo Supercel on a 50 × 50 cm stainless steel Büchner funnel. The cake was washed with 0.5 l water, containing 2 % $NaHSO_3$. To the combined extract (about 3 l), 20 g $CaCl_2$ were added and SO_2 was bubbled through the solution for 10 min. The precipitated proteins were removed by filtration through Supercel. The filtrate was dialysed against running tapwater to give about 4 l of a solution containing about $3.5 \cdot 10^{-6}$ *M* cytochrome *c*.

Stage 2. Boric acid and NaCl were added to this solution, each to a final concentration of 0.05 *M*, and it was then passed through a column of Amberlite IRC 50 (200 mesh) in the sodium form. The column containing the adsorbed cytochrome *c* was washed with water, and the cytochrome eluted with a solution of 1 *M* NaCl, 0.05 *M* borax. The eluate was dialysed against water and filtered. The yield was 170 ml of a solution containing $6.4 \cdot 10^{-5}$ *M* cytochrome *c* (1.8 μmoles cytochrome *c*/kg yeast). The ratio $E_{550} : E_{280}$ at this stage was 0.4.

Stage 3. Further purification was obtained by following the procedure of KEILIN AND HARTREE for horse heart[13]. The combined extracts from 60 kg yeast were diluted with water to 5 l, cooled to 4°, and 3.55 kg ammonium sulphate were added. After filtration, 25 ml 20 % (w/v) trichloroacetic acid were added to each litre of filtrate. The precipitate of cytochrome *c* was collected by centrifugation, washed with saturated ammonium sulphate, and dialysed until free from $SO_4^{=}$. The yield from 120 kg yeast was 550 ml of a solution containing $2.2 \cdot 10^{-4}$ *M* cytochrome *c* (1.0 μmole cytochrome *c*/kg yeast). The ratio $E_{550}:E_{280}$ was 0.80 and the iron content 0.34 %.

Stage 4. This preparation was rechromatographed on a 5 × 15 cm column of Amberlite IRC 50. After adsorption on the column and washing through the column with 1 l water, the cytochrome band was spread over the column with 0.1 *M* ammonium acetate (*cf.* MARGOLIASH's

* We are greatly indebted to the Koninklijke Nederlandse Gist- en Spiritusfabrieken in Delft for carrying out this process for us.

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procedure[14] with horse-heart cytochrome *c*). Unlike horse-heart cytochrome *c*, the yeast cytochrome could not be eluted with 0.25 *M* NH_4OH; apparently it is more firmly bound to the Amberlite. As in stage 2, elution was brought about with 1.0 *M* NaCl, 0.05 *M* borax. The whole cytochrome fraction was collected. $E_{550} : E_{280} = 0.83$.

After dialysis against water, the adsorption was repeated, but the band was eluted with 1.0 *M* NaCl, 0.05 *M* borax, omitting the treatment with ammonium acetate. From the much narrower band of cytochrome *c*, a darker coloured band was separated and discarded. The cytochrome *c* solution was dialysed as before, to yield a solution with an $E_{550}:E_{280}$ of 1.1, containing 0.37% Fe and 15.7% N. Yield = 0.7 μmole/kg yeast.

Stage 5. In free electrophoresis using a Veronal buffer of ionic strength 0.1, pH 8.3, this cytochrome solution separated into two components, with electrophoretic mobilities of 1.9 and $2.35 \cdot 10^{-5}$ cm^2 V^{-1} sec^{-1} respectively. Both components, present in about equal amounts, showed the typical absorption spectrum of cytochrome *c*.

The isolation of the two cytochromes on a larger scale was carried out by paper electrophoresis, as follows. A concentrated solution, containing 6 μmoles of the cytochrome, was applied as a narrow band on a sheet of Whatman No. 3 MM paper, 30 × 30 cm. The paper was previously moistened with 0.02 *M* phosphate, pH 8.4, and the sample was applied at a spot where the fluid convection was at a minimum, found by tests with a neutral substance (glucose). A potential of 400 V was applied for 16 h. No cooling was used. The cytochromes moved very slowly to the cathode and separated clearly, as shown in Fig. 1. The cytochromes were separately eluted from the paper with very dilute acetic acid. The slow- and fast-moving components are referred to as *S* and *F*, respectively.

