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Products of Mitochondrial Protein Synthesis in *Neurospora crassa*

Determination of Equimolar Amounts of Three Products in Cytochrome Oxidase on the Basis of Amino-Acid Analysis

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Cytochrome oxidase isolated from *Neurospora crassa* was resolved into seven protein components by electrophoresis in polyacrylamide gels containing sodium dodecylsulfate. The apparent molecular weights were determined to be 41000, 28500, 21000, 16000, 14000, 11500 and 10000 for the components 1, 2, 3, 4, 5, 6, and 7, respectively. The components 1, 2 and 3 are synthesized on mitochondrial ribosomes as shown by the incorporation of radioactive amino acids in the presence of cycloheximide.

Amino-acid analysis of the isolated components 1, 2 and 3 revealed a high content of apolar amino acids and a low content of basic amino acids compared to an average amino-acid composition of components 4—7.

Components 1, 2 and 3 contribute 27.9%, 18% and 14.2% to the whole cytochrome oxidase protein. This was calculated from the contributions of the single components to the total leucine content of the enzyme and the leucine contents (nmol leucine per mg protein) of the single components as determined by amino-acid analysis. Equimolar relations of the components 1, 2 and 3 are found by dividing the amounts of protein by their apparent molecular weights. A stoichiometry of 1:1:1 results assuming a minimal molecular weight of 150000 for the whole cytochrome oxidase protein.

On the basis of the heme *a* content a molecular weight of about 70000 per heme group was determined, using an absorption coefficient $\Delta\varepsilon_{605}$ (reduced minus oxidized) of 12 mM^{-1} cm^{-1}. It is concluded that the smallest structural unit of cytochrome oxidase contains two heme groups.

In previous papers it was shown that four protein components of cytochrome oxidase from *Neurospora crassa* with apparent molecular weights of 8000 to 170000 are synthesized on extramitochondrial ribosomes [2], whereas a component with a molecular weight of 18000 is provided by the mitochondrial protein synthesis [1]. The site of synthesis of two further components with molecular weights of 28000 and 36000 remained unclear.

It was also demonstrated that the inhibition of mitochondrial protein synthesis by chloramphenicol causes an inhibition of the assembly of cytochrome oxidase. Therefore it was suspected that the inhibition of the extramitochondrial protein synthesis by cycloheximide might also affect the assembly process. Indeed in the present experiments conditions could be elaborated leading to a cycloheximide-resistant amino acid incorporation into all three large components. This is in agreement with experiments carried out with yeast [3,4] where three large components of a cytochrome oxidase isolated by immunoprecipitation contained cycloheximide-resistant label.

In order to characterize the components of mitochondrial origin in more detail these proteins were isolated and their amino-acid compositions determined. Large differences between the components of mitochondrial and extramitochondrial origin were detected with respect to their amino-acid composition, *e.g.* their leucine content. Taking this into account, protein contents and stoichiometric relationships could be derived from the distribution of homogeneous [^{3}H]leucine label among the components after electrophoretic separation.

Enzyme. Cytochrome oxidase or ferrocytochrome *c*: O_2 oxidoreductase (EC 1.9.3.1).

METHODS AND MATERIALS

Cultivation and Labelling of Cells

Neurospora crassa wild type 74A, was grown on Vogel's minimal medium under aeration [1]. Cytochrome oxidase was labelled homogeneously with

[^{3}H]leucine by growing cells of a 15-h culture for 3 h in the presence of the radioactive amino acid prior to isolation of the enzyme. The same ^{3}H-labelled preparations were used for spectral measurements, determinations of heme *a* content, isolation of components, amino-acid analyses and determinations of protein contents in the single components. Special growth conditions in the presence of radioactive amino acids and inhibitors are detailed in the legends to the tables and figures.

Preparation of Cytochrome Oxidase

Whole mitochondrial membrane proteins and whole cytosolic proteins were prepared after disintegration of the cells with a grind mill as described [1]. Cytochrome oxidase was prepared by chromatography on oleyl polymethacrylic acid resin [1]. For large-scale preparations, the enzyme was first enriched by ammonium sulfate precipitation after solubilization with deoxycholate [5]. After concentration on DEAE-cellulose cytochrome oxidase was dialyzed against water and stored at a concentration of about 5 mg/ml at −20 °C.

Preparative-Gel Electrophoresis

For preparative dodecylsulfate-gel electrophoresis a new apparatus was developed, which is described in Fig. 1. With the upper part removed, the column was filled with 120 ml of a sucrose gradient (0−60% sucrose, 50 mM Tris-acetate pH 8, 0.5% sodium dodecylsulfate) through an inlet just above the dialysis membrane. The gradient was underlayered with 75% sucrose, 0.1 M Tris-acetate, 0.5% sodium dodecylsulfate, until the joint was completely filled. The removed upper part of the column was closed at the joint with a dialysis membrane and was filled with the polymerization solution (10% or 15% acrylamide, cross linker 1:30, 50 mM Tris-acetate, pH 8, 0.5% sodium dodecylsulfate). After polymerization of the gel the membrane was removed and the joint was covered with vacuum grease and connected with the lower part of the column. Cooling water (10−14 °C) was run through the jacket. A reservoir was mounted at the top and filled with buffer (50 mM Tris-acetate, 0.5% sodium dodecylsulfate). The lower reservoir was filled with buffer (75% sucrose, 0.1 M Tris-acetate pH 8, 0.5% sodium-dodecylsulfate) and the room below the dialysis membrane was filled by suction through the inlet at the lower side of the membrane. The electrode buffers (0.1 M Tris-acetate pH 8, 0.5% sodium dodecylsulfate) were connected to the column by bridges. 1 mg cytochrome oxidase dissolved in 0.2 ml 50 mM Tris-acetate pH 8.5, 2% sodium dodecylsulfate, 5% mercaptoethanol was layered on top of the gel. At the end of the run (after 24−26 h at 15−20 mA and 300−350 V) the cooling jacket was drained, and the upper reservoir and the upper part of the column containing the gel were removed. The gradient was pumped out through the opening above the dialysis membrane and fractions of 1−1.1 ml were collected. The pooled fractions were dialyzed repeatedly against water. The lyophilized proteins were stored at −20 °C.

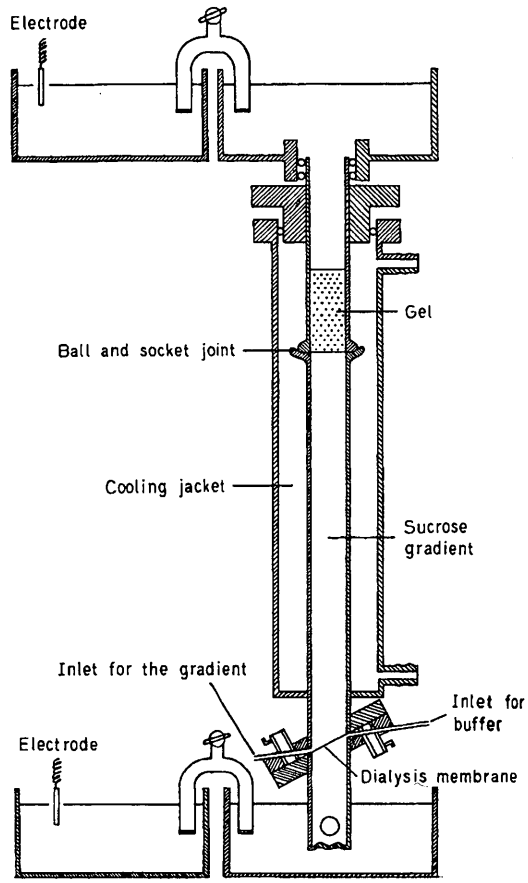

Fig. 1. *Section of the apparatus for preparative polyacrylamide-gel electrophoresis.* Both parts of the central column consist of glass and are connected by a ball and socket joint. The stopper of the cooling jacket is glued to the upper part of the glass column. A dense connection to the jacket is established by a rubber O-ring. The column is intersected below the cooling jacket. Here a dialysis membrane is mounted between two plates, which are glued to the free ends. Immediately above and below the membrane are openings, which can be closed by stopcocks

Analytical Gel Electrophoresis

Analytical gel electrophoresis in the dodecylsulfate [1] or in the phenol-formic acid medium [6] was performed as described. In a modification of former procedures, the dodecylsulfate gels were polymerized in the presence of 0.5% sodium dodecylsulfate and 0.1 M Tris-acetate pH 8.

