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Studies on the Biosynthesis of Cytochrome *c*

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A soluble cytochrome was isolated and purified from the slime mould *Physarum polycephalum* and identified as cytochrome *c* by room-temperature and low-temperature (77°K) difference spectroscopy. A close similarity between *P. polycephalum* and mammalian cytochromes *c* was suggested by a comparison of the initial rates of oxidation of both proteins by mammalian mitochondria. This similarity was further emphasized by redox titrations and gel-electrophoretic studies which indicated that *P. polycephalum* cytochrome *c* has an oxidation–reduction midpoint potential of +257mV at pH7.0 and a molecular weight of 12500±1500 (mean ± maximum deviation for a set of six measurements). *P. polycephalum* exhibits an absolute requirement for protohaemin for growth. The ^{59}Fe-labelled haemin was prepared by chemical synthesis from protoporphyrin. The purified product had a specific radioactivity of 0.8±0.02μCi/mol. Growth of *P. polycephalum* in the presence of [^{59}Fe]haemin resulted in the incorporation of ^{59}Fe into the plasmodial cytochrome *c*. The specific radioactivity of the cytochrome *c* haem was 0.36±0.02μCi/mol. The high specific radioactivity of the cytochrome haem indicates that synthesis of the holoenzyme must proceed by direct attachment of haem to the apoprotein rather than by the intermediate formation of a protoporphyrinogen–apoprotein complex. The observed decrease in the specific radioactivity of the haem group is attributed to exchange of the ^{59}Fe with unlabelled iron in the plasmodia either before or during attachment of the haem group to the apoprotein.

The biosynthesis of cytochrome *c* may be divided into three separate steps: (1) the synthesis of the apoprotein; (2) the synthesis of the prosthetic group; (3) the attachment of the prosthetic group to the apoprotein forming the active holoenzyme. The biosynthetic pathway is further complicated by the different sites of synthesis of apoprotein and prosthetic group.

Bates *et al.* (1960) claimed to have demonstrated the complete synthesis of cytochrome *c* by isolated mitochondria. However, this claim was quickly disproved and later work indicated that the endoplasmic reticulum is the site of synthesis of mitochondrial cytochrome *c* (Gonzalez-Cadavid & Campbell, 1967; Kadenbach, 1969). Gonzalez-Cadavid & Campbell (1967) concluded that the cytochrome is synthesized *in toto* at the ribosomes, including the attachment of the haem group. This conclusion was supported by kinetic studies of the incorporation of ^{59}Fe by Kadenbach (1968) and of δ-[^{14}C]aminolaevulinic acid, a direct precursor of the tetrapyrrole ring, by Davidian and co-workers (Penniall & Davidian, 1968; Davidian *et al.*, 1969). More detailed kinetic studies of the incorporation of ^{59}Fe and [^{14}C]lysine into both 'microsomal' and mitochondrial cytochrome *c* by Kadenbach (1969, 1970, 1971), however, showed that the early-labelled fraction synthesized on the ribosomes is apocytochrome *c*, a probable precursor of mitochondrial cytochrome *c*. Cytochrome *c* of microsomal fractions was shown to be labelled with ^{59}Fe at the same rate as the mitochondrial cytochrome and, in fact, Kadenbach (1969) clearly demonstrated that 90% of the microsomal cytochrome *c* is of mitochondrial origin and becomes attached to the microsomal fractions during homogenization. It was suggested therefore that apocytochrome *c*, after synthesis on the ribosomes, is transferred to the mitochondria as a protein–phospholipid complex and that formation of the holoenzyme occurs within or on the inner mitochondrial membrane.

Sano & Tanaka (1964) synthesized cytochrome *c* *in vitro* by the non-enzymic reaction of apocytochrome *c* with protoporphyrinogen, followed by autoxidation and insertion of iron. Since the reaction occurs readily at room temperature and in aqueous solution, it was suggested that this pathway may represent the biosynthetic sequence of cytochrome *c*. Studies on the incorporation of δ-[^{14}C]aminolaevulinic acid by Kadenbach (1970, 1971) appeared to rule out the possibility that protoporphyrinogen might be attached to the apoprotein at the endoplasmic reticulum.