The paper electrophoresis removed some ninhydrin-positive, non-protein material. This is demonstrated in Fig. 2, which shows a test strip which was cut into three smaller strips after a

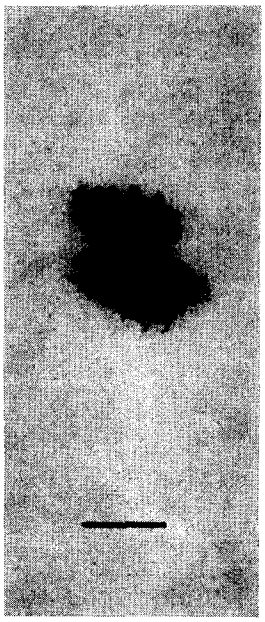

Fig. 1. Paper-electrophoretical separation of yeast cytochrome *c* into two fractions. A 30 cm strip of Whatman No 3 MM paper was used. Phosphate pH 8.4, 0.02 *M*. A potential of 400 V was applied for 16 h. The horizontal line marks the origin. Both fractions move towards the cathode.

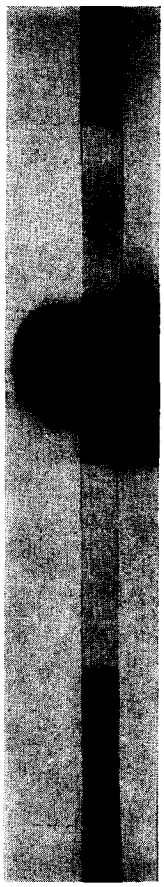

Fig. 2. Paper electrophoresis of the preparation of yeast cytochrome *c* obtained at stage 4. Conditions as in Fig. 1, but the electrophoresis was run for only 2 h. The strip was then cut into three smaller strips. The left-hand strip was unstained, the middle stained with ninhydrin and the right-hand strip stained with bromophenol blue.

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2-h run. The left-hand strip shows the cytochrome not yet separated into its two components. The middle strip was stained with ninhydrin, revealing at least three ninhydrin-positive substances, which were not stained with bromophenol blue (a protein stain) on the third strip.

Amino acid analyses

Hydrolysis. Separate hydrolysates were made for each chromatographic run, according to the procedure of HIRS *et al.*[15]. An amount of the cytochrome *c* solution containing about 0.2 μmole of the cytochrome was freeze-dried on the bottom of a thick-walled Pyrex tube, measuring 10 × 0.8 cm. Glass-distilled HCl (0.5 ml of 6 *N*) was added and the solution frozen in a mixture of solid CO_2 and alcohol. After evacuation by an oil pump, the tube was sealed. The hydrolysis was carried out in an oven at 110 ± 0.5° for 22 h. The tube was then opened, and the contents freeze-dried in a desiccator containing NaOH pellets under a high vacuum.

Analysis. For the chromatographic separation of the acidic and neutral amino acids, the original procedure of MOORE AND STEIN[16] was used, with a few modifications as described by SCHRAM *et al.*[17]. A 100 cm column of Dowex-50 200–400 mesh, 8% cross-linking was used. The basic amino acids were separated on a 15 cm column of Amberlite IR 120, according to a new procedure of MOORE *et al.*[18]. The resin for this column was prepared by grinding one pound of analytical grade Amberlite IR 120 (H) with water in a mechanical mortar for 5 min. By means of a water spray the finest particles were washed through a 200 mesh sieve and the residue returned to the mortar for a further grinding for 5 min. This process was repeated until the whole batch was passed through the sieve. From this preparation the finest particles were removed by fractional sedimentation and discarded. Only that part was used that sedimented from a suspension in water in about 3 min. Cleaning and reconditioning was carried out as prescribed for Dowex-50[16]. The advantages of this method are the greater elution speed (12 ml/h) and the elimination of the difficulties due to changes in the blanks[18].

The elution buffer contained per l: citric acid·$1H_2O$, 28 g; NaOH, 16.5 g; conc. HCl, 7.5 ml; Brij 35* (50% solution), 5 ml. The pH was adjusted to 5.25 ± 0.01 with the glass electrode.