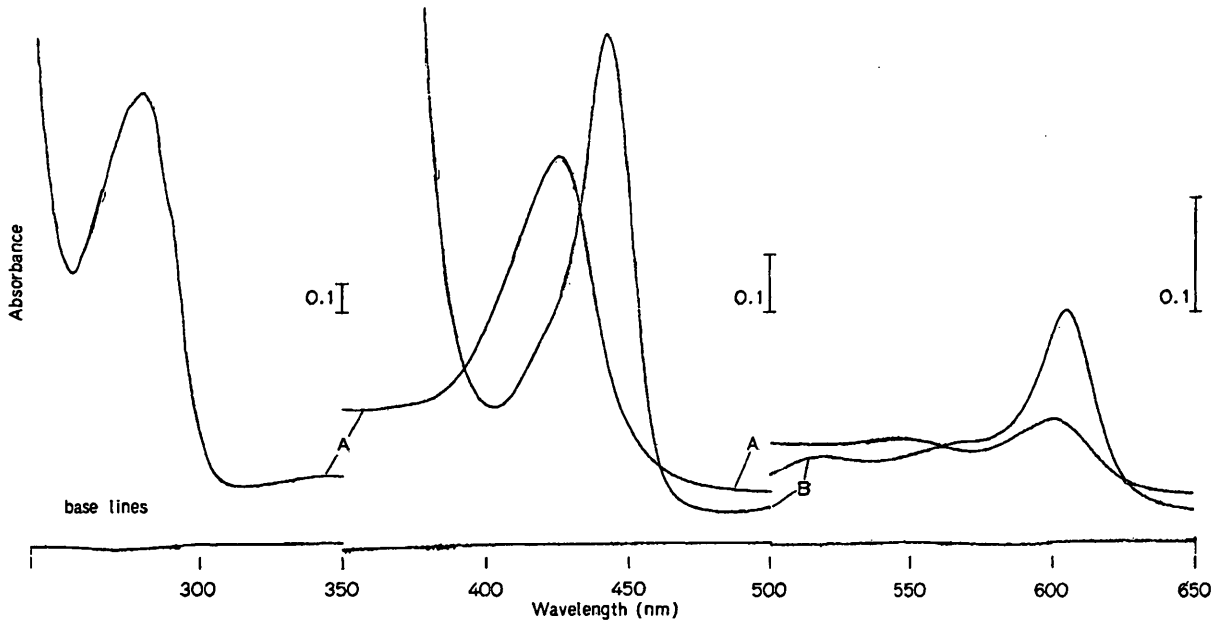

Fig. 2. *Absorption spectra of cytochrome oxidase from* Neurospora crassa. The enzyme was diluted with 0.1 M phosphate buffer pH 7.4 to a concentration of 0.55 mg protein per ml. Spectra were recorded with the (A) air oxidized and the (B) dithionite reduced enzyme. (For evaluation see Table 1, preparation I, No. 2)

Determination of Protein and Radioactivity

Whole mitochondrial membrane proteins and whole cytosolic proteins were precipitated and washed as described [1]. Cytochrome oxidase was precipitated with acetone (final concentration of 90%) from a solution 5 mM in HCl. This procedure removes the heme groups. The dry proteins were dissolved in 1 M NaOH by heating for 10 min at 100 °C. Aliquots were taken for liquid scintillation counting and protein determinations with the biuret method.

Amino-Acid Analysis

Amino-acid compositions were determined in an amino-acid analyzer working at the nanomole range [7]. The lyophilized proteins isolated by preparative dodecylsulfate-gel electrophoresis were dissolved in water containing 0.1% sodium dodecylsulfate. The protein was precipitated with 10% trichloroacetic acid, washed twice with this solution and 3 times with acetone. The protein of cytochrome oxidase was precipitated as described above. The dry proteins were dissolved in formic acid. Aliquots containing about 20 μg were dried in glass tubes. After addition of 0.5 ml 6 M HCl the tubes were flushed with nitrogen and sealed under vacuum. The samples were heated for 24, 48, 72, 120 and 168 h. After drying over KOH in vacuum the amino acids were dissolved in 0.2 M citrate buffer pH 2.2. Amino acids and radioactivity were determined in aliquots containing a total of 8—10 nanomoles. Serine, threonine and tyrosine contents were determined by zero-time extrapolation. Contents of valine and isoleucine were obtained after long hydrolysis times. Methionine was determined as methioninesulfone after peroxidation [8].

Spectral Measurements

Absorption spectra of cytochrome oxidase were recorded with a Cary model 118 spectrophotometer. The heme *a* contents were calculated from the difference in absorbance at 605 nm between the reduced and the oxidized enzyme using a differential absorption coefficient $\Delta\varepsilon_{605} = 12\ \mathrm{mM^{-1} \cdot cm^{-1}}$ [9].

RESULTS

Spectral Properties of Cytochrome Oxidase

The absorption maxima of the oxidized and reduced cytochrome oxidase are found in the same positions as described earlier [1]. Only a peak of the oxidized enzyme is found at a somewhat lower wavelength of 426 nm (Fig. 2). The absorbance recorded with two preparations, each at two different concentrations, are given in Table 1. The ratios of the absorbances at $280_{ox}/426_{ox}$ are 2.4—2.5, in agreement with a cytochrome oxidase preparation from beef heart [10]. The higher ratio occurring in preparation II is accompanied by a somewhat lower heme content.

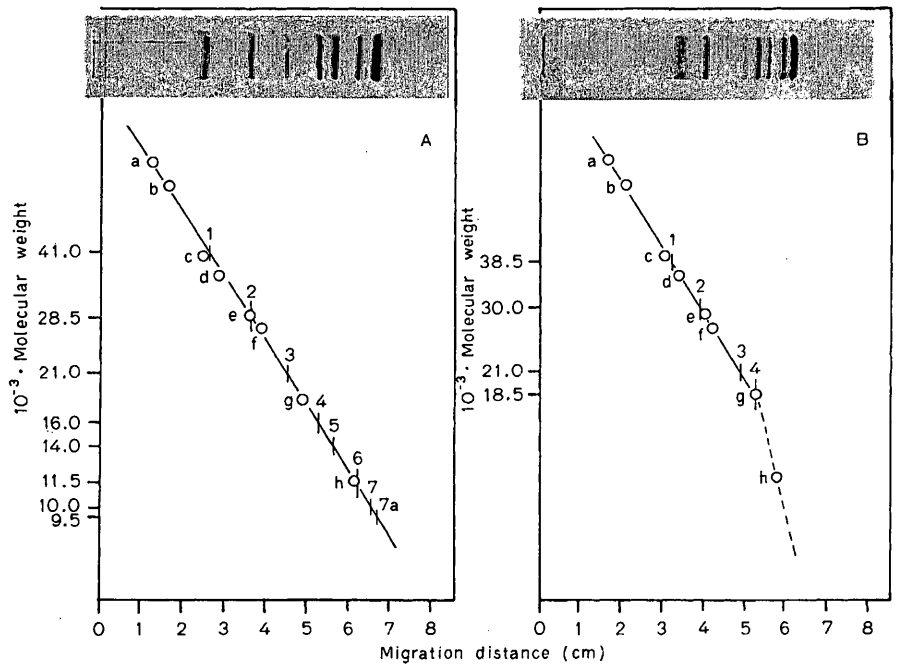

Fig. 3. *Determination of the molecular weights of the protein components of cytochrome oxidase by electrophoresis on (A) 15% and (B) 10% dodecylsulfate gels.* The components are numbered with decreasing molecular weights. The molecular weights were calibrated by running in parallel on the same gel slab proteins of known molecular weights: (a) bovine serum albumin (68000), (b) catalase (60000), (c) aldolase (40000), (d) lactate dehydrogenase (36000), (e) carbonic anhydrase (29000), (f) triosephosphate isomerase (27000), (g) β-lactoglobulin (18500) and (h) cytochrome c (11700)