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Three mechanisms appear possible for the late stages in cytochrome *c* synthesis in eukaryotic organisms. 1. Protohaem, formed by a common route for all haem proteins, is covalently attached to the apoprotein. 2. Protoporphyrinogen, formed on the common pathway, is attached to the apoprotein and the iron is subsequently inserted. 3. A special synthetic route exists for the synthesis of haem *c*, differing from the pathway leading to non-covalently attached haem groups.

To determine the mechanisms and localization of the late stages in cytochrome *c* synthesis and its integration into mitochondrial membranes, it is obviously necessary to determine the substrates for the reactions. A suitable eukaryotic organism for this study is the slime mould *Physarum polycephalum*, which requires protohaem for growth. In the present paper we describe experiments on the purification and biosynthesis of cytochrome *c* in this organism.

Materials and Methods

Culture of P. polycephalum

The strain of *P. polycephalum* (i+1029) used in this work was a kind gift from Dr. M. J. Carlisle, Department of Biochemistry, Imperial College of Science and Technology, London. It was cultured in the dark at 27°C with constant shaking in 1-litre conical flasks containing 125ml of the medium described in Table 1. Plasmodia were harvested after 72h growth and washed free of slime in 10mM-Tes [*N*-tris-(hydroxymethyl)methyl - 2 - aminoethanesulphonic acid] buffer, pH7.5, containing 0.5M-sucrose. The absolute growth requirement for protohaemin, demonstrated by Daniel *et al.* (1962), was confirmed, as shown in Fig. 1. Dry weight was measured after 72h of incubation at 27°C in 250-ml shake-flasks containing 25ml of medium supplemented by various amounts of haemin or protoporphyrin. Haemin (bovine) was from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. Protoporphyrin was prepared by acid hydrolysis of protoporphyrin dimethyl ester.

Table 1. *Semi-defined culture medium for P. polycephalum*

All components other than haemin are dissolved in water, the pH is adjusted to 4.6 with 10% NaOH and the medium autoclaved at 103.4kPa (15lb/in²) for 15min. Stock solutions of haemin, dissolved in 1% NaOH, are autoclaved separately and added to the sterile medium immediately before inoculation. Stock solutions may be stored at 2°C for 1 week.

Glucose	10.0g
Peptone (bacteriological: Oxford)	10.0g
Citric acid,H_2O	3.54g
KH_2PO_4	2.0g
$CaCl_2,6H_2O$	0.9g
$MgSO_4,7H_2O$	0.6g
EDTA (disodium salt)	0.224g
$FeCl_3,4H_2O$	0.06g
$ZnSO_4,7H_2O$	0.034g
Thiamine hydrochloride	0.0424g
Biotin	0.0005g
Haemin (Sigma)	0.005g
Water	1 litre

Isolation and purification of cytochrome c

Cytochrome *c* was isolated from *P. polycephalum* plasmodia by the method of Richardson *et al.* (1970, 1971) as outlined in Scheme 1. Various modifications were introduced in the initial stages of isolation and purification. Homogenization was carried out in 0.25M-KCl at pH4.6 and 2–4°C by using an Ultra-Turrax homogenizer. Ascorbate was added to keep the cytochrome in the reduced form. The homogenate was centrifuged at 20000*g* for 20min and the pellet rehomogenized twice more in 0.25M-KCl. The pooled supernatants were adjusted to pH8.0 with 1M-Tris and filtered through a milk filter to remove in-

