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Purification, Properties and Amino Acid Sequence of Atypical Cytochrome *c* from Two Protozoa, *Euglena gracilis* and *Crithidia oncopelti*

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A basic cytochrome was isolated from the phytomastigophorean protozoan *Euglena gracilis* and a similar protein from the zoomastigophorean protozoan *Crithidia oncopelti*. In both cases chromatography on CM-cellulose in first the reduced and then the oxidized form proved to be an efficient means of purification. The two cytochromes can be classed in the cytochrome *c* family but they have certain atypical features. The α peak of the absorption spectrum is shifted towards the red and is asymmetrical. The pyridine ferrohaemochrome has an α-peak maximum intermediate between that of *c*-type cytochromes and proteins containing protohaem IX. The test for free vinyl groups was positive. The amino acid sequences of the two cytochromes were determined. Attention is drawn in the text to those parts of the evidence that are less satisfactory. Both sequences are homologous with the family of cytochrome *c*, but are unusual in having only one cysteine residue so that the haem is attached through only one thioether bond. Detailed evidence for the amino acid sequences of the two proteins has been deposited as Supplementary Publication SUP 50042 (70 pages) at the British Library (Lending Division) (formerly the National Lending Library for Science and Technology), Boston Spa, Wetherby, Yorks. LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1975) **145**, 5.

Cytochromes *c* from a wide range of eukaryotic sources have been purified. They form a close family of proteins with very similar properties (Margoliash & Schejter, 1966). They contain covalently bound haem, attached to the apoprotein through two thioether bonds involving two cysteine residues (Theorell, 1938). They are readily extracted from cells and are interchangeable in their ability to restore respiration in mammalian mitochondria depleted of their native cytochrome *c* (Byers *et al.*, 1971).

Cytochromes from a few Protozoan sources have been purified. Hill *et al.* (1971) and Kusel *et al.* (1969) have investigated cytochrome *c*-555 from *Crithidia fasciculata*. *Euglena* cytochrome *c*-558 was purified by Perini *et al.* (1964) and Meyer & Cusanovich (1972). It was found that both proteins formed pyridine ferrohaemochromes with atypical spectra, the α-peak maxima being at 553nm. This is in contrast with the 550nm peak of the pyridine ferrohaemochrome of all other *c*-type cytochromes and the 556–558nm peak of all those proteins containing protohaem IX as prosthetic group.

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Experimental

Materials

Organisms. *Crithidia oncopelti* and the Guttman 'cured' strain of *Crithidia oncopelti* were obtained from Dr. B. A. Newton, Molteno Institute of Parasitology, University of Cambridge, Cambridge, U.K., and *Euglena gracilis*, strain z, was obtained from Dr. N. Jardine, Department of Biochemistry, University of Edinburgh, Edinburgh, U.K.

Chromatographic materials and enzymes. These were obtained from the sources listed in Ambler & Wynn (1973). Silicone fluid MS550 was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Chemicals. ε-*N*-Monomethyl-lysine was obtained from a hydrolysate of *Salmonella* flagellin which was a gift from Dr. R. P. Ambler, University of Edinburgh

(Ambler & Rees, 1959). ε-*N*-Dimethyl-lysine was a gift from Dr. G. Leaf, Department of Biochemistry, University of Glasgow, Glasgow, U.K. α-*N*-Acetyl-lysine and cytochrome *c* (horse heart, grade III) were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Evans bacteriological peptone was obtained from BDH Chemicals Ltd.

Methods

Growth of organisms. Euglena gracilis was grown as described by Pettigrew (1974). *Crithidia oncopelti* was grown axenically in 3% bacteriological peptone (Evans), 0.5% glucose, 0.5% NaCl (all w/v) with an initial pH of 7.4. Stock solutions (10ml) were maintained in 25ml conical flasks at 25°C and subcultured every 3–5 days. For bulk growth the organisms were transferred to 100ml volumes of medium, incubated at 25°C for 3 days, and then inoculated into 1.5 litre volumes of medium in 2 litre conical flasks. The flasks were shaken on an orbital shaker at approx. 25°C for 3–5 days and then harvested in a Alfa-Laval centrifuge (LAB 102 B-25). The packed cells were stored frozen at −20°C. The yield was approx. 60g of packed wet cells/10 litres of medium.

Purification of cytochromes. Essentially the same method was used to prepare *Euglena* cytochrome *c*-558 and *Crithidia* cytochrome *c*-557. *Euglena* has a tough pellicle and it was necessary to use a Manton–Gaulin pressure cell to achieve good cell breakage, which was estimated microscopically (Pettigrew, 1974).

Crithidia cells (200g) were frozen and thawed twice and suspended in 4vol. of ice-cold 0.1M-NaH_2PO_4–Na_2HPO_4, pH7.0. Extraction was allowed to proceed for 1h at 0–4°C. Centrifugation at 25000*g* for 30min yielded a cloudy pink supernatant. The cell debris was extracted again with 4vol. of 0.1M-NaH_2PO_4–Na_2HPO_4, pH7.0, and after centrifugation the combined supernatants were acidified to pH5.5 by the addition of 2M-acetic acid with stirring. The heavy white precipitate was separated by centrifugation and the supernatant was subjected to gel filtration at 0–4°C on a column (9cm×40cm) of Sephadex G-25 (medium grade) equilibrated in 0.01M-NaH_2PO_4–Na_2HPO_4, pH7.0. The purification procedure for *Euglena* cytochrome *c*-558 up to the gel-filtration step is very similar and is described by Pettigrew (1974). After the gel-filtration step the *Euglena* extract was passed through a column of DEAE-cellulose in order to adsorb the cytchrome *f*. Thereafter the purification procedures for *Euglena* cytochrome *c*-558 and *Crithidia* cytochrome *c*-557 were identical. The procedure was based on the different chromatographic behaviour of the reduced and oxidized proteins, which allowed a 'diagonal' approach to be applied to their purification (Hartley, 1970). The chromatographic properties on CM-cellulose of reduced and oxidized cytochrome *c* have been described by Dixon & Thompson (1968).

