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Part K

*Membrane Biogenesis: Assembly and Targeting
(Prokaryotes, Mitochondria, and Chloroplasts)*

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Table of Contents

CONTRIBUTORS TO VOLUME 97	ix
PREFACE	xiii
VOLUMES IN SERIES.	xv

Section I. Prokaryotic Membranes

A. General Methods

1. Genetic Analysis of Protein Export in <i>Escherichia coli</i>	JONATHAN BECKWITH AND THOMAS J. SILHAVY	3
2. Isolation and Characterization of Mutants of <i>Escherichia coli</i> K12 Affected in Protein Localization	THOMAS J. SILHAVY AND JONATHAN BECKWITH	11
3. Purification and Characterization of Leader Peptidase from <i>Escherichia coli</i>	P. B. WOLFE, C. ZWIZINSKI, AND WILLIAM WICKNER	40
4. Molecular Genetics of <i>Escherichia coli</i> Leader Peptidase	TAKAYASU DATE, PAMELA SILVER, AND WILLIAM WICKNER	46
5. Pulse-Labeling Studies of Membrane Assembly and Protein Secretion in Intact Cells: M13 Coat Protein	WILLIAM WICKNER, TAKAYASU DATE, RICHARD ZIMMERMANN, AND KOREAKI ITO	57
6. Synthesis of Proteins by Membrane-Associated Polysomes and Free Polysomes	PNANG C. TAI, MICHAEL P. CAULFIELD, AND BERNARD D. DAVIS	62
7. Preparation of Free and Membrane-Bound Polysomes from <i>Escherichia coli</i>	LINDA L. RANDALL AND SIMON J. S. HARDY	70
8. Analysis of Cotranslational Proteolytic Processing of Nascent Chains Using Two-Dimensional Gel Electrophoresis	LARS-GÖRAN JOSEFSSON AND LINDA L. RANDALL	77

B. Outer Membrane

9. Proteins Forming Large Channels from Bacterial and Mitochondrial Outer Membranes: Porins and Phage Lambda Receptor Protein	HIROSHI NIKAIDO	85
10. Phage λ Receptor (LamB Protein) in <i>Escherichia coli</i>	MAXIME SCHWARTZ	100
11. Synthesis and Assembly of the Outer Membrane Proteins OmpA and OmpF of <i>Escherichia coli</i>	IAN CROWLESMITH AND KONRAD GAMON	112
12. Isolation of Mutants of the Major Outer Membrane Lipoprotein of <i>Escherichia coli</i> for the Study of Its Assembly	JACK COLEMAN, SUMIKO INOUE, AND MASAYORI INOUE	124

C. Inner Membrane

13. Analysis of M13 Procoat Assembly into Membranes <i>in Vitro</i>	COLIN WATTS, JOEL M. GOODMAN, PAMELA SILVER, AND WILLIAM WICKNER	130
14. Insertion of Proteins into Bacterial Membranes	PETER MODEL AND MARJORIE RUSSEL	138
15. Influence of Membrane Potential on the Insertion and Transport of Proteins in Bacterial Membranes	ROBERT C. LANDICK, CHARLES J. DANIELS, AND DALE L. OXENDER	146
16. Penicillinase Secretion <i>in Vivo</i> and <i>in Vitro</i>	JENNIFER B. K. NIELSEN	153
17. Lactose Permease of <i>Escherichia coli</i>	J. K. WRIGHT, R. M. TEATHER, AND P. OVERATH	158
18. Cloning of the Structural Genes of the <i>Escherichia coli</i> Adenosinetriphosphatase Complex	DAVID A. JANS AND FRANK GIBSON	176
19. Biogenesis of an Oligomeric Membrane Protein Complex: The Proton Translocating ATPase of <i>Escherichia coli</i>	WILLIAM S. A. BRUSILOW, ROBERT P. GUNSALUS, AND ROBERT D. SIMONI	188
20. Analysis of <i>Escherichia coli</i> ATP Synthase Subunits by DNA and Protein Sequencing	JOHN E. WALKER AND NICHOLAS J. GAY	195
21. Biogenesis of Purple Membrane in Halobacteria	DOROTHEA-CH. NEUGEBAUER, HORST-PETER ZINGSHEIM, AND DIETER OESTERHELT	218
22. Isolation of the Bacterioopsin Gene by Colony Hy- bridization	HEIKE VOGELSANG, WOLFGANG OERTEL, AND DIETER OESTERHELT	226