Table 1. *Absorbances and heme a content of cytochrome oxidase*
The spectra were recorded as described in Fig. 2. The protein contents were calculated from the amino acid analyses presented in Table 4. Cysteine and tryptophan, which were not determined, were assumed to amount to 0.95 and 3.8 mol per 100 mol amino acids as in beef heart cytochrome oxidase [10]. The spectral data and the protein contents obtained by amino-acid analysis could be related to each other *via* radioactivity measurements, because the same enzyme preparations labelled with [^{3}H]leucine (see Methods) were used

Preparation	Probe No	Absorbances						Protein contents determined by		Heme *a* contents related to	
		Oxidized at			Reduced at		Reduced minus oxidized 605 nm	Amino-acid analysis	Biuret	Amino-acid analysis	Biuret protein
		280 nm	426 nm	600 nm	443 nm	605 nm					
								mg/ml		nmol/mg	
I	1	1.886	0.790	0.112	1.044	0.229	0.122	0.681	0.635	14.9	16.0
	2	1.596	0.680	0.109	0.896	0.203	0.099	0.550	0.512	15.0	16.1
II	1	1.496	0.592	0.082	0.751	0.159	0.083	0.492	0.471	14.1	14.7
	2	1.294	0.520	0.080	0.666	0.152	0.076	0.462	0.442	13.7	14.3

Based on a protein determination *via* amino acid analysis, a heme content of 14—15 nmol heme *a* per mg protein is calculated, using an absorption coefficient $\Delta\varepsilon_{650}$ (reduced minus oxidized) of 12 mM^{-1} cm^{-1}. The protein determined by the biuret method is less by about 5%, consequently the calculated heme contents are higher.

Protein Components of Cytochrome Oxidase

The protein part of cytochrome oxidase is separated into seven fractions by dodecylsulfate-gel electrophoresis. On a 15% gel (Fig. 3A) molecular weights of 41000, 28500, 21000, 16000, 14000, 11500 and 10000 are determined. The components are numbered with decreasing molecular weights. Fraction 7 contains two overlapping bands. In addition, the presence of several weak bands is observed. These bands are more prominent in preparations of lower heme content, and can thus be used as a criterion of purity. On the 10% gel (Fig. 3B) resolution of components 4—7 is worse, and calibration of molecular weights is not yet possible in the low-molecular-weight range.

Table 2. *Change of cycloheximide-resistant labelling of cytochrome oxidase after stimulation of mitochondrial protein synthesis*

Hyphae grown for 17 h in a 2-l culture were labelled for 1 h with [^{3}H]leucine, [^{3}H]isoleucine and [^{3}H]phenylalanine each 0.25 mCi/l. Thereafter the culture was divided into two equal portions. One half was incubated with 4 mg chloramphenicol per ml for 30 min. Then both cultures were washed separately three times with fresh culture medium, and aerated in 1 l medium for another 15 min. To both cultures 0.1 mg cycloheximide per ml was added, and 2 min later [^{14}C]leucine, [^{14}C]isoleucine and [^{14}C]phenylalanine, each 0.0125 mCi/l. After 60 min the cells were mixed with ice, and the different cell fractions were isolated. The specific ^{3}H radioactivities of whole cytosolic and whole mitochondrial membrane proteins were in both cultures between 680000 and 710000 counts min^{-1} mg^{-1}. The specific ^{3}H radioactivities of the cytochrome oxidase preparations were 78000 and 83000 counts $\times$ min^{-1} $\times$ nmol heme a^{-1}

Cellular fractions	^{14}C/^{3}H ratios	
	In control	After a transitory incubation with chloramphenicol
Cytosolic proteins	0.27	0.49
Whole mitochondrial membrane proteins	5.9	9.2
Total protein of cytochrome oxidase	6.3	15.9
Components of cytochrome oxidase		
1	2.0	16.0
2	8.0	17.0
3 + 4	21.0	32.5
5	1.0	1.0
6	1.5	2.0
7	0.8	2.5

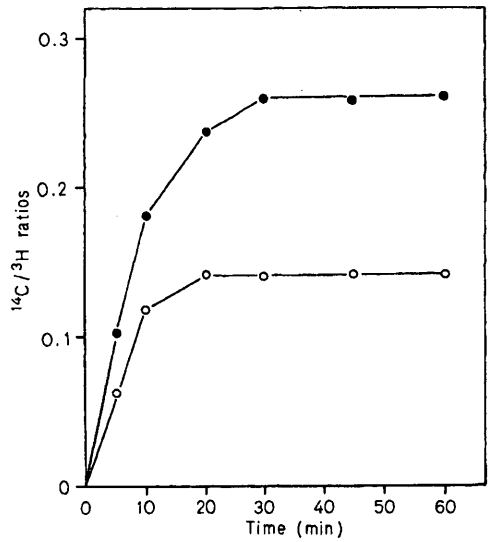

Fig. 4. *Kinetics of cycloheximide-resistant amino-acid incorporation after a transitory incubation with chloramphenicol.* Hyphae grown for 16 h in a 2-l culture were labelled with [^{3}H]leucine (0.1 mCi/l) for 2 h. Thereafter the culture was divided into two equal portions. One half was incubated with 4 mg chloramphenicol per ml for 30 min. Then the cells in both cultures were washed separately three times with fresh culture medium and aearated in 1 l of medium for another 15 min. To both cultures 0.1 mg cycloheximide per ml was added and 2 min later [^{14}C]leucine (0.02 mCi/l). At the times indicated, aliquots were mixed with ice, and the whole mitochondrial membrane proteins were isolated. All ^{14}C values were related to the ^{3}H label, which was 102000 ($\pm$ 5%) counts $\times$ min^{-1} $\times$ mg^{-1} at all times. (●——●) ^{14}C/^{3}H ratios after chloramphenicol preincubation, (○——○) ^{14}C/^{3}H ratios in the control experiment

It may be mentioned that in previous experiments [1,2,15] slightly different molecular weights were determined, due to a different procedure for polymerization of the gels (see Methods).

Cycloheximide-Resistant Labelling of Polypeptides of Cytochrome Oxidase after a Transitory Incubation with Chloramphenicol

After incorporation of radioactive amino acids in the presence of cycloheximide the specific radioactivity of the isolated cytochrome oxidase, as indicated by the ^{14}C/^{3}H ratio, slightly exceeds that of the whole mitochondrial membrane proteins (Table 2). The majority of the incorporated radioactivity is found in the component 3. In addition a lower labelling of component 2 is observed (Table 2, Fig. 5A). The other protein components contain negligible radioactivity, as published earlier [1].

As described for yeast [11], mitochondrial protein synthesis in *Neurospora crassa* is stimulated after a transitory incubation with chloramphenicol. The cycloheximide-resistant amino acid incorporation proceeds over a longer time and at a higher rate, when the cells have previously been incubated with chloramphenicol for 30 min and thereafter washed free of the inhibitor (Fig. 4). After this treatment the cytochrome oxidase shows a higher enrichment of specific radioactivity (Table 2). Besides component 3, the two larger components 1 and 2 are highly labelled (Table 2, Fig. 5B).

Recovery of Polypeptides of Cytochrome Oxidase with Cycloheximide-Resistant Labelling after Washing-Out the Inhibitor and Further Growth of the Cells

After incorporation of [^{14}C]leucine in the presence of cycloheximide for 1 h the inhibitior was washed out with fresh culture medium containing a chase of unlabelled leucine, and the cells were grown for further 4 h. In addition to the labelling of the whole mitochondrial membrane proteins, a substantial amount of radioactivity appears also in the whole cytosolic proteins (Table 3), in contrast to the experiment described in Table 2. This indicates, that the chase does not suppress completely the incorporation of residual free [^{14}C]leucine. Nevertheless the ^{14}C