The cytochrome, in solution in 0.01M-NaH_2PO_4–Na_2HPO_4, pH7.0, was adsorbed on to a column (2cm×10cm) of CM-cellulose (Whatman CM-23) which had been equilibrated in the same buffer, and the cytochrome was eluted in a single step with the phosphate buffer containing 0.5M-NaCl. After gel filtration on Sephadex G-25 equilibrated in 0.01M-NaH_2PO_4–Na_2HPO_4, pH7.0, to remove the salt, the cytochrome was fully reduced by the addition of a tenfold molar excess of neutralized ascorbic acid and then adsorbed on to a column (1.0cm×10cm) of CM-cellulose (CM-52) equilibrated with 0.01M-NaH_2PO_4–Na_2HPO_4, pH7.0. Elution was carried out with 0.04M-NaH_2PO_4–Na_2HPO_4, pH7.0. The pink fractions were diluted with 1vol. of water, the cytochrome was oxidized with the minimum amount of ferricyanide and then re-adsorbed on CM-cellulose under the same conditions as for the chromatography in the reduced form. Chromatography was again carried out with 0.04M-NaH_2PO_4–Na_2HPO_4, pH7.0.

The eluate was diluted with 1vol. of water and the cytochrome was concentrated by adsorption on a small column (0.5cm×3cm) of CM-cellulose (CM-52) and a single-step elution with 0.01M-NaH_2PO_4–Na_2HPO_4, pH7.0, containing 0.5M-NaCl. The cytochrome was usually stored at 0–4°C in this solution or alternatively precipitated in 90%-satd. $(NH_4)_2SO_4$ and stored as a slurry. The yields are given in the Results section.

Electrophoresis in polyacrylamide gels. Polyacrylamide-gel columns (0.5cm×7cm) were prepared by the methods of Davis (1964). The conditions used were 10% (w/v) acrylamide, 0.2% (w/v) *NN*-methylenebisacrylamide in 0.05M-acetic acid–sodium acetate, pH5.0. Electrophoresis was carried out at 5mA/gel with loadings of protein of 3nmol or 0.1nmol. Staining was by 0.2% (w/v) Amido Black in methanol–water–acetic acid (5:5:1, by vol.) and the same solvent was used to destain the gel columns.

Approximate estimation of molecular weight. A formula weight can be derived from the proposed amino acid sequence (Pettigrew, 1972) and a minimum molecular weight can be obtained from the amino acid composition of the proteins based on iron content. Gel filtration in a column (50cm×1.5cm) of Sephadex G-75 (superfine grade) equilibrated with 0.1M-Tris–HCl, pH7.5, containing 0.5M-NaCl was used to estimate molecular size (Andrews, 1964). A standard curve was obtained by plotting elution volumes of known proteins relative to that of ε-Dnp-lysine against log(mol.wt.).

Electrophoresis in 10% (w/v) polyacrylamide gels containing sodium dodecyl sulphate was carried out as described by Dunker & Rueckert (1969). Cytochrome *c* (horse heart) and myoglobin were run as

molecular-weight markers in co-electrophoresis with the unknown.

Iron determination. The method of Cameron (1965) was used.

Spectrophotometry. Spectra were recorded on a Unicam SP. 1800 spectrophotometer or manually on a Unicam SP. 500 spectrophotometer.

Pyridine ferrohaemochromes were prepared by the method of Falk (1964). The final concentration of pyridine was 2.1 M and of NaOH was 0.17 M.

Test for free vinyl groups. When vinyl groups on haem molecules are saturated with hydrazine hydrate a shift in the pyridine ferrohaemochrome spectrum can be observed (Fischer & Gibian, 1941). The spectrum of the protein or haemin in 0.75 ml of pyridine and 3 ml of hydrazine hydrate was recorded. The solution was heated at 100°C for 15 min and the spectrum was recorded again.

Removal of haem from cytochrome. (1) Haem was removed from protein by the method of Sletten *et al.* (1968). The protein (approx. 1 μmol) was dissolved in 0.01 M-HCl and finely ground $HgCl_2$ was added to a concentration of 12 mg/ml. The solution was incubated overnight in the dark at approx. 18°C.

After this incubation the solution was sometimes turbid but clarified after addition of an equal volume of 1.8% (v/v) HCl in acetone. Diethyl ether was used to extract the haem (three times with equal volumes of ether) and during this process the apoprotein precipitated. The ether solution of haem (which probably contains most of the acetone and $HgCl_2$) was washed several times with water and concentrated under a N_2 stream. Any experiments to be performed on the haem were usually planned for the same day, as the haemin was unstable and aged preparations gave different chromatographic patterns.

(2) The method described by Ambler & Wynn (1973) was used to remove the haem from cytochrome *c*-557 and *c*-558 in preparation for amino acid sequence studies. Protein concentration was 0.5–1.0 μmol/ml. $HgCl_2$ was added as a fine powder to give a concentration of 10 mg/ml. Protein, freeze-dried from 5% (v/v) formic acid, was normally used as starting material but considerable difficulty was sometimes experienced in removing the haem from cytochrome *c*-558. More consistent results were obtained if the protein was precipitated from solution at pH 7.0 by the addition of ethanol to give an 80% (v/v) solution (Margoliash *et al.*, 1962). The precipitate was washed with ethanol, dried and dissolved in the urea solution for removal of the haem group.

Chromatography of the isolated haemin. The chromatography system used was that described by Chu & Chu (1955). Whatman no. 1 filter paper was dipped in light petroleum (b.p. 40–60°C) containing silicone fluid MS550 (10%, v/v) and allowed to dry horizontally. Ascending chromatography on this support was carried out in water–propan-1-ol–pyridine (55:1:4, by vol.). The chromatograms were stained for haem with the *o*-tolidine spray (Flatmark, 1964). Haematohaemin was prepared by $HgCl_2$ cleavage of cytochrome *c* (horse heart), and protohaem IX was obtained by precipitation of haemoglobin with acetone containing 2% (v/v) HCl and extraction of the haem into ether.

Amino acid analysis. The method of Spackman (1963) was used, with a Locarte amino acid analyser. Samples for analysis (approx. 50 nmol of protein or 10 nmol of peptide) were prepared and hydrolysed by the methods of Ambler & Brown (1967).

Trimethyl-lysine was prepared from α-*N*-acetyl-lysine by the method of De Lange *et al.* (1969). It was homogeneous as judged by amino acid analysis and paper electrophoresis at pH 6.5. On the Locarte 20 cm single-column programme, trimethyl-lysine was not usually visible owing to the ammonia peak. On the 50 cm column operated at 63°C, equilibrated and run in 1.0 M-sodium citrate, pH 6.65, all methylated lysines were completely separated.