The dried contents of the hydrolysis tubes were dissolved in 0.5 ml of citrate buffer with the same sodium concentration as the elution buffer, but adjusted to pH 2.5 for the 100 cm column and 4.2 for the 15 cm column**. This solution was brought on to the column, which was then washed with three 0.3 ml quantities of the same buffer used to dissolve the hydrolysate. Elution was then commenced, 1 ml samples being collected in an automatic fraction-cutter, already described[20]. The amino acid content of the fractions was determined by the new photometric ninhydrin method of MOORE AND STEIN[21]. The photometric readings were converted to concentrations of amino acids, by means of factors previously determined for each amino acid. The values for serine and threonine were corrected by 16% and 8% respectively, to allow for destruction of the amino acids during the hydrolysis. The figures for ammonia, given in the tables, were not corrected for the unknown amounts of ammonia evolved during the partial destruction of serine and threonine and the complete destruction of tryptophan.

The chromatographic separations obtained with both columns, using a hydrolysate of yeast cytochrome *c*, are shown in Figs. 3 and 4. It should be noted that owing to the higher sodium concentration of the buffer, histidine and lysine are eluted in the opposite order, compared with the Dowex-50 method. Since no methionine sulphoxide was found, no correction of the methionine values (*cf.* ref.[17]) was necessary.

RESULTS

Isolation of yeast cytochrome c

The yeast used was specially grown for its high cytochrome content, a choice being made from four batches. Visual spectroscopy showed that it contained about 2.5–4 μmoles/kg (*cf.* maximum of about 5 μmoles/kg reported by BOREI AND SJÖDÉN[22]) so that the initial extraction (2.3 μmoles/kg yeast) gave a good yield. Losses occurred in the later steps (see Table I), particularly during the trichloroacetic acid

* Polyoxyethylene lauryl ester, Atlas Powder Company, Wilmington, Delaware.

** In the early experiments, the hydrolysate was treated according to the procedure of HIRS *et al.*[15], in order to oxidize cysteine to cystine. This step was later omitted, since no cystine was dectected in the chromatograms. This is in agreement with the finding that cysteine is not detached from the porphyrin by acid hydrolysis[1] and that cytochrome *c* contains only 4 sulfur atoms, two from the cysteines and two from the methionines[19].

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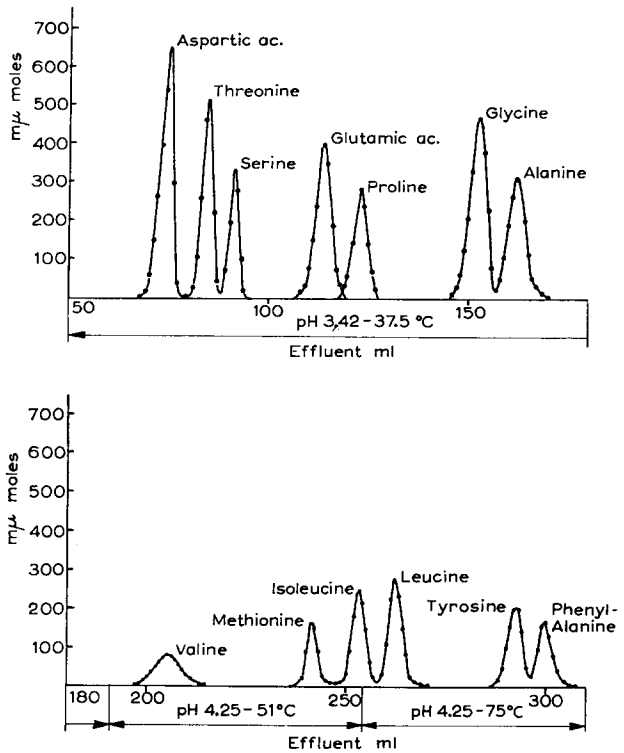

Fig. 3. Separation on a 100 cm column of Dowex-50 of the acidic and neutral amino acids, present in a hydrolysate of yeast cytochrome *c*, fraction *S*.