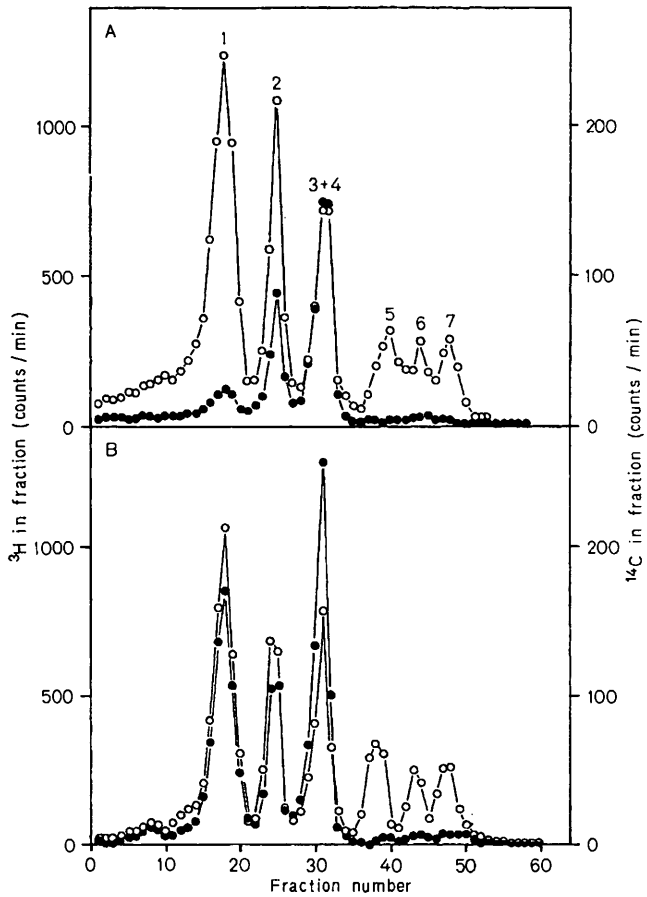

Fig. 5. *Changes in the cycloheximide-resistant labelling of the protein components of cytochrome oxidase after a transitory incubation with chloramphenicol.* The cytochrome oxidase preparations described in Table 2 were separated by gel electrophoresis in the presence of dodecylsulfate. (○) ^{3}H control label; (A) (●) ^{14}C radioactivity incorporated in the presence of cycloheximide; (B) (●) ^{14}C radioactivity incorporated in the presence of cycloheximide after a transitory incubation with chloramphenicol

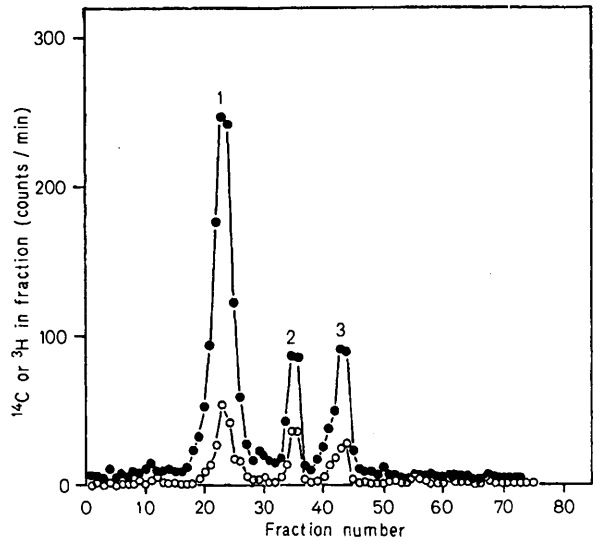

Fig. 6. *Appearance of cycloheximide-resistant label in components of cytochrome oxidase after washing out the inhibitor and further growth of the cells.* The cytochrome oxidase preparation described in Table 3. was separated by dodecylsulfate-gel electrophoresis. (●) ^{14}C radioactivity added 2 min after the cycloheximide; (○) ^{3}H radioactivity added 60 min later

Table 3. *Appearance of cycloheximide-resistant label in cytochrome oxidase after washing out the inhibitor and further growth of the cells*

Hyphae grown for 18 h were incubated for 2 min with 0.1 mg cycloheximide per ml. Then [^{14}C]leucine (0.05 mCi/l)was added and 60 min later [^{3}H]leucine (0.5 mCi/l). 5 min after application of the ^{3}H-labelled amino acid the cells were washed 5 times with fresh culture medium containing unlabelled leucine 2 mM. The cells were aerated in the chase medium another 4 h before isolation of the different cell fractions

Cellular fractions	Specific radioactivities		^{14}C/^{3}H ratios
	^{14}C	^{3}H	
	counts × min^{-1} × mg^{-1}		
Cytosolic proteins	12000	33000	0.36
Whole mitochondrial membrane proteins	56000	30000	1.70
Total protein of cytochrome oxidase	148000	26300	5.36

label is highly enriched in the isolated cytochrome oxidase.

In order to assess the amount of the ^{14}C label incorporated during and after the washing-out of cycloheximide, [^{3}H]leucine was added 5 min before the beginning of the washing procedure. The [^{3}H]-leucine is taken up by the cells, but as can be seen (*e.g.* in Fig. 1) hardly any incorporation takes place in cells treated for 1 h with cycloheximide. After washing out the inhibitor, the [^{3}H]leucine is incorporated exactly like the residual free [^{14}C]leucine.

The ^{14}C/^{3}H ratios are different in the different cell fractions. The lowest ratio of 0.36 is observed (Table 3) with the cytosolic proteins, which corresponds to the ratio of [^{14}C]leucine to [^{3}H]leucine added. Cycloheximide-resistant labelling of cytosolic proteins is negligible. Hence, this ratio may be used to calculate from ^{3}H label present in other proteins the cycloheximide-resistant part of the ^{14}C label, according to the following formula:

$$[^{14}C]_{\text{cycloheximide}} = [^{14}C]_{\text{total}} - [^3H]\,\frac{[^{14}C]_{\text{cytosol}}}{[^3H]_{\text{cytosol}}}.$$

In the case of the whole protein of cytochrome oxidase, 94% of the total ^{14}C radioactivity is calculated to be incorporated in the presence of cycloheximide. The ^{14}C label appears in components 1, 2 and 3 (Fig. 6). From the low ^{3}H control label it follows that

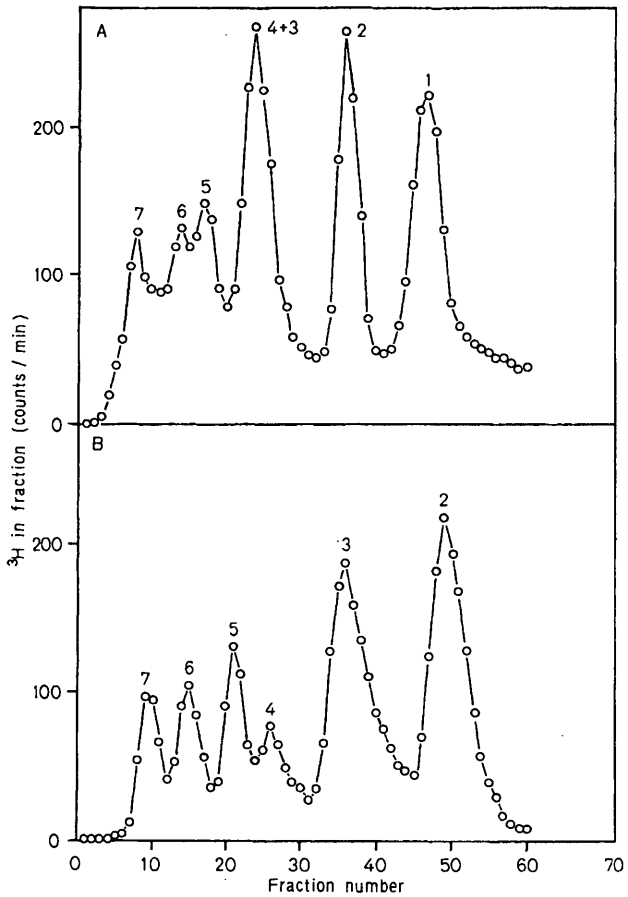

Fig. 7. *Separation of cytochrome oxidase by preparative polyacrylamide-gel electrophoresis in the presence of dodecylsulfate.* 1 mg of a cytochrome oxidase labelled homogeneously with [^{3}H]leucine (see Methods) was separated. Fractions of 1.1 ml were collected after electrophoresis, and aliquots of 0.05 ml were used for determination of radioactivity. Separations are shown obtained (A) on a 10% gel of length 6 cm and (B) on a 15% gel of length 4 cm

most of the ^{14}C label present in these components has been incorporated in the presence of cycloheximide. This is in agreement with the results presented in the previous paragraph and indicates a mitochondrial site of synthesis of components 1, 2 and 3.