Tryptophan was measured by the spectrophotometric method of Goodwin & Morton (1946) by taking readings at 294.4 and 280 nm. Because of interference from the haem group, solutions of apoprotein were used, but it must be borne in mind that the protein had then been exposed to acidic conditions which might be expected to result in partial destruction of the tryptophan. A portion of the protein solution, used for the spectrophotometric examination, was taken for amino acid analysis and the number of molecules of tyrosine and tryptophan per molecule could be calculated by using the formulae of Goodwin & Morton (1946). Tryptophan was not usually quantified in peptides. A qualitative indication of tryptophan was obtained by the Ehrlich reaction on paper (Smith, 1953) and chymotryptic peptides containing tryptophan were often digested with carboxypeptidase A, and the products were analysed by paper electrophoresis at pH 2.0.

Cysteine was measured as cysteic acid after oxidation of apoprotein or peptide with performic acid (Hirs, 1967) or was reduced and carboxymethylated by the method of Crestfield *et al.* (1963). The mercaptoethanol and iodoacetic acid were present in 100-fold molar excess over protein (30 μmol to 0.3 μmol). The carboxymethylated protein was separated from excess of reagents by gel filtration on a column (2 cm × 25 cm) of Sephadex G-25 (fine grade), equilibrated and run in 0.2 M-acetic acid in the dark.

Cysteic acid and carboxymethylcysteine were determined by amino acid analysis.

N-Terminal and C-terminal regions of proteins. Proteins were labelled with dansyl chloride essentially as described by Gray (1972*a*). Dried protein (20 nmol) was dissolved in 0.5 ml of 0.5 M-$NaHCO_3$ containing

8M-urea, and 0.5ml of dansyl chloride in acetone (20mg/ml) was added. After incubation at 37°C for 2h the protein was precipitated by the addition of acetone. After centrifugation the precipitate was washed with acetone and then hydrolysed in 6M-HCl at 105°C overnight. The dried hydrolysate was examined by electrophoresis at pH4.36 (Gray, 1972*a*; Ambler & Brown, 1967).

The method of Porter (1957) was used for dinitrophenylation, and dinitrophenyl-amino acids were separated by chromatography on polyamide layers (Wang & Wang, 1968). One cycle of the Edman degradation (Gray, 1972*b*) was performed on cytochrome *c*-557.

Digestion of proteins or peptides with carboxypeptidase A was carried out as described by Ambler (1972).

Methods of sequence determination. Established methods were used for the digestion of proteins and the separation of peptides (Ambler, 1963; Ambler & Wynn, 1973). The amino acid sequences of pure peptides were investigated by using the Edman degradation (Gray, 1972*a*). The dansyl method (Gray, 1972*b*) was used for identification of *N*-terminal residues. Dansyl-amino acids were separated on polyamide layers by the system of Ramshaw *et al.* (1970).

Acetyl groups were identified by hydrazinolysis of a peptide and treatment of the acetylhydrazine with dansyl chloride (Schmer & Kreil, 1969). Acetyl-dansyl-hydrazine was identified by chromatography on polyamide layers (Ramshaw *et al.*, 1970).

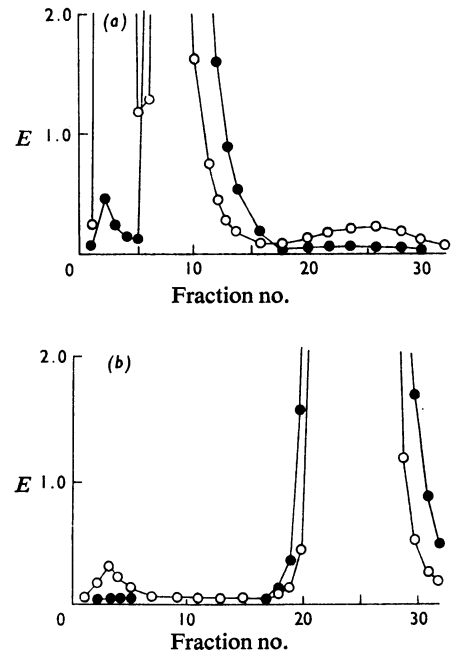


Fig. 1. *Chromatography of (a) ferrocytochrome c-557 and (b) ferricytochrome c-557 on CM-cellulose*

The conditions were as described in the text with a column (10cm × 1cm) of Whatman CM-52. Fractions of volume 1ml were collected. In (*a*) fractions 5–15 were pooled for oxidation and rechromatography. ○, E_{280}; ●, E_{412}.

Results

Purification of cytochromes

Profiles of the chromatographic separations of the reduced and oxidized species of cytochrome *c*-557 are shown in Figs. 1(*a*) and 1(*b*). A similar pattern was obtained for cytochrome *c*-558 under the same conditions. The approximate yields of pure cytochrome *c*-557 and cytochrome *c*-558 per 100g wet weight of cells were 2μmol and 0.2μmol respectively.

Both cytochromes gave single bands after electrophoresis in polyacrylamide gels. Two gels were run for each cytochrome, one containing 3nmol of protein and the other 0.1nmol of protein. The latter quantity was easily visible after staining.

Preparations of both proteins showed less than 4% binding of CO, although this percentage was increased slightly if the protein had been previously freeze-dried for storage.

Margoliash & Schejter (1966) have emphasized that multiple chromatographic forms of cytochrome *c* can occur in preparations of the protein. Tests of purity must therefore not only distinguish non-cytochrome components but also detect non-native cytochrome fractions.

Crithidia cell extract which had been kept at pH4.5 overnight contained two forms of cytochrome *c*-557. One of these forms, cytochrome *c*-557(A), did not adsorb on to CM-cellulose in 0.02M-NaH_2PO_4–Na_2HPO_4, pH7.0, whereas the second, cytochrome *c*-557 (B), behaved identically with the single species isolated after extraction at pH7.0. The spectral parameters of forms A and B were very similar and neither combined with CO.

Glutamic acid or glutamine was identified as the *N*-terminal amino acid of cytochrome *c*-557 (A) and the amino acid composition was consistent with the loss of the four *N*-terminal amino acids (G. W. Pettigrew, unpublished work).