Section II. Mitochondria

23. Assessing Import of Proteins into Mitochondria: An Overview	SUSAN M. GASSER AND RICK HAY	245
24. Molecular Cloning of Middle-Abundant mRNAs from <i>Neurospora crassa</i>	ADELHEID VIEBROCK, ANGELA PERZ, AND WALTER SEBALD	254
25. Biogenesis of Cytochrome <i>c</i> in <i>Neurospora crassa</i>	BERND HENNIG AND WALTER NEUPERT	261
26. Biosynthesis and Assembly of Nuclear-Coded Mitochondrial Membrane Proteins in <i>Neurospora crassa</i>	RICHARD ZIMMERMANN AND WALTER NEUPERT	275

27. Isolation and Properties of the Porin of the Outer Mitochondrial Membrane from <i>Neurospora crassa</i>	HELMUT FREITAG, ROLAND BENZ, AND WALTER NEUPERT	286
28. Synthesis and Assembly of Subunit 6 of the Mitochondrial ATPase in Yeast	SANGKOT MARZUKI AND ANTHONY W. LINNANE	294
29. Preparation and Use of Antibodies against Insoluble Membrane Proteins	MORDECHAI SUISSA AND GRAEME A. REID	305
30. Processing of Mitochondrial Polypeptide Precursors in Yeast	PETER C. BÖHNI AND GÜNTHER DAUM	311
31. Pulse Labeling of Yeast Cells and Spheroplasts	GRAEME A. REID	324
32. Import of Polypeptides into Isolated Yeast Mitochondria	SUSAN M. GASSER	329
33. A Yeast Mitochondrial Chelator-Sensitive Protease That Processes Cytoplasmically Synthesized Protein Precursors: Isolation from Yeast and Assay	PHYLLIS C. MCADA AND MICHAEL G. DOUGLAS	337
34. Selection and Characterization of Nuclear Genes Coding Mitochondrial Proteins: Genetic Complementation of Yeast <i>pet</i> Mutants	KAREN O'MALLEY AND MICHAEL G. DOUGLAS	344
35. Transformation of Nuclear Respiratory Deficient Mutants of Yeast	CAROL L. DIECKMANN AND ALEXANDER TZAGOLOFF	355
36. Analysis of Yeast Mitochondrial Genes	CAROL L. DIECKMANN AND ALEXANDER TZAGOLOFF	361
37. Genetics and Biogenesis of Cytochrome <i>b</i>	PHILIP S. PERLMAN AND HENRY R. MAHLER	374
38. Synthesis and Intracellular Transport of Mitochondrial Matrix Proteins in Rat Liver: Studies <i>in Vivo</i> and <i>in Vitro</i>	GORDON C. SHORE, RICHARD A. RACHUBINSKI, CAROLE ARGAN, RIMA ROZEN, MARCEL POUCHELET, CAROL J. LUSTY, AND YVES RAYMOND	396
39. Biosynthesis of Cytochrome <i>c</i> and Its Posttranslational Transfer into Mitochondria	TAKASHI MORIMOTO, SHIRO MATSUURA, AND MONIQUE ARPIN	408
40. Isolation of Mammalian Mitochondrial DNA and RNA and Cloning of the Mitochondrial Genome	DOUGLAS P. TAPPER, RICHARD A. VAN ETTEN, AND DAVID A. CLAYTON	426
41. Analysis of Human Mitochondrial RNA	GIUSEPPE ATTARDI AND JULIO MONTOYA	435
42. Isolation of a Hexokinase Binding Protein from the Outer Mitochondrial Membrane	JOHN E. WILSON, JANICE L. MESSER, AND PHILIP L. FELGNER	469

-
- | | | |
|--|---|-----|
| 43. Protein Synthesis by Isolated Plant Mitochondria | C. J. LEAVER, E. HACK,
AND B. G. FORDE | 476 |
|--|---|-----|