Isolation of the Components with Mitochondrial Site of Synthesis by Preparative Dodecylsulfate-Gel Electrophoresis

The three large components of cytochrome oxidase can be separated by preparative dodecylsulfate-gel electrophoresis. In the device described under Methods the sucrose gradient preserves the separation of the proteins obtained in the gel, because the protein · dodecylsulfate complexes were found to exhibit the same electrophoretic mobility in the carrier-free electrophoresis. The apparatus avoids an elution chamber [12], and the separated proteins are recovered in small volumes without loss of material [13]. The system has the disadvantage that the migration of the proteins out of the gel cannot be recorded during the separation. Hence, the optimal separation times have to be determined in preruns.

The three large components 1, 2 and 3 are clearly separated from each other on a 10% gel (Fig. 7 A). But whereas the isolated components 1 and 2 appear as single bands on analytical gels (Fig. 8 B, C), component 3 is still contaminated with component 4 (Fig. 8 E). On a 15% gel (Fig. 7 B) the low molecular weight proteins are well separated, but for isolation of component 1 too-long separation times are required. The pure component 3 shown in Fig. 8 D was obtained by submitting the fraction isolated on a 10% gel (Fig. 8 E) to reelectrophoresis on a 15% gel.

Reelectrophoresis of Components 1, 2 and 3 under Different Conditions and Comparison with the Mitochondrial Products Present in the Whole Mitochondrial Membrane

The isolated components run as single bands on dodecylsulfate gels (Fig. 9). The component 4 still present with component 3, isolated on a 10% gel, appears as a shoulder. The molecular weight of the proteins is not changed after a 15-min incubation in 0.2 M NaOH [4]. A small amount of protein of lower mobility is observed with component 3. This band represents most probably an aggregate, because it vanishes nearly completely after preincubation at alkaline pH.

On phenol-formic-acid gels, components 1 and 2 run again as single bands. Components 3 exhibits a shoulder under these conditions, and the contaminating component 4 is now clearly separated. In both the acidic and the dodecyl-sulfate gels component 4 has the same mobility as β-lactoglobulin whereas the mitochondrial products, especially components 1 and 3 show a strongly reduced electrophoretic mobility in the acidic system. In both media, control label and cycloheximide-resistant label are nearly superimposable suggesting that the three components are not contaminated with proteins of cytosolic origin.

The molecular weights of components 1, 2 and 3 are very similar to those of the mitochondrial translation products found in the whole mitochondrial membrane (Fig. 11). To see wether there are any differences, the isolated components of cytochrome oxidase were compared by coelectrophoresis with the separated mitochondrial products from the whole membrane (Fig. 10). Component 1 exhibits the same mobility as the membrane fraction of corresponding molecular weight in both the dodecylsulfate and the acidic system. Component 2 coincides with a shoulder of the membrane fraction and component 3 exhibits

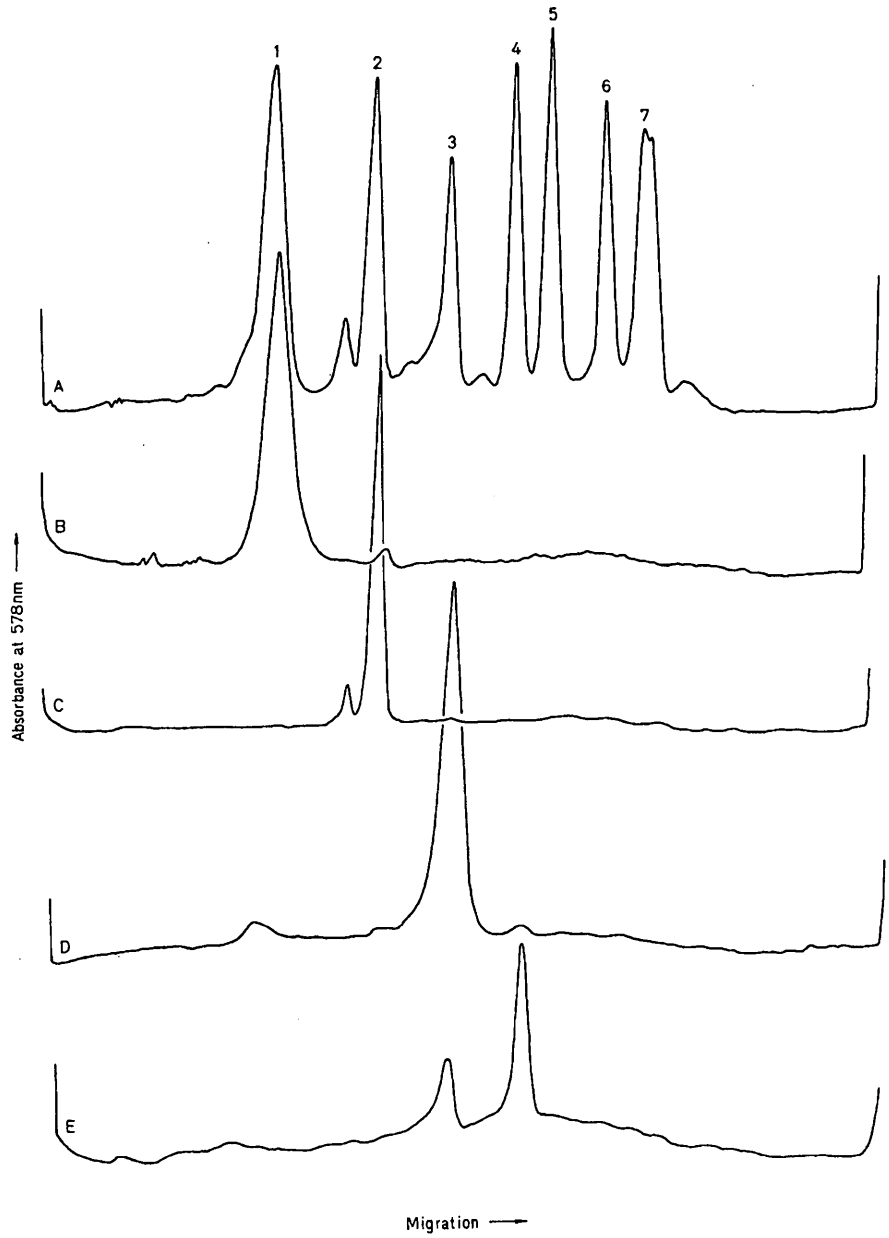

Fig. 8. *Densitometric tracings of (A) cytochrome oxidase and components (B) 1, (C) 2 and (D) 3 after dodecylsulfate-gel electrophoresis and coomassie-brillant-blue staining.* The tracings B, C and E were obtained with components isolated by preparative electrophoresis on a 10% gel (Fig. 7A). Component 3 (D) was obtained by submitting the fraction (E) isolated on a 10% gel to reelectrophoresis on a 15% gel (Fig. 7B)

a slightly higher mobility than the membrane protein, indicating the presence of still other proteins of mitochondrial origin in the membrane.

Amino-Acid Composition of Cytochrome Oxidase and Components 1, 2 and 3

Amino-acid analysis of cytochrome oxidase was performed with two preparations. Their spectral properties are shown in Table 1. The amino-acid composition of the *Neurospora* cytochrome oxidase (Table 4) is quite similar to that of the beef heart enzyme [10]. Major differences are observed in the case of threonine, methionine, isoleucine and lysine. A polarity [14] of 38.5% is calculated for the *Neurospora* enzyme not considering cysteine and tryptophan, which were not determined.