Because of the difference in amino acid compositions of form (A) and form (B), and because form (A) was recovered in decreasing yield as the exposure to acid pH was shortened, we consider that form (A) is an artifact of the preparation (henceforth referred to as modified cytochrome *c*-557) and that form (B) is native protein.

Molecular weight

Assuming that the proteins contain only haem and amino acids, minimum molecular weights can be calculated from the amino acid composition related to the iron content, and formula weights can be calculated from the proposed sequences.

Cytochrome *c*-557 and *c*-558 have formula weights 12510 and 11856 respectively and because of the good correspondence of amino acid compositions based on iron content and on sequence (Table 4), the minimum molecular weights approximate to these values.

An estimate of molecular size was made for each protein by using the gel-filtration method. Cytochrome *c*-558 had an apparent molecular weight of 13000, but cytochrome *c*-557 was considerably more excluded from the gel matrix, and a value of 16000 was obtained. This latter result is similar to that obtained by Hill *et al.* (1971) for cytochrome *c*-555 from *Crithidia fasciculata*.

Because of this disagreement with the formula weight in the case of cytochrome *c*-557, electrophoresis in sodium dodecyl sulphate–polyacrylamide gels was used to obtain a second estimate of the molecular weight. In this system, cytochrome *c*-557 migrated with a slightly greater mobility (1.04) than cytochrome *c* (1.0). Myoglobin had a mobility of 0.87. Dunker & Rueckert (1969) found that in this system cytochrome *c* (horse heart) had an apparent molecular weight of 13600 compared with the formula weight of 11700. If this value of 13600 is used for cytochrome *c* (horse heart), then the apparent molecular weight of cytochrome *c*-557 in the sodium dodecyl sulphate–polyacrylamide system approximates to its formula weight.

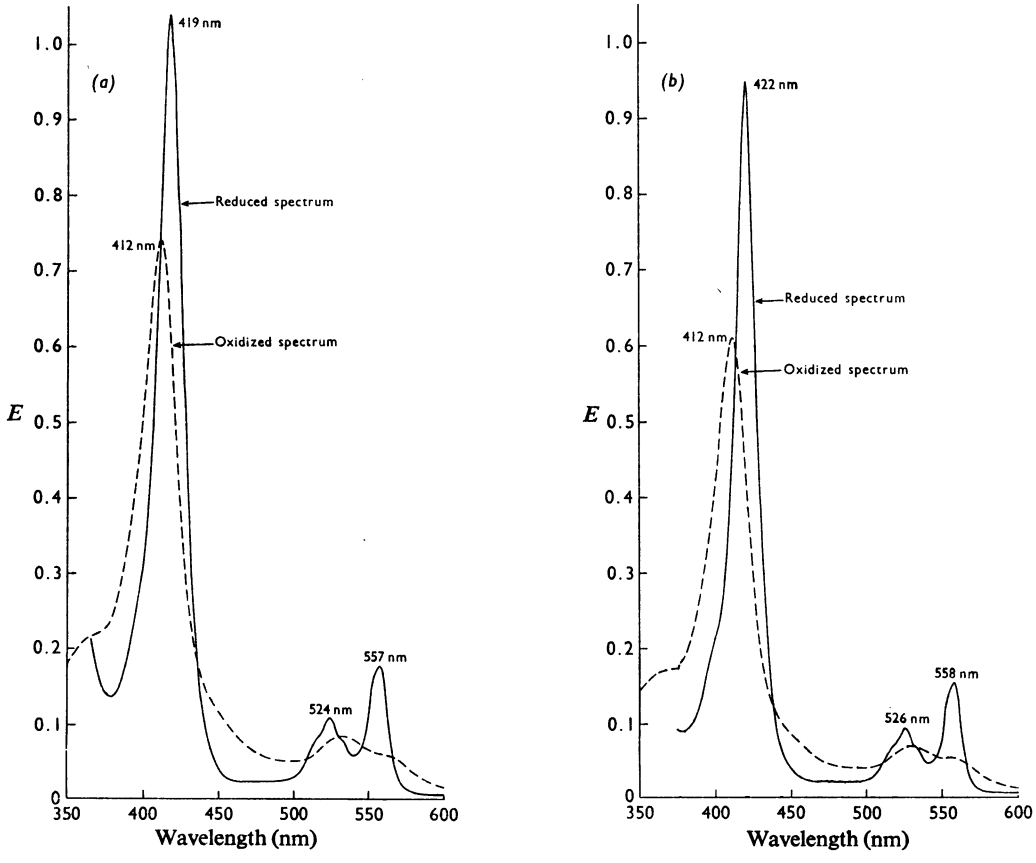


Fig. 2. *Visible spectra of reduced and oxidized cytochrome* c*-557 (a) and cytochrome* c*-558 (b)*

Concentrations of cytochrome used were (*a*) 7μM and (*b*) 6μM. Reduction was with sodium dithionite, oxidation with potassium ferricyanide.

Table 1. *Spectral parameters of cytochrome c-557, c-558 and c*

Extinction coefficients are based on iron content of the solutions.

Cytochrome *c*-557			Cytochrome *c*-558			Cytochrome *c* (horse)		
	Maxima (nm)	Extinction (mM)		Maxima (nm)	Extinction (mM)		Maxima (nm)	Extinction (mM)
Reduced	557	24.7	Reduced	558	26.3	Reduced	550	29
	524	15.0		526	15.5		521	15.5
	419	140		422	158		415	135
Oxidized	532	12.5	Oxidized	530	10.5	Oxidized	528	11.2
	412	100		412	101		408	109
	275	24.1		280	30.0		280	23.2
	Ratio			Ratio			Ratio	
$E_{\text{Soret (red.)}}/\alpha$	5.65		$E_{\text{Soret (red.)}}/\alpha$	6.0		$E_{\text{Soret (red.)}}/\alpha$	4.65	
α/E_{275}	1.02		α/E_{280}	0.88		α/E_{280}	1.25	

Table 2. *α-Peak maxima of pyridine ferrohaemochromes of cytochromes and haemins*

The solutions contained pyridine (2.1 M) and NaOH (0.17 M).