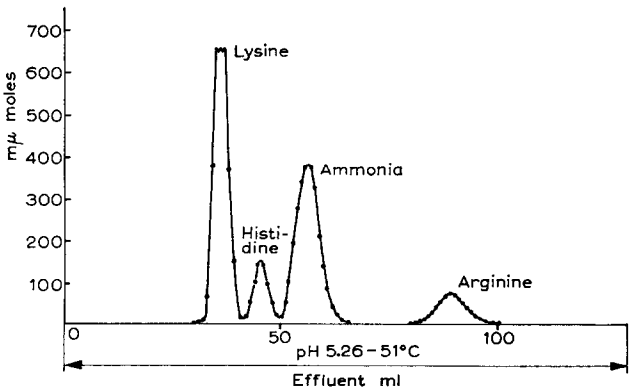

Fig. 4. Separation on a 15 cm column of Amberlite IR 120 of the basic amino acids, present in a hydrolysate of yeast cytochrome *c*, fraction *S*.

precipitation and the paper electrophoresis. The final yield of both fractions (*S* and *F*) was about 10%.

During the course of this work, the preparation of crystalline cytochrome *c*, extracted from yeast with ethyl acetate, was described by OKUNUKI and co-workers[9].

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TABLE I

ISOLATION AND PURIFICATION OF YEAST CYTOCHROME c

Stage	Yield (μmole/kg yeast)	$D_{550}:D_{280}$	% Fe
1. After SO_2 treatment	2.3	—	—
2. Eluate from Amberlite column	1.8	0.4	—
3. After trichloroacetic acid precipitation	1.0	0.80	0.34
4. Fractionation on Amberlite	0.7	1.10	0.37
5. Paper electrophoresis Fraction S	0.3	1.12	0.42
Fraction F		1.08	0.35

The yield of crystals (about 4 μmoles/kg) was very high, which suggests that the yeast used by the Japanese workers contained an exceptionally high concentration of cytochrome c. In our hands, ethyl acetate did not extract any more cytochrome c from the Delft yeast than other procedures tried. We were also unsuccessful in crystallizing our cytochrome c, when the procedure of the Japanese workers was applied to our preparation, stage 4.

Properties of the cytochrome preparations

Electrophoretic properties. The electrophoretic mobilities of yeast cytochrome c, stages 4 and 5, and horse-heart cytochrome c are shown in Table II. The electrophoretic patterns of horse-heart cytochrome c and of yeast fractions S and F were those of solutions containing a single component. The mobilities of horse-heart cytochrome c are essentially the same as those found by THEORELL AND ÅKESON for beef-heart cytochrome c[1].

TABLE II

ELECTROPHORETIC MOBILITIES OF YEAST AND HORSE-HEART CYTOCHROME c

Sample	Buffer	pH	Ionic strength	Electrophoretic mobility ($cm^2\ V^{-1}\ sec^{-1}$) $\cdot 10^5$
Yeast cytochrome, stage 4	veronal	8.3	0.1	1.90
				2.35
Yeast cytochrome, stage 5, fraction S	veronal	8.0	0.1	2.28
fraction F	veronal	8.0	0.1	3.08
Horse-heart cytochrome	phosphate	7.73	0.1	2.80
	phosphate	8.3	0.1	2.87

Enzymic activities. The rate of auto-oxidation and the catalytic activities of fractions S and F of yeast cytochrome c are about the same (Fig. 5). Compared with horse-heart cytochrome c, yeast cytochrome c had a somewhat lower catalytic activity and a much greater rate of auto-oxidation (Fig. 6).

Absorption spectra. As ordinarily measured with a quartz prism in the Hilger Uvispek spectrophotometer, no difference could be detected between the spectrum of yeast cytochromes fractions S and F and horse-heart cytochrome c. Accurate measurements with the glass prism in the region of 550 mμ revealed, however, that

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the maximum of the α-band of both yeast fractions was 0.5 mμ closer to the blue compared with the horse-heart cytochrome *c* (549.5 mμ compared with 550.0 mμ; see Fig. 7).

Amino acid composition. When yeast cytochrome *c*, stage 4, was subjected to the

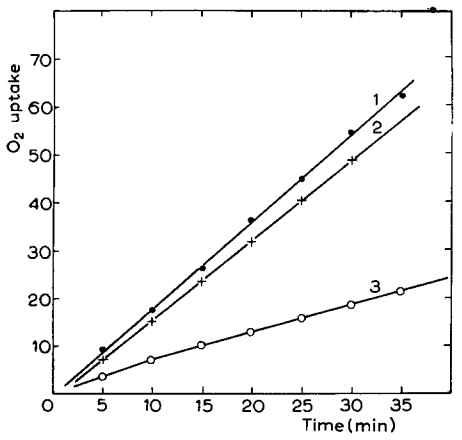

Fig. 5. Enzymic activity of yeast cytochrome *c*, fraction *S* (curve 1) and *F* (curve 2) and the autooxidation of both *S* and *F* (curve 3). For the composition of the reaction mixture see METHODS.