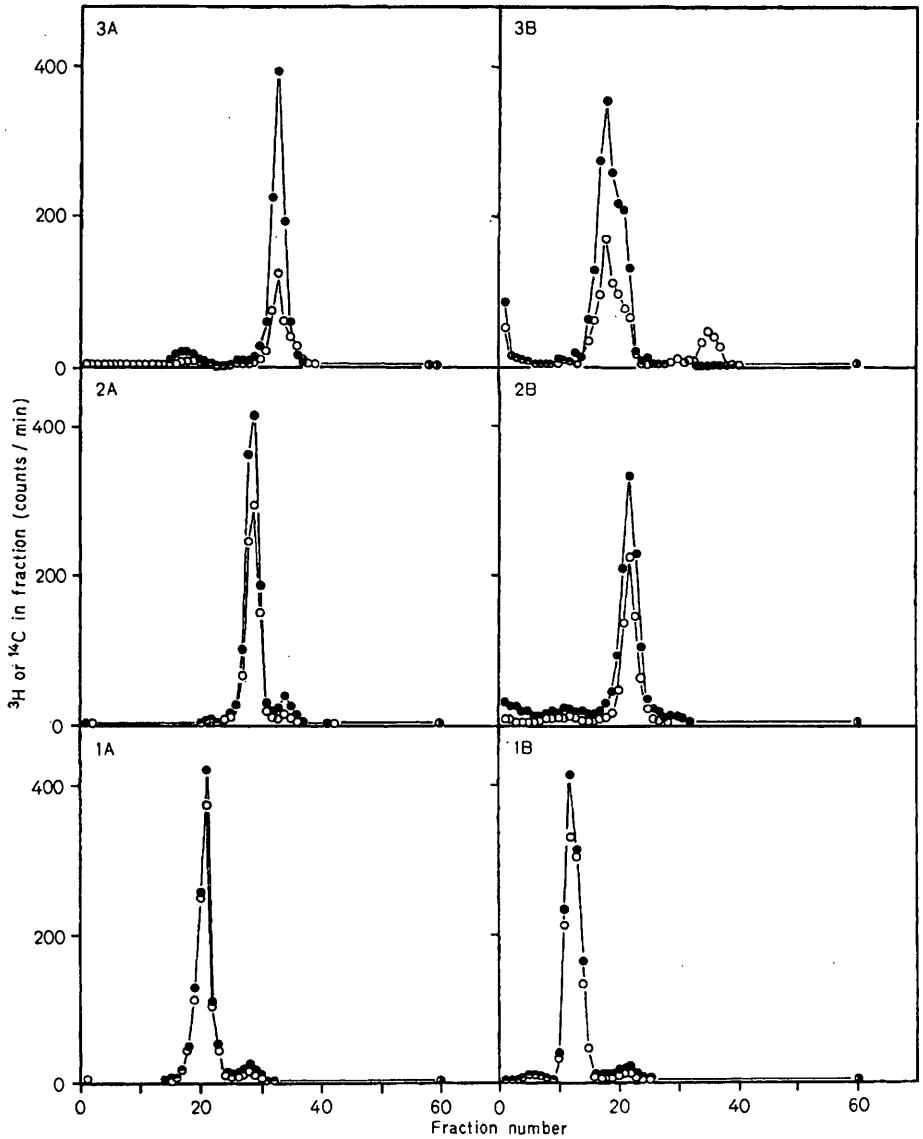

Fig. 9. *Distribution of (○) control label and (●) cycloheximide-resistant label of isolated component 1, 2 and 3 after gel electrophoresis in the presence of (1A, 2A, 3A) dodecylsulfate or (1B, 2B, 3B) phenol-formic acid.* Cycloheximide-resistant labelling was performed after a transitory incubation with chloramphenicol (Table 2). Components 1, 2 and 3 were isolated by preparative electrophoresis on a 10% gel (Fig. 7 A)

The preparations of components 1, 2 and 3 used for amino-acid analysis are shown in Fig. 8 B, C and D.

The amino-acid compositions of the three mitochondrial products exhibit large differences in the contents of certain amino acids. Common to all three proteins is the high content of apolar amino acids and the low content of basic amino acids, especially of lysine. A polarity of 32.5%, 40.1% and 35.8% is calculated for components 1, 2 and 3, respectively.

The average amino-acid composition of components 4—7 (Table 4, last column) can be calculated from the amino-acid contents of whole cytochrome oxidase and of components 1, 2 and 3 on the basis of the contributions of the three mitochondrial products to the total amino acids of the enzyme protein. The contents of the apolar amino-acids leucine, isoleucine, valine and phenylalanine are up to 100% higher in the mitochondrial products. The polar amino-acid serine, however, is also relatively abundant in components 1, 2 and 3. On the other hand the polar amino-acids glutamic acid, lysine and arginine, but also the hydrophobic amino-acids ala-

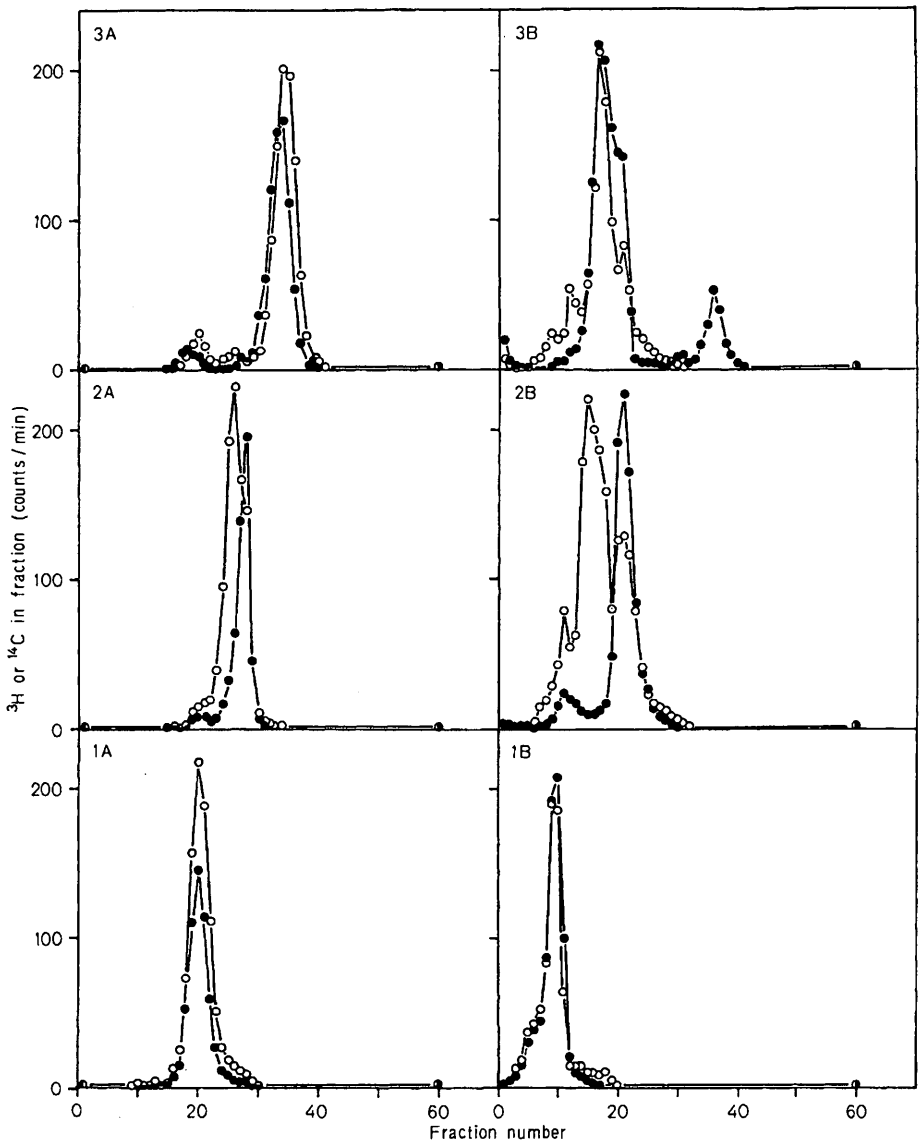

Fig. 10. *Coelectrophoresis of components 1, 2 and 3 isolated from cytochrome oxidase and of mitochondrial translation products separated from the whole mitochondrial membrane proteins.* (●) Components 1, 2 and 3 labelled homogeneously with [^{3}H]leucine (see Methods) and (○) mitochondrial membrane proteins labelled with [^{14}C]leucine in the presence of cycloheximide (Fig. 11) were isolated by preparative electrophoresis on a 10% gel (Fig. 7 A). Components 1, 2 and 3 were mixed with the membrane proteins corresponding to fractions 17—26, 29—38 and 40—48 in Fig. 11, respectively, and then applied to gels containing (1A, 2A, 3A) dodecylsulfate or (1B, 2B, 3B) phenol-formic acid

nine and methionine are found to be higher in components 4—7. In general, cytochrome oxidase seems to be composed of two types of proteins: one part, which is a product of mitochondrial protein synthesis, has a hydrophobic character, in contrast to the other more polar part provided by the extramitochondrial protein synthesis.