	α-Peak maximum of pyridine ferrohaemochrome (nm)	ε_{mM}	Reference
Cytochrome *c* (horse heart)	550	29.1	Morton (1958)
Proteins containing protohaem IX	557–558	34.4	Morton (1958)
Cytochrome *c*-557	553	29.95	This work
Cytochrome *c*-558	553	—	This work
Haemin from cytochrome *c*	547–548		This work
Haemin from cytochrome *c*-557	552		This work
4(2)-Hydroxyethyl-2(4)-vinyldeuterohaem IX	552		Porra & Jones (1963)
Haematohaemin	549		Porra & Jones (1963)

Spectral parameters of the cytochromes

The visible spectra of cytochromes *c*-557 and *c*-558 are shown in Figs. 2(*a*) and 2(*b*). Maxima and extinction coefficients are listed in Table 1 and are based on iron content of the solution used for measurement.

The spectrum of the α peak of cytochrome *c*-557 does not change through the purification. Cells homogenized three times in 0.25% (v/v) HCl in acetone and resuspended in 0.01 M-NaH_2PO_4–Na_2HPO_4, pH 7.0, showed the same α-peak absorption maximum and shoulder as pure protein.

The characteristics of the α peak of the pyridine ferrohaemochromes of cytochrome *c*-557 and cytochrome *c*-558 are compared in Table 2 with those of cytochrome *c* (horse heart) and cytochromes *b*.

The position of the α-peak absorption maximum of the pyridine ferrohaemochrome before and after heating with hydrazine hydrate and pyridine are shown in Table 3 for cytochrome *c* (horse heart), haemoglobin, cytochrome *c*-557 and cytochrome *c*-558.

Table 3. *α-Peak maxima of pyridine ferrohaemochromes in hydrazine hydrate*

The solutions contained 0.75 ml of pyridine and 3 ml of hydrazine hydrate. Heating was at 100°C for 15 min.

	α peak of pyridine ferrohaemochrome in hydrazine hydrate (nm)	
	Before heating	After heating
Cytochrome *c*	551	551
Haemoglobin	557	548–549
Cytochrome *c*-557	554	551
Cytochrome *c*-558	553	550
Haemin from cytochrome *c*	548	548
Protohaem IX	557	548–549
Haemin from cytochrome *c*-557	553	549

Properties of the haemin isolated from cytochrome c-557

The absorption peaks of the haemin in ether were at 640 nm, 535 nm and a double Soret peak with

maxima at 404 and 370nm. The corresponding spectrum of haemin isolated from cytochrome *c* (horse heart) had absorption maxima at 637, 532, 400 and 370nm. The pyridine ferrohaemochrome of the haemin from cytochrome *c*-557 had an α-peak absorption maximum at 551–552nm, compared with that of cytochrome *c* (horse heart) at 547–548nm (Table 2). The corresponding parameters for 4(2)-hydroxyethyl-2(4)-vinyl deuterohaem IX and haematohaemin are 552 and 549nm respectively (Porra & Jones, 1963).

Typical R_F values obtained for chromatography of the isolated haemins were 0.91 for haematohaemin (horse heart cytochrome *c*), 0.84 for the haemin from cytochrome *c*-557 and 0.60 for protohaem IX. In several repeated experiments these values were not reproducible, but the pattern of separation remained the same. In aged preparations of the haemins from cytochrome *c*-557 and cytochrome *c*, a slower-streaking material was observed with approx. R_F 0.3.

The presence of free vinyl groups on the isolated haemin was tested by using hydrazine hydrate. The results are shown in Table 3.

Amino acid composition

The amino acid compositions of cytochrome *c*-557 and *c*-558 are shown in Table 4, where they are compared with the residues identified in the proposed sequences. The composition of *Euglena* cytochrome *c*-558 is very similar to that given by Meyer & Cusanovich (1972).

Cysteic acid was determined in separate hydrolysates of both cytochromes after removal of the haem group and oxidation with performic acid. For cytochrome *c*-557, 1.07 and 1.08mol of cysteic acid/7mol of leucine and for cytochrome *c*-558, 0.92mol of cysteic acid/5mol of leucine were recovered. After carboxymethylation of apocytochrome *c*-557, 0.85mol of carboxymethylcysteine/7mol of leucine was found.

Tryptophan and tyrosine were estimated by the method of Goodwin & Morton (1946). The results were 3.19mol of tyrosine and 0.97mol of tryptophan per mol of cytochrome *c*-557 and 5.2mol of tyrosine and 2.07mol of tryptophan per mol of cytochrome *c*-558. After removal of the haem group of cytochrome *c* (horse heart) under the same conditions a ratio of tyrosine to tryptophan of 3.81 was obtained. There are four tyrosine and one tryptophan residues in the horse heart sequence.

Cytochrome *c*-557 contained 1.72mol of trimethyllysine/10mol of lysine and cytochrome *c*-558 contained 1.04mol of trimethyl-lysine/12mol of lysine. Small amounts of monomethyl- and dimethyl-lysine

Table 4. *Amino acid compositions of Crithidia cytochrome c-557 and Euglena cytochrome c-558*

Hydrolyses for 20 and 90h were performed on portions of a solution of each protein. The iron content of each solution was determined. The analysis results are derived from average values of the 20 and 90h hydrolysis, except for those of threonine and serine, which were extrapolated to zero time of hydrolysis, and those of isoleucine and valine, for which the 90h values were taken. The results in the analysis (1) columns were calculated relative to the iron content of the parent solutions. The results in the analysis (2) columns were calculated relative to the sums of the amounts of leucine, phenylalanine, histidine and arginine divided by 18 for cytochrome *c*-557 and by 14 for cytochrome *c*-558. Values in the sequence columns are residue totals from the proposed sequences. Analyses of cysteine and tryptophan were performed separately and the methods are described in the text.

	Cytochrome *c*-557			Cytochrome *c*-558		
	Analysis (1)	Analysis (2)	Sequence	Analysis (1)	Analysis (2)	Sequence
Asp	9.8	10.2	10	9.5	9.2	9
Thr	4.0	4.1	4	5.0	4.9	5
Ser	3.9	4.0	4	7.1	6.9	7
Glu	8.7	8.9	9	9.3	9.0	9
Pro	9.1	9.3	9	4.4	4.3	4
Gly	13.7	14.0	14	9.9	9.7	10
Ala	12.4	12.8	13	12.2	11.8	12
Val	6.7	6.9	7	5.2	5.0	5
Met	1.8	1.9	2	2.0	1.9	2
Ile	3.9	4.0	4	4.1	4.0	4
Leu	6.7	6.9	7	5.1	5.0	5
Tyr	2.7	2.8	3	5.0	4.8	5
Phe	5.0	5.1	5	3.1	3.0	3
His	1.9	2.0	2	2.0	1.9	2
Lys	9.8	10.1	10	12.3	12.0	12
Me_3Lys	1.7	1.7	2	1.0	1.0	1
Arg	3.8	4.0	4	4.2	4.1	4
Cys	1.1		1	0.9		1
Trp	1.0		1	2.1		2

were detected in the hydrolysate of cytochrome *c*-557 and these results are discussed below.