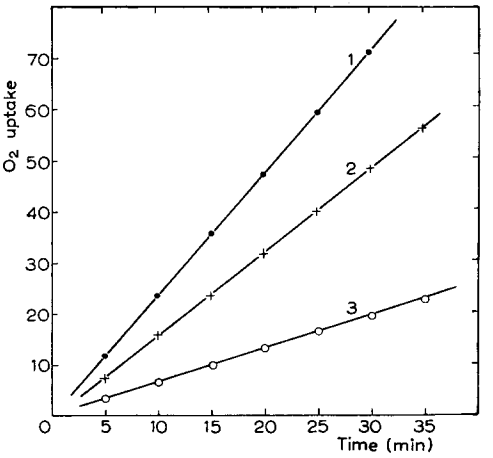

Fig. 6. Enzymic activity of horse-heart cytochrome *c* (curve 1) and yeast cytochrome *c*, fraction *S* (curve 2) and the autooxidation of the yeast cytochromes (curve 3). The autooxidation of horse cytochrome *c* under these conditions was zero. For the composition of the reaction mixture see METHODS.

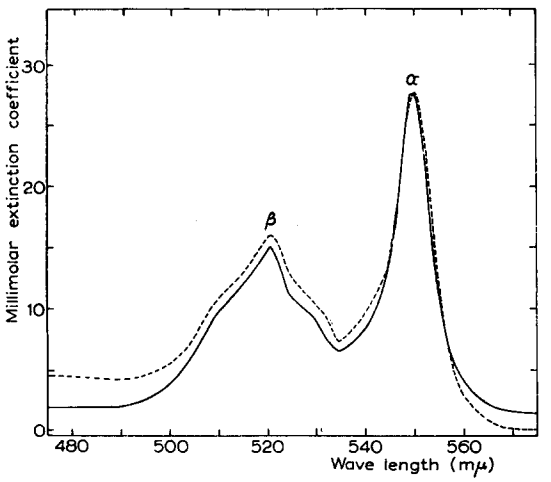

Fig. 7. Absorption spectrum of reduced horse-heart cytochrome *c* (dashed line) and yeast cytochromes *S* and *F* (solid line), measured with a Hilger Uvispek spectrophotometer fitted with as glass prism. The effective band-width in the region of 550 mμ was 0.25 mμ, and the three samples were measured immediately one after the other with the same reference setting and with the same setting of the wavelength drum.

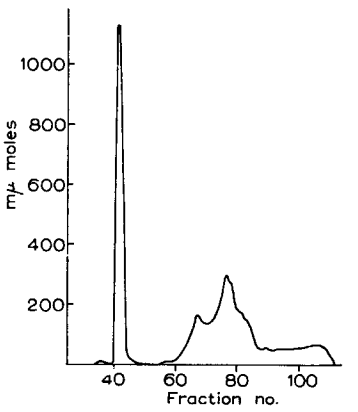

Fig. 8. Amino acid analysis of the yeast cytochrome *c* preparation before electrophoretical purification (stage 4). Only the first part of the separation on a 100 cm Dowex-50 column is shown and should be compared with Fig. 3. The high peak at the 40th fraction contains carbohydrate.

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procedure of MOORE AND STEIN (see METHODS) for the analysis of amino acids, using the 100 cm column, a large amount of foreign material was revealed. The first part of the chromatogram is shown in Fig. 8, which should be compared with Fig. 3, which shows the chromatogram obtained with more highly purified cytochrome (stage 5). It is seen that the usual separation was not obtained and that a new sharp peak appeared, giving the normal blue ninhydrin colour. The analysis of stage 4 cytochrome also revealed an unexpectedly high number of histidine molecules (five).

Some information concerning the nature of these impurities was obtained with the help of paper chromatography. In these experiments, one half of each fraction collected in the chromatogram was developed with ninhydrin, and the other half desalted on a Dowex-2 column[13] and used for paper chromatography. In addition to the amino acids that usually appear in this region of the chromatogram (aspartic acid, threonine, serine and methionine sulphoxide), there were found the rare hydroxyproline, an unidentified ninhydrin-positive substance, and two substances fluorescing in ultraviolet light (possibly a purine or pyrimidine). The sharp peak at about the 40th fraction separated on paper (using buffered phenol and butanol–ethanol–water mixtures) into three fractions. The major component appeared to be amino-sugars (not glucosamine), but no definite identification was attempted.