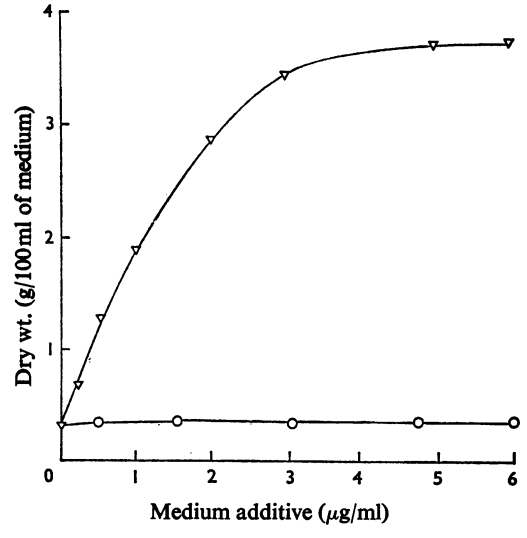
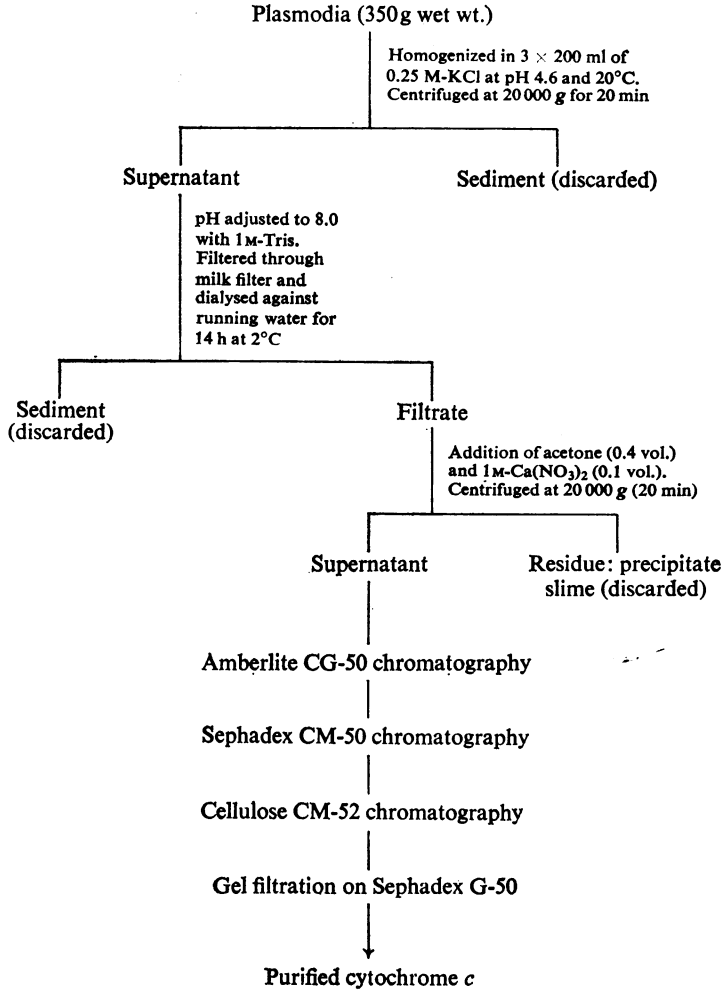


Fig. 1. *Growth requirement of P. polycephalum (strain i+1029) for added protohaem*

Growth was measured (g dry wt./100ml of medium) after 72h incubation at 27°C in 250-ml shake-flasks containing 25ml of culture medium (average of three flasks). ○, Protoporphyrin; ▽ protohaemin. For further details see the text.



Scheme 1. *Isolation of cytochrome c from P. polycephalum*

soluble materials (personal communication from R. T. M. Poulter, Department of Genetics, Leicester). After dialysis at 2–4°C for 14h against running water, the crude extracts were applied to columns of Amberlite CG-50 [NH_4^+ (pH10) form] as described by Richardson *et al.* (1970). However, it was found that the extracellular slime still present in the *P. polycephalum* extracts drastically decreased the column flow rate. The slime was precipitated by the addition of acetone (0.4vol.) in the presence of 0.1 M-$Ca(NO_3)_2$ at pH8.0, and removed by centrifugation. The supernatant was again dialysed against running water for 14h at 2–4°C and applied to the Amberlite resin. Further purification steps involved chromatography on Sephadex CM-50 and cellulose CM-52 and gel filtration on Sephadex G-50. These were carried out as described by Richardson *et al.* (1970).

Characterization of cytochrome c

Absorption spectra of cytochrome *c* were recorded on a split-beam spectrophotometer constructed according to the principles of Yang & Legallais (1954). The monochromator used was a Hilger D.330 and slit-widths were 0.5mm. Cytochrome *c* concentration was determined from its extinction after reduction with dithionite [$\Delta(\epsilon_{550}-\epsilon_{540}) = 20.4 mM^{-1} \cdot cm^{-1}$; Margoliash & Frohwirt, 1959].