N-Terminal groups of cytochrome c-557 and c-558

Dansyl-glutamate was identified after dansylation of the modified form of cytochrome *c*-557 discussed above. The only α-dansyl-amino acid spot identified in the hydrolysate of native cytochrome *c*-557 treated with dansyl chloride was a faint spot of dansyl-arginine. No α-dinitrophenyl-amino acid was identified in the hydrolysate of dinitrophenylated native cytochrome *c*-557. There was, however, a decrease of approx. 1 mol of arginine/mol in the hydrolysate of the dinitrophenylated protein. A single Edman cycle was carried out on apoprotein and amino acid analysis showed a loss of 0.3 mol of arginine/mol.

The amino acid sequence evidence discussed below suggests that the *N*-terminus of the protein is blocked. It is possible that these positive results for arginine may be due to 'raggedness' at the *N*-terminus. If, during isolations of the protein limited proteolysis had occurred at the amino side of arginine (–6) as well as the observed cleavage at the carboxyl side that gives rise to 'modified' form of cytochrome *c*-557, then the resultant fraction of modified protein may not have been separated from intact protein during purification and some *N*-terminal arginine would be detected. This cannot, however, account for the loss of one arginine residue per molecule in the hydrolysate of the dinitrophenylated protein.

No α-dansyl-amino acid was identified in the hydrolysate of cytochrome *c*-558 treated with dansyl chloride.

C-Terminal regions of cytochrome c-557 and c-558

The results of digestion of the two proteins with carboxypeptidase A are given in Table 5.

Determination of the amino acid sequences of cytochrome c-557 and c-558 and deposition of supplementary data

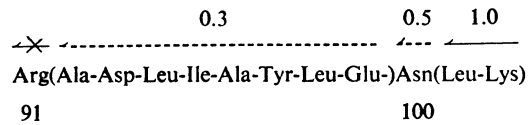
The peptides from tryptic and chymotryptic digestion of cytochrome *c*-557 and tryptic, chymotryptic and peptic digestion of cytochrome *c*-558 have already been discussed (Pettigrew, 1972, 1973; Lin *et al.*, 1973). This evidence has now been extended to include thermolytic digestion of the two proteins. The detailed evidence has been deposited in the British Library (Lending Division) (formerly the National Lending Library for Science and Technology) Boston Spa, Yorks. LS23 7BQ, U.K., for storage on microfiche as Supplementary Publication SUP 50042.

Table 5. *Carboxypeptidase A digestion of cytochrome c-557 (a) and c-558 (b)*

Apocytochrome *c*-557 (approx. 10 nmol) and a portion of a solution of apocytochrome *c*-558, the protein content of which was accurately known from amino acid analysis, were digested with 10 μg of carboxypeptidase A for 60 min as described by Ambler (1972). The solutions were acidified after incubation by addition of a few drops of 1 M-acetic acid and dried *in vacuo*. The residues were taken up in sodium citrate buffer, pH 2.0, and applied to the analyser column. For cytochrome *c*-557 digestion, the residues per molecule were calculated relative to lysine released, equal to 1. For the cytochrome *c*-558 digestion the residues per molecule are based on the total amount of protein digested (12 nmol).

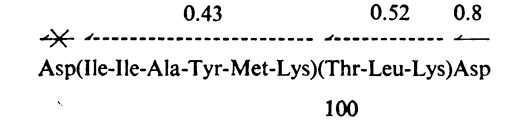
(*a*)	Amino acids released (nmol)	Residues/mol
Asp	1.5	0.23
Ser + Asn + Gln	3.1	0.48
Glu	2.0	0.31
Ala	3.9	0.60
Ile	1.9	0.30
Leu	10.4	1.6
Tyr	1.8	0.28
Lys	6.5	1.0

Interpretation:



(*b*)		
Asp	9.4	0.8
Thr	7.0	0.59
Ala	5.2	0.44
Met	5.5	0.47
Ile	10.0	0.85
Leu	5.8	0.49
Tyr	5.3	0.45
Lys	11.2	0.95

Interpretation:



The evidence comprises: (1) a description and definition of the conventions and abbreviations used in recording the information; (2) electrophoretic maps of the digestion mixtures; (3) tables summarizing the amino acid compositions of peptides and certain parameters of purification, i.e. the electrophoretic mobility at pH 6.5, the relative elution volume on gel filtration through Sephadex G-25 and the final yield; (4) the descriptions of individual

Fig. 3. *Comparison of the amino acid sequences of Crithidia cytochrome c-557, Euglena cytochrome c-558 and horse cytochrome c*

Sequences are numbered from glycine-1 of horse cytochrome *c* (Margoliash, 1962) with a deletion at residue 26 for *Euglena* cytochrome *c*-558. The *N*-terminus of *Crithidia* cytochrome *c*-557 is blocked but the blocking group has not been characterized. Residues common to all mitochondrial cytochromes *c* studied are boxed. Previously unvaried residues which are variant in the case of cytochrome *c*-557 or *c*-558 are boxed only in the horse cytochrome *c* sequence. The one-letter notation for amino acids is A, Ala; B, Asp or Asn; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; X, *ε*-*N*-trimethyl-lysine; Y, Tyr; Z, Glu or Gln. Deletion is –.

peptides for each enzymic digestion including dansyl–Edman evidence, secondary digestion of peptides and assignment of amide groups.

The sequences of the two proteins are shown in Fig. 3 where they are aligned with the sequence of horse cytochrome *c*.

In a few regions, the evidence for the amino acid sequence is poor or incomplete. These points are emphasized below.

Evidence for the sequence of cytochrome c-557

Studies on the *N*-terminus of this cytochrome are described above. They indicate that at least a fraction of the purified preparation contained *N*-terminal arginine. This result is difficult to explain in view of the peptide evidence discussed below and also because the four arginine residues expected from the analysis of whole protein are already positioned in the sequence.