These substances were not found after paper electrophoresis (stage 5). The sugar-containing peak had completely disappeared and the separation of the amino acids was not disturbed (Fig. 3).

Table III compares the amino acid compositions (expressed as moles amino acid/mole cytochrome *c*) of horse-heart cytochrome *c* and yeast cytochrome *c*, fractions *S* and *F*. In the same table the analyses of beef-heart cytochrome *c* made by EHRENBERG AND THEORELL[5] are given for comparison. No analyses were performed

TABLE III

AMINO ACID COMPOSITION OF CYTOCHROME *c*

Results of the analyses are given in molecules of each amino acid per molecule of cytochrome *c*. The results for beef-heart cytochrome are those of EHRENBERG AND THEORELL[5]. No analyses for tryptophan or cysteine in the horse or yeast cytochromes were carried out. In the Table, the values found in beef cytochrome[1] are assumed to apply to the horse and yeast cytochromes.

	Beef	*Horse*	*Yeast (S)*	*Yeast (F)*
Aspartic acid	9.4	7.8	10.9	11.8
Glutamic acid	11.9	11.8	8.8	9.2
Proline	3.9	3.8	4.2	4.8
Glycine	15.5	12.1	11.6	11.9
Alanine	6.6	6.5	7.3	8.1
Valine	3.3	3.0	3.0	3.3
Isoleucine	6.2	5.7	3.8	4.8
Leucine	6.2	5.9	6.9	5.5
Serine	8.5	0.4	4.3	5.8
Threonine		9.8	8.1	8.9
Cysteine	2	(2)	(2)	(2)
Methionine	2	2.1	1.8	2.5
Tyrosine	3.8	3.4	4.0	4.7
Phenylalanine	3.3	3.6	3.4	3.5
Tryptophane	1	(1)	(1)	(1)
Histidine	3.1	2.6	3.3	2.9
Lysine	18.3	18.3	14.8	16.0
Arginine	3.4	1.7	2.7	2.8
Ammonia	—	8.8	11.1	13.0

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by either ourselves or EHRENBERG AND THEORELL for cysteine and tryptophan, and it has been assumed that THEORELL AND ÅKESON's earlier values for beef-heart cytochrome c[1] apply also to the other cytochromes.

Table IV shows the results of calculations of the protein weight from the amino acid analyses. The nitrogen content, calculated from the amino acid analyses, was in good agreement with that directly determined by the Kjeldahl method.

TABLE IV

CALCULATION OF PROTEIN WEIGHT

		Horse heart	*Yeast (S)*	*Yeast (F)*
Dry weight	(g/mole)	12,385	13,213	15,770
Protein weight*				
No. amino acids/mole		101	102	109
Total amino acid residues	(g/mole)	11,246	11,200	12,037
Haematin	(g/mole)	618	618	618
Total protein	(g/mole)	11,864	11,818	12,655
	(% dry weight)	96	89	80
% nitrogen				
Amino acid N + NH_3 N	(g/mole)	1,942	2,008	2,153
Porphyrin N	(g/mole)	56	56	56
Total N	(g/mole)	1,998	2,064	2,209
% dry weight	(calculated)	16.13	15.62	14.01
% dry weight	(Kjeldahl)	16.13	15.45	13.45**
Total N	(atoms/mole)	143	147	158
Iron	% dry weight	0.454	0.424	0.355
	% protein weight	0.472	0.474	0.443
$E_{550} : E_{280}$		1.24	1.12	1.08

* Protein here means conjugated protein, *i.e.* apoprotein + prosthetic group.
** This determination was not as accurate as the others.

DISCUSSION

Purity of cytochrome c *preparations*

Before discussing the amino acid analyses, it is necessary to discuss the purity of the cytochrome c preparations that were analysed. It appears likely that the horse-heart cytochrome c was very nearly pure. Its iron content was about the same as the purest preparations of MARGOLIASH[14] and the $E_{550} : E_{280}$ (1.24) was slightly higher. The Japanese workers have reported a ratio of 1.29 for crystalline beef-heart cytochrome c, and 1.28 for pig-heart cytochrome c[24], TSOU AND LI have reported that the ratios $E_{550} : E_{278}$ were 1.22 (horse), 1.23 (beef) and 1.24 (pig)[25], and BODO[26] reports a ratio of 1.15 for crystalline cytochrome c from the King Penguin. Table IV suggests the presence of a small amount of non-protein material, but this may be within the experimental error.