Protein Contents of the Components with a Mitochondrial Site of Synthesis

After long-time incorporation of [^{3}H]leucine the specific radioactivity of leucine (counts $\times$ min^{-1} $\times$ nmol^{-1}) is nearly the same in whole cytochrome oxidase and in components 1, 2 and 3 (Table 5). This is to be expected for exponentially growing

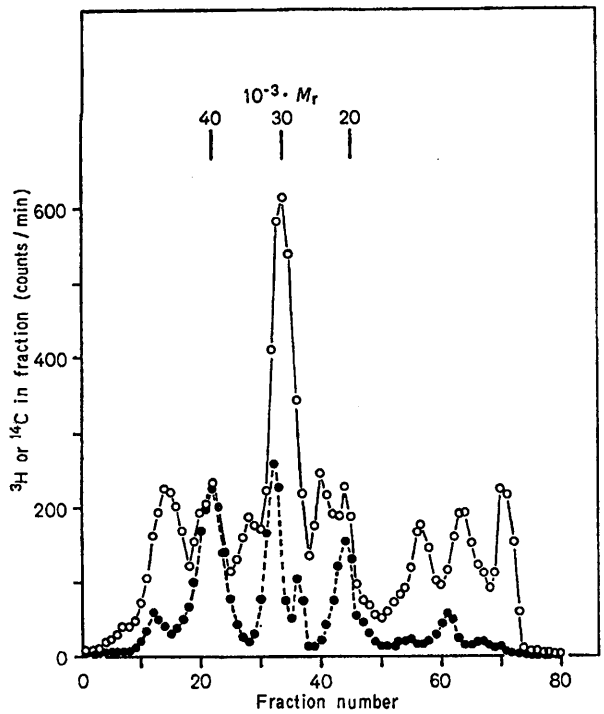

Fig. 11. *Distribution of (○) ^3H control label and (●) cycloheximide-resistant ^{14}C label among electrophoretic fractions of whole-mitochondrial-membrane proteins.* Hyphae grown for 16 h were incubated for 2 h with [^{3}H]leucine (0.1 mCi/l). Then 0.1 mg cycloheximide per ml was added and 2 min later [^{14}C]leucine (0.02 mCi/l). After 1 h the cells were mixed with ice, and the mitochondrial membrane proteins were isolated and then separated by dodecylsulfate-gel electrophoresis

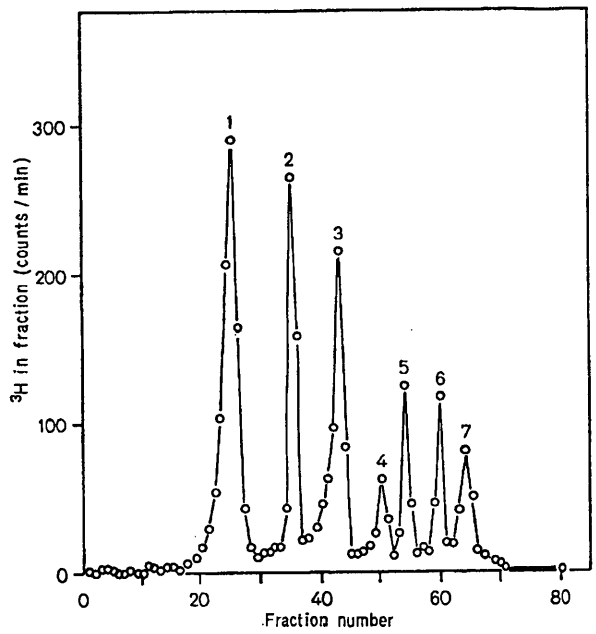

Fig. 12. *Distribution of [^{3}H]leucine label among the protein components of cytochrome oxidase.* Cytochrome oxidase labelled homogeneously with [^{3}H]leucine (see Methods) was separated by gel electrophoresis in the presence of dodecylsulfate

cells, where the rates of synthesis should be proportional to the amounts of the proteins present in the cell [15]. Consequently, the distribution of [^{3}H]-leucine among the components as obtained after dodecylsulfate-gel electrophoresis (Fig. 12), reflects the distribution of leucine among the single components. On the other hand, the leucine content of cytochrome oxidase and of components 1, 2 and 3 is determined by the amino-acid analysis (Table 4). From both experimental data the protein contents of the mitochondrial products were calculated. The components 1, 2 and 3 contain together 60% of the whole cytochrome oxidase protein (Table 5). The amounts of the individual proteins follow nearly the same proportions as the molecular weights determined on dodecylsulfate gels, indicating a 1:1:1 stoichiometry of components 1, 2 and 3. On the basis of these molecular weights it is concluded that the minimal molecular weight of cytochrome oxidase is 150000.

In the case of components 4—7, for which the individual leucine contents are still unknown, the sum of the proteins amounts to 40% of the whole enzyme protein. This corresponds to a molecular weight of 60000 in a cytochrome oxidase with a molecular weight of 150000. Remarkably, the added molecular weights of components 4—7 computed from the dodecylsulfate gels result in nearly the same value.

The distribution of [^{3}H]leucine does not indicate an equimolar relation of these components. From the distribution of the coomassie-blue staining, however, such a relation seems likely (Table 6). The products of extramitochondrial protein synthesis are stained twice as strongly by coomassie blue compared to the other components. Thus, the relative amounts of dye present in each component correspond more closely to the molecular weights determined on dodecylsulfate gels when components 1—3 and 4—7 are treated separately. The amount of dye present in component 7 corresponds to the amount of two proteins, whose existence was already suspected by the appearance of two bands in this fraction (Fig. 3).

DISCUSSION

Synthesis and Processing of Mitochondrial Translation Products in the Cycloheximide-Inhibited Cell

Cycloheximide-resistant incorporation of radioactive amino acids is an experimental approach [16—19] now widely used for the detection of proteins synthesized on mitochondrial ribosomes. In

Table 4. *Amino-acid composition of cytochrome oxidase and of components 1, 2 and 3*

Amino acid	Amount in						
	cytochrome oxidase from			components			
	beef heart[a]	*Neurospora* preparation					
		I	II	1	2	3	4—7[b]
	mol/100 mol						
Aspartic acid	7.36	8.06	7.67	6.45	8.55	7.21	8.32
Threonine	7.92	4.92	5.10	4.80	4.15	6.51	5.24
Serine	7.78	7.80	7.39	10.09	9.36	7.55	4.47
Glutamic acid	7.56	8.09	8.45	4.28	9.80	6.13	11.71
Proline	6.97	7.18	6.63	6.84	7.33	4.69	6.86
Glycine	7.85	8.05	8.56	10.20	6.81	9.56	7.80
Alanine	8.22	8.02	8.20	7.53	4.90	7.46	10.45
Cysteine	1.03	n.d.	n.d.	n.d.	n.d.	n.d.	
Valine	6.75	6.43	6.28	7.14	7.71	6.38	4.97
Methionine	2.05	2.82	2.84	1.27	2.05	1.28	4.91
Isoleucine	5.65	7.34	7.29	8.72	9.16	9.41	4.63
Leucine	11.37	10.66	10.64	13.37	11.68	12.59	7.47
Tyrosine	4.26	4.40	4.45	4.18	4.89	3.65	4.74
Phenylalanine	6.24	6.55	6.59	8.25	5.38	9.15	5.00
Lysine	4.04	3.16	3.26	1.51	2.50	1.24	5.61
Histidine	2.79	3.12	2.97	2.56	2.64	4.62	2.82
Arginine	2.94	3.42	3.67	2.81	3.07	2.56	4.97
Tryptophan	4.04	n.d.	n.d.	n.d.	n.d.	n.d.	
NH_3	9.17	n.d.	n.d.	n.d.	n.d.	n.d.	
Total	105.1	99.99	100.02	100.0	99.98	99.99	99.97

[a] From [10]. The mean values of two preparations were calculated.

[b] Calculated from the amino-acid compositions of components 1, 2, and 3 and of cytochrome oxidase (preparation II) on the basis of the contributions of components 1 (28.6%), 2 (17.9%) and 3 (14.2%) to the total amino acids of cytochrome oxidase (see Table 5).