No terminal amino group could be detected in the tryptic peptide [$Pro_{(-9)}$–$Arg_{(-6)}$] or the chymotryptic peptide [$Pro_{(-9)}$–$Phe_{(10)}$] by the ninhydrin, isatin, dansyl or dansyl–Edman methods. The electrophoretic mobility of this tryptic peptide at pH 6.5 was 0.9 (relative to lysine = 1). This indicates that the peptide has a net positive charge of two rather than one (Offord, 1966), a surprising result in view of the apparently blocked α amino group.

Peptide [$Pro_{(-9)}$–$Arg_{(-6)}$] was digested with thermolysin and one ninhydrin-positive peptide was recovered with composition (Ala-Arg). No other staining material was detected with the chlorination method (Rydon & Smith, 1952). A peptide containing proline and trimethyl-lysine and with an electrophoretic mobility at pH 6.5 of 0.64 was recovered from the paper by cutting the whole sheet into strips. After hydrazinolysis of this material (Akabori *et al.*, 1952) and treatment with dansyl chloride, dansyl-trimethyllysine was identified.

Carboxypeptidase B released arginine from peptide [$Pro_{(-9)}$–$Arg_{(-6)}$].

A ninhydrin-positive peptide with composition (Pro-Lys) was isolated in low yield (4%) from the tryptic digestion of cytochrome *c*-557. No *N*-terminal amino acid was identified with dansyl chloride but ε-Dns-lysine was recorded. The electrophoretic mobility at pH 6.5 is not very much lower than that of a dipeptide (0.65 compared with 0.75 for Ser-Lys).

This result is consistent with the pattern of partial methylation found in the amino acid analysis of tryptic peptide [$Pro_{(-9)}$–$Arg_{(-6)}$] and chymotryptic peptide [$Pro_{(-9)}$–$Phe_{(10)}$]. The analysis of the latter peptide indicated that residue (–8) is approx. 7% monomethyl-lysine, 14% dimethyl-lysine, 63% trimethyl-lysine and 16% lysine. Therefore the tryptic

peptide (Pro-Lys) may derive from that fraction of the protein which was not methylated at lysine (~8).

Thus the evidence for the sequence of the *N*-terminal region is unsatisfactory and the nature of the blocking group is unknown.

One other result with cytochrome *c*-557 should be emphasized and that is the recovery of a dipeptide (Ala-Leu) in 2% yield from the thermolysin digestion of the protein. This peptide could not be positioned in the proposed sequence and it was thought that it may derive from transpeptidation of amino acids. No such dipeptide was found, however, in an incubation mixture of alanine (2μmol), leucine (2μmol) and thermolysin (1mg).

The evidence for the purity of the protein preparation indicates that levels of contamination with a single protein of greater than about 3% are unlikely. Assuming that the dipeptide (Ala-Leu) was recovered in approx. 20% yield after purification a recovery of 2% would represent a 10% molar level of (Ala-Leu) in the digestion mixture. The possibility that the proposed sequence is incorrect in this respect is considered unlikely as the amino acid composition of the protein indicates 7 residues of leucine per molecule and 7 leucine residues have been located on the basis of good evidence. A dimorphism at either an alanine or a leucine in the sequence cannot be ruled out but no evidence from amino acid analyses or dansyl–Edman results could be found to support this hypothesis.

Evidence for the sequence of cytochrome c-558

Some difficulty was encountered in the dansyl–Edman degradation of the tryptic peptide [$Gly_{(23)}$–$Arg_{(38)}$] of cytochrome *c*-558 (Pettigrew, 1973). In three experiments, only one degradation gave clean results up to tryptophan-33. In the other two experiments, results were obtained which indicated that acidolysis of the peptide chain at residue 28 had occurred. Three separate secondary digestions of peptide T5 were performed and there was no evidence to suggest that there is a mistake in the sequence.

However, only one thermolysin peptide from this region was recovered, namely [$Val_{(24)}$–$Ser_{(31)}$]. In this respect both the thermolysin and the pepsin digestions of cytochrome *c*-558 were unsatisfactory. The protein used in these digestions still contained haem although it had been subjected to cleavage with $HgCl_2$. Both digestion mixtures contained brown material of high molecular weight which was not fractionated and which interfered with the electrophoresis of peptides. However, thermolysin peptides were obtained in reasonable yield from the region containing cysteine-17, indicating that the treatment with $HgCl_2$ did in fact cleave the thioether bond.

Thus it is possible that the observed loss of a large section of the molecule may be due to the attachment of the haem group to a secondary site on the peptide chain.

Acetyl-dansyl-hydrazine was identified after treatment of tryptic peptide [$Gly_{(1)}$–$Arg_{(5)}$] with hydrazine. In a separate experiment, this peptide was incubated with 6M-HCl at 100°C for 30min and the mixture was fractionated by electrophoresis at pH6.5 and 2.1. The results were consistent with the sequence (acetyl-Gly-Asp-Ala-Glu-Arg), but no peptide containing the glycine residue was identified nor was acetyl-glycine.

Discussion

The method of chromatography on an ion-exchange column in the reduced and then the oxidized form yielded protein of a high degree of purity. When cytochrome *c* (horse heart) with a E_{550}/E_{280} ratio of 0.94 was subjected to the same procedure a product with E_{550}/E_{280} ratio of 1.23 was obtained by using chromatography in 0.07M-NaH_2PO_4–Na_2HPO_4, pH7.0. This is within the range 1.2–1.25 quoted by Margoliash & Walasek (1967) for pure protein. The most stringent test of purity was considered to be the correspondence between the amino acid compositions of the two cytochromes derived from acid hydrolysis of the whole proteins and those derived from the amino acid sequences. The values in Table 4 can be manipulated by using individual amino acids rather than iron as a reference and a closer approximation to integral values can be obtained (analysis 2). The elucidation of a unique sequence with no unfitted peptides is not, in this case, considered an indication of purity, since contaminants of up to 10% would probably not be detected as peptides with the methodology used. CO binding by the pure preparations was low, indicating the presence of only a small fraction of deamidated or polymeric forms, and the gel electrophoresis supports this conclusion. For sequence work this is a relatively unimportant consideration (unless the percentage of deamidation is very high) but for other purposes, e.g. crystallization and enzymic studies, the presence of such modified forms can greatly affect results.