The effectiveness of slime-mould cytochrome *c* as a substrate for mammalian cytochrome oxidase was compared with that of horse-heart cytochrome by the following method. Mitochondria were prepared by conventional methods from rat liver (Chappell & Hansford, 1969). Slime-mould and horse-heart cytochromes *c* [Boehringer Corp. (London) Ltd., London W5 2TZ, U.K.] were reduced with ascorbate and excess of ascorbate was removed by passage through a Sephadex G-50 column. The initial rate of oxidation of both cytochromes *c*, at a concentration of 4μM, by cytochrome oxidase at pH7.0 and 37°C was measured in a dual-wavelength spectrophotometer at $E_{550}-E_{540}$.

Determination of mid-point potential

The anaerobic procedures for the assay of oxidation–reduction mid-point potentials were those of Dutton *et al.* (1970). Titrations were carried out, at $E_{550}-E_{540}$, by using sodium dithionite as reductant, in 20mM-Mes [2-(*N*-morpholino)ethanesulphonic acid] buffer, pH7.0, containing 0.2mM-potassium ferricyanide and 10μM-diaminodurol to facilitate electron equilibration between the platinum electrode and the cytochrome *c*.

Determination of molecular weight of cytochrome c

Determination of the molecular weight of *P. polycephalum* cytochrome *c* was carried out by sodium dodecyl sulphate–polyacrylamide gel electrophoresis by using the method of Tanner & Gray (1971). The markers used were ovotransferrin (subunit molecular weight, 80000), bovine serum albumin (subunit molecular weight, 67000), glutamate dehydrogenase (subunit molecular weight, 56000) and glyceraldehyde 3-phosphate dehydrogenase (subunit molecular weight, 36000).

Preparation of [^{59}Fe]protohaem

The ^{59}Fe-labelled protohaem was prepared by chemical synthesis from protoporphyrin. The ^{59}Fe (as $FeCl_3$ in HCl, specific radioactivity 10mCi/mg of iron) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Protoporphyrin dimethyl ester (100mg) was hydrolysed in 25% (w/v) HCl according to the method of Falk (1964, p. 126). After hydrolysis the protoporphyrin was extracted into diethyl ether (Falk, 1964, p. 110), washed with 0.36% (w/v) HCl and finally extracted into 5% (w/v) HCl. The pH of the solution was adjusted to 4.0 and the protoporphyrin precipitated out of solution after being left to stand overnight at room temperature in the dark. The precipitate was then washed with water and the crystals were collected by centrifugation and dried over KOH in a vaccum desiccator.

The insertion of ^{59}Fe followed the method of Warburg & Negelein (see Falk, 1964, p. 135) with some modifications. Protoporphyrin (20mg) was dissolved in 2ml of pyridine, diluted to 100ml with acetic acid and gently refluxed in the presence of boiling chips. The $^{59}FeCl_3$ (80μCi) was added to a solution of 10mg of unlabelled $FeCl_3$ in 6ml of acetic acid–pyridine (1:1, v/v). The mixture was heated to boiling, a trace of sodium ascorbate added to reduce the Fe^{3+} and the boiling solution transferred directly to the protoporphyrin reflux. Traces of the $^{59}FeCl_3$ solution were washed into the reflux with a further 3ml of boiling unlabelled $FeCl_3$ solution. After refluxing for 5min, the reflux vessel was cooled rapidly in ice and the contents were diluted with an equal volume of water. The haemin formed was extracted directly into ether at pH4.0 and washed exhaustively with 5% (w/v) HCl to remove unchanged protoporphyrin. The ether phase (300ml) was then washed with 0.1M-$FeCl_3$ solution (5×200ml) followed by water (5×400ml). This washing procedure should effectively remove contaminating traces of inorganic ^{59}Fe. The ether phase was then filtered through a fluted filter paper and the filtrate concentrated to dryness in a rotary evaporator. The haemin was finally taken up in 1% NaOH, precipitated out of solution by acidification with 3M-HCl and collected by centrifugation. After thorough washing with water the haemin was dried by vacuum desiccation over KOH.

Counting of radioactivity of ^{59}Fe-labelled samples

Solutions of inorganic ^{59}Fe, [^{59}Fe]haemin and cytochrome *c* were counted at infinite thinness in an end-window Geiger counter. The efficiency of counting was 16%. Samples of cytochrome *c* counted after a period of weeks were corrected for radioactive decay.