Both yeast cytochrome c's, however, contained a considerable amount of non-protein material. The preparation at stage 4 contained substances (among which hydroxyproline* and amino sugars were identified), which interfered seriously with the amino acid analyses. Paper electrophoresis completely removed these substances,

* Up to stage 4 of the purification, the hydroxyproline appeared to be selectively purified with the cytochrome. Insufficient was present in the hydrolysate of whole yeast to be detected.

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but it is clear from the calculations in Table IV that a considerable amount of non-protein material, presumably carbohydrate, remained. It is not known whether this carbohydrate is a part of the protein structure *in vivo*, or becomes firmly attached to the protein during purification. Carbohydrate is known to be present in preparations of other purified haemproteins[27]. Fortunately, the presence of carbohydrate does not interfere with the amino acid analyses.

Both yeast cytochrome *c* fractions were homogenous by electrophoresis. The ratio $E_{550}:E_{280}$, which is a measure of the purity of the preparation with respect to contaminating proteins, reached a constant value at stage 4 (after fractionation on Amberlite IRC-50) and was not increased by the paper electrophoresis which clearly removed other impurities. This suggests, therefore, that the ratio found for fraction *S* (1.12) is close to the true ratio for our yeast cytochrome *c*. The difference between this ratio and that found for the horse-heart cytochrome *c* (1.24) is to a large extent accounted for by the higher content of tyrosine + phenylalanine in the preparation of yeast cytochrome *c*. Thus, if the actual ratios found for the two yeast fractions are multiplied by the fractions (tyrosine + phenylalanine in yeast cytochrome)/(tyrosine + phenylalanine in horse heart cytochrome), corrected $E_{550}:E_{280}$ ratios of 1.18 (fraction *S*) and 1.26 (fraction F) are obtained. There is, therefore, little evidence of appreciable amounts of other substances (such as nucleic acids) absorbing at this wavelength. Higher $E_{550}:E_{280}$ ratios for yeast cytochrome *c* have, however, been reported by OKUNUKI *et al.*[9] and LI AND TSOU[8], namely 1.28 and 1.21, respectively. The calculation of the protein weight of fraction *S* in Table IV suggests that this fraction cannot be seriously contaminated by other proteins, unless the molecular weight of yeast cytochrome *c* is appreciably less than that of horse-heart cytochrome *c*. The difference between our results and those of the Japanese workers remains difficult to explain. Table IV suggests that the yeast fraction *F* might still be contaminated with a little extraneous protein.

The somewhat lower catalytic activities of fractions *F* and *S* in the horse-heart cytochrome *c* oxidase system and the higher rate of auto-oxidation compared with mammalian cytochrome *c* could represent a real difference in properties of the cytochromes from the two sources. However, in view of the fact that LI AND TSOU[8] and OKUNUKI and co-workers[10] have reported that their yeast cytochrome *c*'s have a higher and the same catalytic activity, respectively, in comparison with the mammalian cytochrome, it is more likely that our yeast preparation is partly denatured during the extensive purification procedure. This point was not further examined, since denaturation would have no effect on the amino acid analysis, which was the main purpose of this investigation.

Amino acid analyses

The agreement between our analyses of horse-heart cytochrome *c* and those of EHRENBERG AND THEORELL[5] for beef-heart cytochrome *c* shown in Table III is quite striking. We find somewhat less aspartic acid, glycine and much less arginine. Both sets of analyses are quite different from those of DE BARBIERI AND ZAMBONI[28]. EHRENBERG AND THEORELL gave a combined figure for serine and threonine. Our procedure clearly separated between these two amino acids, and showed that very little serine was present. The amount of serine is much less than 1 molecule per molecule of protein, which is puzzling. The small peak, appearing in the usual position of

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serine in the chromatogram, was not in fact definitely identified as serine, and the possibility that it represented the sulphone of methionine was not definitely excluded. The determination of the number of histidine molecules in horse cytochrome *c* was repeated several times, in view of MARGOLIASH's report that four were present[11]. We found about three, in agreement with the results of EHRENBERG AND THEORELL with beef-heart cytochrome.