Section III. Chloroplasts

- | | | |
|---|---|-----|
| 44. Synthesis and Assembly of Thylakoid Membrane Proteins in Isolated Pea Chloroplasts | RICHARD S. WILLIAMS AND
JOHN BENNETT | 487 |
| 45. <i>In Vitro</i> Reconstitution of Synthesis, Uptake, and Assembly of Cytoplasmically Synthesized Chloroplast Proteins | JOHN E. MULLET AND
NAM-HAI CHUA | 502 |
| 46. Structure and Synthesis of Chloroplast ATPase | NATHAN NELSON | 510 |
| 47. Cloning in Physical Mapping of Maize Plastid Genes | LAWRENCE BOGORAD,
EARL J. GUBBINS,
ENNO KREBBERS,
IGNACIO M. LARRINUA,
BERNARD J. MULLIGAN,
KAREN M. T.
MUSKAVITCH,
ELIZABETH A. ORR,
STEVEN R. RODERMEL,
RUDI SCHANTZ,
ANDRE A. STEINMETZ,
GUIDO DE VOS, AND
YUKUN K. YE | 524 |
| 48. Role of Thylakoid Polypeptide Phosphorylation and Turnover in the Assembly and Function of Photosystem II | M. WETTERN, J. C. OWENS,
AND I. OHAD | 554 |

Section IV. Summary of Membrane Proteins

- | | | |
|--|---|-----|
| 49. Membrane Proteins: A Summary of Known Structural Information | DAVID R. NELSON AND
NEAL C. ROBINSON | 571 |
|--|---|-----|

Addendum

- | | | |
|--------------------------|---|-----|
| Addendum to Article [39] | TAKASHI MORIMOTO,
SHIRO MATSUURA, AND
MONIQUE ARPIN | 621 |
| AUTHOR INDEX | | 625 |
| SUBJECT INDEX | | 661 |

[25] Biogenesis of Cytochrome *c* in *Neurospora crassa*

By BERND HENNIG and WALTER NEUPERT

Cytochrome *c* is an exceptionally well-studied protein with respect to its primary and tertiary structure, function in the mitochondrial respiratory chain, topological arrangement,¹ and molecular genetics and evolution.² This protein is therefore particularly suitable for investigating the molecular mechanism of mitochondrial assembly. Several steps of the biogenesis of cytochrome *c* have already been elucidated.³ Its structural gene(s) are contained in nuclear DNA and transcribed into poly(A)-mRNA. The mRNA is translated on free cytoplasmic ribosomes. The primary translation product is an extramitochondrial precursor which, unlike most other mitochondrial precursor proteins, does not carry an N-terminal or C-terminal transient sequence. Apparently it is identical to apocytochrome *c*, which can be prepared from holocytochrome *c* by chemical means. Apocytochrome *c* binds to specific sites on the surface of mitochondria. During the transfer into mitochondria, the heme group becomes covalently linked to the apoprotein via two thioether bonds. The mature protein, holocytochrome *c*, binds to the inner membrane and can reversibly dissociate into the intermembrane space. Antibodies can be obtained against apo- and holocytochrome *c* that do not cross-react, presumably because apocytochrome *c* differs strongly from holocytochrome *c* in its conformation. These antibodies allow one to trace precursor and mature protein during the assembly process.

A further important aspect is that practically unlimited amounts of apocytochrome *c* can be chemically prepared, in contrast to the minute amounts of precursors, which can be obtained by synthesis in cell-free systems. This is a great advantage in investigating (*a*) the specificity, affinity, and identity of the putative receptor sites on the mitochondrial surface; (*b*) the mechanism of translocation of apocytochrome *c* across the membrane; and (*c*) the conversion to holocytochrome *c* by the putative enzyme, cytochrome *c* heme lyase.

¹ R. Timkovich, in "The Porphyrins" (D. Dolphin, ed.), p. 241. Academic Press, New York, 1979.

² E. Margoliash, *Proc. Int. Conf. Theor. Phys. Biol.* 3rd, 1971 p. 175 (1973).

³ B. Hennig and W. Neupert, *Horiz. Biochem. Biophys.* 7 (in press).