Table 5. *Contributions of components 1, 2 and 3 to the total protein of cytochrome oxidase*

The individual contributions of components 1—7 to the total [^{3}H]leucine of cytochrome oxidase were evaluated from electrophoretic separations as shown in Fig. 12. The values represent the average of 10 separations of two different preparations. The cytochrome oxidase was labelled homogeneously with [^{3}H]leucine as described under Methods. The specific radioactivities of leucine in cytochrome oxidase and components 1, 2 and 3 were 184, 180, 175 and 189 (counts · min^{-1} · nmol $leucine^{-1}$) respectively, as determined *via* amino acid analysis. The leucine contents (μmol/mg protein) of cytochrome oxidase and components 1, 2 and 3 were found to be 0.97, 1.25, 1.05 and 1.16, respectively, (from Table 4). Tryptophan and cysteine, which had not been determined, were not included in the calculation of these values. The protein contents of the components were calculated as a percentage of total enzyme protein according to the following formula:

$$\%\ \text{protein/component} = \frac{\%\ [^3\text{H}]\text{leucine/component} \times \mu\text{mol leucine/mg of cytochrome oxidase}}{\mu\text{mol leucine/mg of component}}$$

Component	[^{3}H]leucine	Protein	Protein per molecular mass of 150000 g	Molecular weights determined on dodecylsulfate gels
	% of total		g	
1	35.9	27.9	42000	41000
2	19.6	18.0	27000	28500
3	16.9	14.2	21500	21000
4	4.9	39.9	60000	51500[a]
5	8.0			
6	7.1			
7	7.5			

[a] Sum of components 4—7.

Table 6. *Relative amounts of coomassie brillant blue bound by the individual protein components of cytochrome oxidase* The amounts of stain in the individual components were evaluated from densitometric tracings as shown in Fig. 8. They were related to the total amount present in all components as 100%

Component	Absorbance at 578 nm			Molecular weights determined on dodecylsulfate-gels
	cf. total	Calculated per a total of		
		212000	105000	
	%			
1	21.4	45400		41000
2	12.5	26500		28500
3	8.7	18400		21000
4	11.7		12300	16000
5	15.3		16100	14000
6	12.3		12900	11500
7	18.9		19800	10000
7a				9500

the present investigation, however, refinements of this method were necessary to establish a mitochondrial origin of components of cytochrome oxidase. Applying the usual labelling procedure, a reasonable amount of cycloheximide-resistant label is found only in component 3 of the isolated cytochrome oxidase. After washing out the inhibitor and further growth of the cells, however, definite cycloheximide-resistant label appears also in components 1 and 2. This incidates that under the action of the inhibitor precursor proteins are synthesized but not integrated into the functional enzyme.

The different labelling behaviour of the three components is explained by the previous observation [15], that also in the absence of the inhibitor the labelling of components 1, 2 and 3 follows a different time course, due to different pool sizes of the individual precursor proteins. The labelling of the components 1, 2 and 3 observed in the presence of cycloheximide is similar to that observed 5—10 min after a pulse labelling without the inhibitor [15]. Apparently, the formation of cytochrome oxidase proceeds only for a limited time period under the action of cycloheximide. This inhibition of the assembly of a functional cytochrome oxidase is probably caused by the exhaustion of precursor protein(s) of cytosolic origin. The limiting pool could be that of the precursor of component 7, for which a half-life of 7 min has been determined [15]. Concerning previous labelling experiments [2], a similar conclusion was drawn to explain the inhibition of the assembly of cytochrome oxidase by chloramphenicol. There it was suggested that the small pool size of the precursor of component 3 (half-life 3.5 min) limits the formation of the enzyme after inhibition of mitochondrial protein synthesis. On the other hand, cycloheximide-resistant amino acid incorporation proceeds only 15—20 min in exponentially growing cells of *Neurospora crassa* (Fig. 4). Possibly the synthesis of a component with mitochondrial origin ceases before exhaustion of the precursor protein(s) of cytosolic origin and thus, the formation of the functional enzyme is prevented. Interestingly, the influence of cycloheximide on the assembly process was found to be less pronounced in experiments performed with yeast cells during respiratory adaptation [25] or in the early stationary phase [3].

A cycloheximide-resistant amino acid incorporation into components 1 and 2 is also obtained in cells previously incubated for 30 min with chloramphenicol and washed free again from the inhibitor. As already described with yeast [11], cycloheximide-resistant amino acid incorporation proceeds longer and at a higher rate after this treatment. During the chloramphenicol incubation the precursor proteins of cytosolic origin accumulate [2]. It is unclear, if there is any causal connection between the accumulation of the precursor proteins and the stimulation of the cycloheximide-resistant amino acid incorporation. Nevertheless it is tempting to speculate, that a mitochondrial protein synthesis can be only detected if the completed peptide chains are immediately assembled with precursor proteins of cytosolic origin, either to form whole cytochrome oxidase or "pre-cytochrome oxidases". An enlargement of the precursor pools by the chloramphenicol treatment would allow a longer assembly and consequently a prolonged synthesis of the mitochondrial translation products. According to this hypothesis it seems possible, that under the influence of cycloheximide the synthesis of the precursors of components 1, 2 and 3 ceases at different times, because the precursors combine with "pre-cytochrome oxidases" of different pool sizes. Such an uncoupling of mitochondrial protein synthesis is suggested by the relatively high cycloheximide-resistant label of component 1 obtained after washing out the inhibitor and further growth of the cells (Fig. 6).

Stoichiometric Subunit Composition of Cytochrome Oxidase

The results of the present and of the previous [2] labelling experiments strongly suggest that precursor proteins synthesized in the presence of cycloheximide or chloramphenicol do not appear in whole cytochrome oxidase until precursor proteins, whose synthesis was inhibited, are available again. Hence, it may be concluded, that the seven components observed on dodecylsulfate gels are essential building stones of the same structural unit. Further evidence for the conclusion that these components are integral parts of one polypeptide complex is provided by the observation of stoichiometric relationships. In the literature, the amounts of protein present in the individual components have been tentatively esti-

mated from the distribution of coomassie blue [22] or homogeneous [^{3}H]leucine label [21] after electrophoretic separation. With the *Neurospora* enzyme these approaches would lead to erraneous results, because the leucine contents of components 1, 2 and 3 are nearly twice as high as the average content of the other components, whereas the coomassie blue stains the components 4—7 in average twice as strongly as the components 1, 2 and 3. Within each of these groups, leucine contents and binding capacity for the dye seem to be more uniform. The true protein contents of components 1, 2 and 3 are obtained in the present experiments taking into account the individual leucine contents determined by amino acid analysis.

The 1:1:1 stoichiometry calculated for components 1, 2 and 3 as well as the minimal molecular weight of 150000 calculated for the whole cytochrome oxidase are based on the molecular weights determined by dodecylsulfate-gel electrophoresis. The molecular weights of soluble proteins determined by this method are claimed to be accurate with an error of $\pm$ 10% [23], because the binding capacity of such proteins for dodecylsulfate is quite constant [24]. The dodecylsulfate-binding capacities of hydrophobic proteins, such as components 1, 2 and 3, are still unknown. Hence the molecular weights determined for these proteins must be considered tentative. Despite the uncertainties, the molecular weight of 150000 calculated for the whole cytochrome oxidase is in good agreement with the molecular weight of 130000—140000 calculated from the heme aa_3 content. In the literature heme contents of highly purified cytochrome-oxidase preparations are described ranging from 10—15 nmol heme *a* per mg protein [1,4,10,21]. These high discrepancies may not only be due to preparations of different purity, but also due to differences in the methods used for determination of protein and heme concentrations. Further, the possibility has to be considered, that different heme contents occur in cytochrome oxidases from different species. The high heme content determined in the *Neurospora* enzyme described here, is based on a protein determination by amino-acid analysis. This determination of protein seems to be more reliable than the biuret or Lowry method, which gave values lower by respectively 5% and 30%. The heme *a* concentrations were calculated from the difference in absorbance at 605 nm between the oxidized and the reduced enzyme, using an absorption coefficient of 12 mM^{-1} cm^{-1}. This coefficient has been determined with beef heart cytochrome oxidase [9], and the possibility has to be considered that this is not the correct value for the *Neurospora* enzyme.

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