The [^{59}Fe]haemin was dissolved in 1% NaOH before being included in the growth medium of *P. polycephalum*. The concentration of protohaematin was determined by diluting portions in alkaline pyridine and measuring the $E_{418.5}$ after reduction with dithionite ($\epsilon_{418.5} = 191.5 mM^{-1} \cdot cm^{-1}$; Falk, 1964, p. 240). The specific radioactivity determined was 0.80±0.02 (mean ± maximum deviation for a set of five measurements) μCi/μmol of haemin.

Contamination of the [^{59}Fe]haemin by trace amounts of inorganic ^{59}Fe was ruled out by the following method. Paper chromatography of a mixture of unlabelled haemin and $^{59}FeCl_3$ in pyridine–propan-2-ol–aq. 8M-NH_3 (1:2:2, by vol.) moved the haemin a considerable distance up the paper while the $^{59}FeCl_3$ remained at the origin. After similar chromatography of a sample of the [^{59}Fe]-haemin, the haemin spot was cut out and the haemin was eluted off the paper with 20% pyridine in 0.1M-NaOH. The calculated specific radioactivity

($0.8 \pm 0.02\,\mu$Ci/μmol) was identical with the specific radioactivity obtained before chromatography.

Growth of P. polycephalum in the presence of [^{59}Fe]-haemin

The [^{59}Fe]haemin, dissolved in 1% NaOH and sterilized separately, was added to 1-litre shake-flasks of *P. polycephalum* culture medium to a final concentration of 3 μg of [^{59}Fe]haemin/ml of culture medium. This concentration of haemin results in almost maximum growth of plasmodia (Fig. 1). After growth for 72h the plasmodia were harvested and stored at −20°C until sufficient quantities were obtained for cytochrome *c* isolation.

Results and Discussion

Of the possible mechanisms proposed in the introduction for the attachment of the haem prosthetic group to the apoprotein of cytochrome *c*, the more widely accepted theory envisages a direct reaction between the vinyl groups of the haem and the apoprotein. However, the formation of cytochrome *c* (Sano & Tanaka, 1964) in a non-enzymic reaction of protoporphyrinogen and apocytochrome *c*, followed by autoxidation of the porphyrinogen 'prosthetic group' and insertion of iron, lent considerable support to the theory that the biosynthesis of cytochrome *c* is a process involving the formation of an intermediate protoporphyrin–apoprotein complex.

The slime mould *P. polycephalum* exhibits a requirement for protohaemin as a growth factor. This requirement is absolute since protohaemin cannot be replaced by protoporphyrin or by a number of haem precursors and derivatives [see Daniel *et al.* (1962) and Fig. 1 of the present paper]. Preliminary investigations in this laboratory failed to detect the presence of ferrochelatase (Barnes *et al.*, 1973). A study of the incorporation of [^{59}Fe]haemin into *P. polycephalum* cytochrome *c* was therefore undertaken to resolve the controversy about the mechanism of formation of the cytochrome *c* holoenzyme.

Purification and characterization of P. polycephalum cytochrome c

Before investigating the incorporation of ^{59}Fe into this organism, a procedure was first developed for the isolation and partial purification of the plasmodial cytochrome *c*. Full details of the purification procedure are given in the Materials and Methods section. The early steps in the purification procedure were complicated by the presence of quite large amounts of extracellular polysaccharide (slime). The slime is a sulphated galactose polymer containing trace amounts of rhamnose (McCormick *et al.*, 1970) and is produced during exponential growth in shaken flasks. A considerable amount of the slime may be removed during harvesting, since it forms a readily separable layer over the plasmodial pellet. However, it was found that application of a crude plasmodial extract to columns of Amberlite CG-50 was unsatisfactory since sufficient slime remained in the extract to cause a drastic decrease in flow rate through the resin. The most effective means of removing the slime was by precipitation with acetone (0.4 vol.). $Ca(NO_3)_2$ (0.1 M) was included to minimize the interaction (and consequent loss) of cytochrome *c* with the sulphate groups of the polysaccharide.

P. polycephalum is also rich in phenolic compounds which impart a bright-yellow colour to the plasmodia. This yellow material interfered with the spectrophotometric estimation of cytochrome *c* in crude extracts, but was effectively separated from the cytochrome during chromatography on Amberlite CG-50.