Features of the spectra of cytochromes c-557 and c-558

The peaks of the visible spectra of both cytochromes *c*-557 and *c*-558 are shifted to the red in comparison with horse heart cytochrome *c*. Such shifts are not unknown in *c*-type cytochromes (Kamen & Horio, 1970), but all mitochondrial cytochromes *c* studied until now possess a symmetrical α peak at 550nm. The asymmetry of the α peaks of the two protozoan cytochromes is of interest, as cytochrome *c*-555 from *Crithidia fasciculata* possesses a symmetrical α peak (Hill *et al.*, 1971; Kusel *et al.*, 1969). Cytochrome *c*-555 is presumably a close relative of cytochrome

c-557, and comparative studies may reveal a basis for this spectral dissimilarity. On the other hand, the similarities in the properties of the cytochromes from the two species of *Crithidia* rules out the possibility that cytochrome *c*-557 is derived from the bacterial endosymbiote of *Crithidia oncopelti* (Chang & Trager, 1974).

Some preliminary experiments were performed on the Guttman strain of *Crithidia oncopelti*, 'cured' of its endosymbiote, but the cytochrome isolated from these organisms had an α peak at 555nm and an amino acid composition resembling that of cytochrome *c*-555 from *Crithidia fasciculata*. This supports suspicions that the 'cured' strain of *Crithidia oncopelti* is, in fact, *Crithidia fasciculata* (Newton, 1968).

The low purity indices [ratios of $E_{(\alpha \text{ peak reduced})}$ to E_{280}] of cytochromes *c*-557 and *c*-558 are due in both cases to a decreased α-peak absorption and also, with cytochrome *c*-558, to a greatly increased absorption at 280nm. This is consistent with the presence of a second tryptophan residue in cytochrome *c*-558.

The pyridine haemochrome spectrum of a haemoprotein is diagnostic of the type of haem it contains (Enzyme Nomenclature Recommendations, 1965). The absorption maximum of the pyridine ferrohaemochrome α peak of both cytochromes *c*-557 and *c*-558 lies between that of all *c*-type cytochromes and all proteins containing protohaem IX. This led Meyer & Cusanovich (1972) to postulate that the haem of cytochrome *c*-558 was not haem *c* but 4(2)-hydroxyethyl-2(4)-vinyldeuterohaem and that it was bound to the protein through only one thioether bond.

The shifts in the pyridine ferrohaemochrome spectra of both the cytochrome *c*-557 and *c*-558 proteins and their derived haemins on treatment with hydrazine hydrate are consistent with the presence of single vinyl functions on the haem groups. The haem cannot be removed from either protein by treatment with acetone containing 2% (v/v) HCl and the successful cleavage with heavy-metal salt indicates the presence of a thioether bond.

The prosthetic group in both cytochromes *c*-557 and *c*-558 is therefore probably monosubstituted protohaem IX with one unsaturated vinyl group. However, rigorous proof of the haem structure would require chemical characterization of the isolated haemin. The evolutionary conservatism of coenzymes and the likelihood of a common origin for cytochromes *c*-557, *c*-558 and the mitochondrial cytochromes *c* from plants, animals and fungi argue in favour of the simple difference outlined above.

Structure of the haem-binding site of cytochromes c-557 and c-558

Both cytochrome *c*-557 and *c*-558 lack the cysteine residue at position 14 which forms a thioether link with the haem group in cytochromes *c*. This result is compatible with the body of evidence presented above for the presence of an unsaturated vinyl group on the haem. The alanine at residue 14 implies at least two base changes in the protozoan cytochromes *c* since their divergence from the other eukaryotic cytochrome *c* genes.

The possibility that alanine-14 is an artifact should be considered. Cysteine can give rise to dehydroalanine under certain conditions (Patchornik & Sokolovsky, 1964). However, when cytochrome *c*-557 was treated with sodium sulphite, which converts dehydroalanine into cysteic acid (Wiener *et al.*, 1966) no cysteic acid was found after acid hydrolysis *in vacuo*. Also, the spectrum of *Crithidia* cells after extraction with acid acetone shows an asymmetrical α peak at 557nm, so that the spectrum is not modified during purification.

Lastly, enzymic studies indicate that cytochrome *c*-558 is very similar to horse cytochrome *c* in its reaction with mammalian cytochrome *c* reductase and cytochrome oxidase preparations (Davis *et al.*, 1972). Thus, if the atypical haem-binding is an artifact, it is an artifact that has no effect on the biological activity of the molecule.

Amino acid sequences of cytochrome c-557 and c-558

The aligned sequences are shown in Fig. 3. The novel changes that occur in these two proteins have been discussed (Pettigrew, 1972, 1973). The totals of residues located in both sequences show good correspondence with the amino acid composition (Table 4).

Approx. 100mg of cytochrome *c*-557 and 75mg of cytochrome *c*-558 were used to determine the amino acid sequences. These are relatively small quantities compared with many of the sequence investigations of cytochromes *c*, but are much larger than the amounts used in the characterization of some plant cytochrome *c* sequences (Thompson *et al.*, 1971).

Because of the small quantities obtained, some of the analytical systems used, in particular peptide purification on paper, were employed near the limits of their sensitivity. Amino acid-analysis values tended to be less satisfactory than those of studies using the same techniques but on larger quantities of protein (e.g. Ambler & Brown, 1967).

Because of the small amounts of pure peptide obtained, thorough characterization was often not possible, and thus corroborative evidence from several sources was necessary. Dansyl–Edman evidence was relied on a great deal and thus a relatively small percentage of the peptide bonds were shown to be suspectible to enzymic hydrolysis (53 out of 110 in the case of cytochrome *c*-557 and 59 out of 101 in the case of cytochrome *c*-558).

The evidence of the amino acid sequences supports the conclusion that both cytochrome *c*-557 and *c*-558 are members of the cytochrome *c* family although these are exceptions to the rule that the haem-binding site of *c*-type cytochromes follows the pattern Cys-X-Y-Cys-His.

In the case of cytochrome *c*-558 the novel changes in the sequence have not impaired reactivity with mammalian reductase and oxidase preparations. In the case of cytochrome *c*-557, Keller *et al.* (1973), using n.m.r. spectroscopy, have shown that the structure and properties of the haem crevice are very similar to that of horse cytochrome *c*. The n.m.r. spectra contain features consistent with the absence of a thioether bond and the presence of a free vinyl group.

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