The general pattern of the amino acid composition of yeast cytochrome *c* is also rather similar to that of mammalian cytochrome. In comparison with horse cytochrome, fraction *S* contains more aspartic acid, less glutamic acid, and much more serine. There are no important differences between the two yeast cytochrome fractions. In view of the considerably greater difficulty of eluting the yeast cytochrome from IRC-50 in comparison with the mammalian cytochrome, which suggests that the former is more strongly basic, a greater difference in amino acid composition was expected. In fact, the total numbers of basic and acidic amino acids are about the same in all samples. This suggests that forces other than electrostatic (*e.g.* van der Waals) play an important rôle in the adsorption of the protein on the resin.

The significance of the two different cytochrome fractions in yeast is not known. The iron content (on a protein basis) of fraction *F* (0.443%) is lower than that of fraction *S* (0.474%), which is the same as that of horse-heart cytochrome *c*. The amount of non-protein material in this fraction is also higher. It is impossible to say whether two different cytochromes exist in yeast, or whether fraction *F* is formed only during the isolation procedure. LI AND TSOU have also isolated two different cytochrome *c* fractions from yeast, but the difference in electrophoretic mobilities of their two fractions is much greater than ours, and they could be partially separated by ammonium sulphate fractionation[8]. ESTABROOK has reported that horse-heart cytochrome *c* also consists of several (three) components distinguished by slight differences in absorption spectra at low temperatures[29]. Slight heterogeneity of the preparations may explain why the number of amino acids often deviates from a whole number (Table III). In some cases we feel that the deviations are definitely outside the possible experimental error. The values given in Table III, rounded off to the nearest 0.1 of a unit, are the means of four closely agreeing figures.

The similarity of the amino acid composition of cytochrome *c* from such widely different sources as horse heart and yeast is very striking, and recalls the similar close resemblance between glyceraldehydephosphate dehydrogenase isolated from rabbit-skeletal muscle and yeast[30]. These similarities could perhaps have an evolutionary significance, or they may suggest that the possibility of variations in composition of a protein with the specific catalytic activity of cytochrome *c* are not great. The agreement between the number of histidine molecules is particularly interesting. The requirement for two histidines can be explained by THEORELL's view that these are the haemochromogen-forming groups on the iron atom. Our analysis suggests that a third molecule might also be necessary for the catalytic activity. Although differences in composition between cytochrome *c*'s isolated from different sources are not great, some differences in the amino acid sequence in the region of the iron atom have been found by TUPPY and co-workers[3,4]. Chicken-heart cytochrome *c* was found not to be identical with beef-, salmon- or horse-heart cytochrome *c*. It will be recalled that very minor differences in the structure of insulin obtained from different sources have also been reported[31].

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SUMMARY

1. Two cytochromes *c* with different electrophoretic mobilities have been isolated from yeast in about equal amounts. The overall yield of purified cytochrome *c* (0.3 μmole/kg compressed yeast) represents about 10% of that present in the yeast, which was grown under conditions to give a high cytochrome *c* content.

2. The electrophoretic mobilities of the two cytochromes were slightly higher and slightly lower, respectively, than that of beef- or horse-heart cytochrome *c*.

3. The maximum of the α-band of both yeast cytochromes lies at 549.5 mμ, compared with 550.0 mμ for the heart cytochrome.

4. The two yeast cytochromes were electrophoretically homogeneous and the purity, judged by other physico-chemical and chemical criteria, was sufficient to justify an analysis for the amino acids and a comparison of the results with those obtained for highly purified horse-heart cytochrome *c*. The main impurity was carbohydrate.

5. The amino acid content of the horse-heart cytochrome *c* was very similar to that reported by EHRENBERG AND THEORELL[5] for the beef-heart cytochrome. On the whole, there was also a rather striking resemblance between the composition of the two yeast and the mammalian cytochromes *c*.

6. All cytochromes contained three histidine residues.

7. Yeast cytochrome *c* is much more strongly adsorbed on Amberlite IRC-50 than horse-heart cytochrome. This cannot be explained by the difference in amino acid composition, and may be due to differences in van der Waals' forces.

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