The yield and purity of cytochrome *c* at the different stages in the purification procedure are shown in Table 2. The ratio E_{410}/E_{280} is used as an index of purity (Richardson *et al.*, 1970). The purity index at the final stage of purification fell within the range 3.0–3.4. This indicates quite a high degree of purity

Table 2. *Properties of cytochrome c at the different steps of purification from P. polycephalum plasmodia*

The purity index is the E_{410}/E_{280} ratio, and the ratio of γ-reduced form/α-reduced form is $E_{416(red.)}/E_{550(red.)}$. For further details see the text.

Purification step	Yield (mg)	Purity index	Ratio of γ form/α form
Crude extract after removal of slime	5.5	Unmeasurable	Unmeasurable
Crude preparation after Amberlite CG-50	4.0	0.6–0.9	4.8
After first run on Sephadex CM-50	3.2	1.8–2.2	4.8
After second run on Sephadex CM-50	2.3	2.4–2.6	4.8
After cellulose CM-52	1.7	2.8–3.0	4.9
After Sephadex G-50	1.3	3.0–3.4	4.9

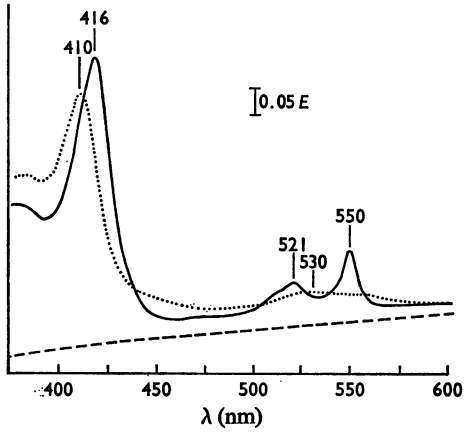


Fig. 2. *Absolute absorption spectra of P. polycephalum cytochrome c*

For details see the text. ····, Spectrum of oxidized cytochrome *c*; ——, spectrum of cytochrome *c*, reduced with dithionite; ---, baseline.

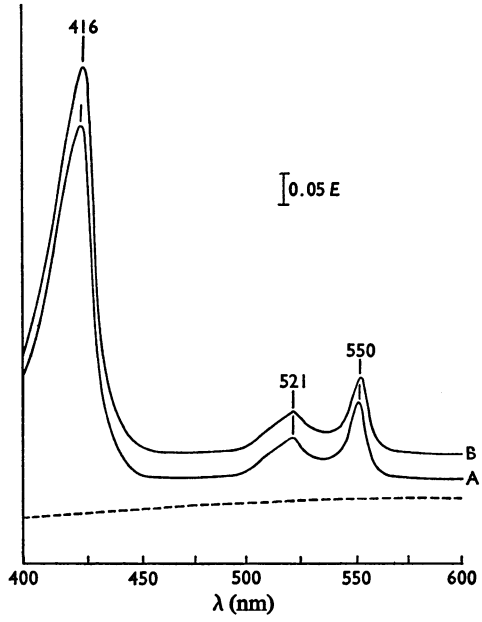


Fig. 3. *Absolute absorption spectra of reduced cytochrome c from P. polycephalum and horse heart*

For details see the text. Curve A, cytochrome *c* from *P. polycephalum*, reduced with dithionite; curve B, cytochrome *c* from horse heart, reduced with dithionite; ---, baseline. (For curve B the baseline is offset by 0.05*E*.)

compared with the value of 3.9–4.2 obtained by Richardson *et al.* (1970) for pure cytochrome *c* from various species of higher plants.

The ratio, $E_{550(red.)}/E_{280(ox.)}$ for purified *P. polycephalum* cytochrome *c* was between 0.75 and 0.85. Margoliash & Frohwirt (1959) report a value of 1.20 for pure horse heart cytochrome *c* and Freeman *et al.* (1967) obtained a similar value (1.21) for pure cytochrome *c* from mouse. For higher plants the ratios fall between 0.88 and 1.22 (Richardson *et al.*, 1970).

The ratio, γ-reduced form (E_{416})/α-reduced form (E_{550}), of *P. polycephalum* cytochrome *c* is somewhat higher than the ratio observed for this protein from animal sources (4.4), but not as high as those reported by Richardson *et al.* (1970) for higher plants (5.0–5.5) or by Hirata & Fukui (1968) for bacteria (5.0–7.1).

The absolute absorption spectra of oxidized and reduced cytochrome *c* in the 375–600nm range are shown in Fig. 2. The absorption maxima are at 410nm and 530nm in the oxidized form and 416, 521 and 550nm in the reduced form. The spectrum of reduced cytochrome *c* from *P. polycephalum* is indistinguishable from that of horse-heart cytochrome *c* in the 400–600nm range, as shown in Fig. 3.

The pyridine haemochrome spectra of *P. polycephalum* and horse-heart cytochrome *c* (shown in Fig. 4) are also identical, with absorption maxima at 414, 520 and 550nm in the reduced form.

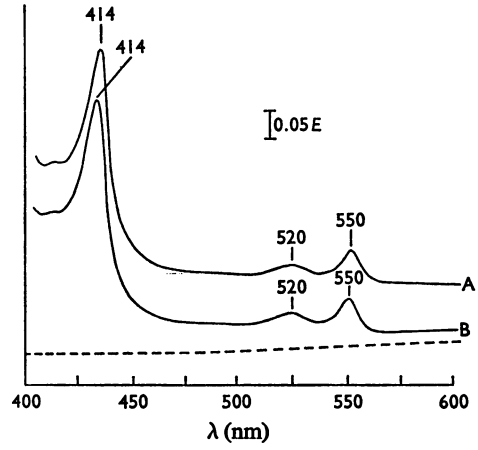


Fig. 4. *Absolute absorption spectra of the pyridine haemochromes of haem c from P. polycephalum and horse heart*

Cytochromes were dissolved in 20% pyridine in 0.1 M-NaOH and reduced with dithionite. Curve A, *P. polycephalum* haem *c*; curve B, horse heart haem *c*; ---, baseline. (For curve A the baseline is offset by 0.075*E*.)

A close similarity between slime-mould and mammalian cytochrome *c* was further suggested by a comparison of the initial rates of oxidation of both proteins by mammalian mitochondria. With a cytochrome concentration of 4 μM in the standard oxidase assay, the initial rate of oxidation of *P. polycephalum* cytochrome *c* was 0.128 μmol/min per mg of mitochondrial protein as compared with 0.132 μmol/min per mg of protein for horse-heart cytochrome *c*.

The oxidation–reduction midpoint potential of *P. polycephalum* cytochrome *c* was found to be +257 mV at pH 7.0. This is directly comparable with the known midpoint potentials of mammalian cytochromes *c* (Dutton *et al.*, 1970).

Electrophoretic studies on polyacrylamide gel of purified *P. polycephalum* cytochrome *c* indicated a molecular weight of 12500±1500. The plasmodial cytochrome *c* coincided with horse-heart cytochrome *c* in 7.5% (w/v) polyacrylamide. No impurities were detected, indicating a high degree of purity of the slime-mould cytochrome.

Low-temperature (77°K) difference spectroscopy of intact *P. polycephalum* mitochondria indicated the presence of two species of cytochrome *c*. Two bands at E_{548} and E_{553} were resolved. These correspond to the known α-bands of mammalian cytochrome *c* at E_{548} and cytochrome *c* at E_{553} (Chance *et al.*, 1964).

A purified preparation of the soluble cytochrome isolated from *P. polycephalum* was also subjected to low-temperature difference spectroscopy. An α-band at E_{548} was recorded confirming that the purified cytochrome is indeed mitochondrial cytochrome *c*.

Incorporation of [^{59}Fe]haemin into P. polycephalum cytochrome c

Since the procedures outlined in Scheme 1 permitted the isolation and purification of cytochrome *c* from relatively small quantities of harvested plasmodia they were used to measure the extent of incorporation of protohaem into the cytochrome. Conical flasks (36×1 litre) containing 0.3 μg of [^{59}Fe]haemin/ml of medium were inoculated with haem-depleted plasmodia, grown for 72h under the usual conditions, and cytochrome *c* was isolated and purified from the plasmodial pellet after harvesting. The purity index and specific radioactivity of cytochrome *c* after each step in the purification procedure are shown in Table 3.

Growth of *P. polycephalum* in the presence of [^{59}Fe]haemin resulted in the incorporation of ^{59}Fe into the plasmodial cytochrome *c*. This incorporated radioactivity can be assumed to be present only as cytochrome *c* haem iron, since contamination by non-haem ^{59}Fe appeared to be ruled out by the lack of variation in the calculated specific radioactivity throughout the various purification steps. The observed specific radioactivity was 0.36±0.02 μCi/μmol of cytochrome *c* as compared with a specific radioactivity of 0.8±0.02 μCi/μmol of starting [^{59}Fe]-haemin.

Direct combination of [^{59}Fe]haemin with apocytochrome *c* should, theoretically, result in the formation of a ^{59}Fe-labelled holoenzyme with a specific radioactivity very similar, if not identical, with that of the starting [^{59}Fe]haemin. On the other hand, breakdown of the [^{59}Fe]haemin, followed by reduction of protoporphyrin, attachment of the unlabelled protoporphyrinogen to the cytochrome apoprotein and reinsertion of iron, should result in the formation of a holoenzyme with a very low and probably undetectable specific radioactivity, since under the conditions of the experiment the ratio of unlabelled to labelled iron in the plasmodia is likely to be very high. The high specific radioactivity of the cytochrome haem (45% of that of the starting [^{59}Fe]haemin) indicates that the latter mechanism of holoenzyme formation is most unlikely.

If synthesis of the holoenzyme proceeds by direct attachment of haem to the apoprotein an explanation must be sought for the observed decrease in the specific radioactivity of the haem group. Contamination of the [^{59}Fe]haemin by inorganic ^{59}Fe can be ruled out since chromatographic analysis of a sample of the [^{59}Fe]haemin showed it to be free of traces of $^{59}FeCl_3$. Similarly, it is unlikely that the culture medium contained significant amounts of unlabelled

Table 3. *Specific radioactivity of cytochrome c from P. polycephalum at various stages of purification*

P. polycephalum was cultured on a medium containing 3 μg of [^{59}Fe]haemin/ml (specific radioactivity, 0.8±0.02 μCi/μmol). For details see the text. The values are given as means±maximum deviation.

Purification step	Purity index	Specific radioactivity of cytochrome *c* (μCi/μmol)
Amberlite CG-50	0.8	0.36±0.02
First run on Sephadex CM-50	2.2	0.36±0.02
Second run on Sephadex CM-50	2.6	0.36±0.02
Cellulose CM-52	2.9	0.36±0.02
Sephadex G-50	3.2	0.36±0.02

haemin since the flasks used in the [^{59}Fe]haem growth experiments were inoculated with plasmodia grown for four successive growth phases in limiting haem (0.25 μg/ml of medium) and the only undefined constituent of the medium (i.e. bacteriological peptone) did not contain a detectable amount of either protohaemin or haem-containing proteins.

The decrease in specific radioactivity could, however, be affected by exchange of the ^{59}Fe with unlabelled iron in the medium or within the plasmodia. Since iron exchange is not known to occur in haem proteins, it must be assumed that any exchange taking place occurs before or during synthesis of the holoenzyme. It is unlikely that any significant exchange occurs in the culture medium since the ^{59}Fe is present in the Fe^{3+} form and does not readily undergo exchange under these conditions (Falk, 1964, p. 39).

However, either before or during attachment to the apoprotein, the Fe^{3+} may be reduced to the Fe^{2+} form. It seems reasonable to speculate that during reduction and/or the attachment of the haem group to the apoprotein, the haem iron may be more labile and exchangeable with the unlabelled iron of the mitochondria. This assumption is supported by the demonstration that ferrochelatase can catalyse exchange of Fe^{2+} from protohaemin (Porra & Jones, 1963).

Kadenbach (1969, 1970, 1971) has suggested that, after synthesis on the ribosomes, apocytochrome *c* is transferred as a protein–phospholipid complex to the mitochondria. It is suggested that the thiol groups of the transferred apoprotein may be protected in this complex (Kadenbach, 1970) and that transfer of the complex occurs after chance contact with individual mitochondria (Kadenbach, 1971). Formation of the holoenzyme is completed either within or on the inner mitochondrial membrane. The results of the present investigation on the biosynthesis of cytochrome *c* in *P. polycephalum* suggest that formation of the holoenzyme is a process involving the direct attachment of the haem prosthetic group to the apoprotein.

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