Isolation and Purification of Cytochrome *c* from *Neurospora crassa*

Neurospora cells are grown in liquid culture according to established procedures.⁴ Vogel's minimal medium supplemented with 2% sucrose is used. A maximal yield of 0.4 mg of cytochrome *c* per gram of protein of crude cell extract is obtained when wild-type (No. 262, Fungal stock center, Arcata, California) is harvested at mid log phase (Fig. 1). This stage (optical density (OD) at 410 nm = 1.2) is reached 14–16 hr after inoculating the culture with 1×10^9 conidia per liter (OD at 410 nm = 0.1) and growth under vigorous aeration at 25°. Longer growth leads to a decline of the cytochrome *c* content in cells to about half the value obtained at mid log phase. Cells are harvested by suction filtration on cellulose filter paper in a

⁴ R. H. Davis and F. J. Serres, this series, Vol. 17A, p. 79.

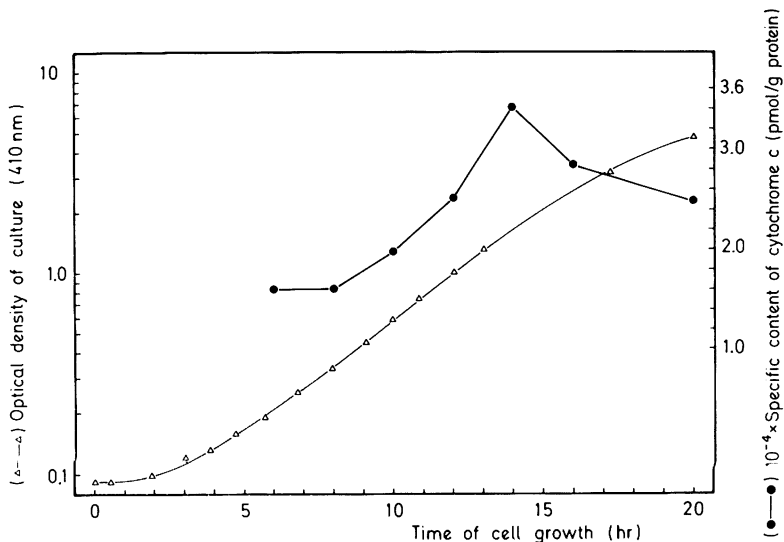


FIG. 1. Specific cytochrome *c* content during growth of *Neurospora* cells. Eight bottles containing 1 liter of Vogel's minimal medium supplemented with 2% sucrose were inoculated with 10^9 conidia and aerated at 25°. Samples were taken from one culture at the indicated times, and growth was determined by measuring the optical density of the culture at 410 nm. The other cultures were harvested at the indicated times, and the cytochrome *c* content of cells was determined in the following way. Cells were suspended in 150 mM KP_i and disrupted with a homogenizer and by sonication as described in the text. The homogenate was centrifuged in a SS-34 rotor for 20 min at 20,000 rpm. In the supernatant, total protein of the crude cell extract was determined. After high-spin centrifugation in a Ti-50 rotor for 60 min at 50,000 rpm, cytochrome *c* was determined in the resulting supernatant by difference spectroscopy of the dithionite-reduced vs ferricyanide-oxidized samples ($E_{550\text{ nm}-533\text{ nm}} = 21.6 \times 10^3 \text{ cm}^2/\text{mmol}$).

Büchner funnel and washed with distilled water. They can then be kept frozen at -20° for up to several months.

Two different procedures are recommended for isolation of cytochrome *c*. Procedure A is preferred for small amounts of cells (less than 100 g wet weight); procedure B should be employed with large amounts of cells (1–2 kg).

Procedure A. This procedure allows nearly quantitative recovery of cytochrome *c* and has been successfully used to purify amounts of less than 1 mg. It is a modified version of the microscale preparation described by Hennig.⁵

Cells are thawed in 2 ml of 0.15 M KP_i , pH 7.2, per gram of cell wet weight. Then 5 μ l of phenylmethylsulfonyl fluoride (PMSF) (from a 200 mM stock solution in absolute ethanol) are added per milliliter of homogenate, and cells are homogenized with an Ultra-Turrax (Janke and Kunkel, Staufen, Federal Republic of Germany) for 1–5 min at 0° . After addition of 1 μ l of 2-mercaptoethanol per milliliter, the homogenate is sonicated at maximum power in an ice bath. Temperature is kept below 10° by frequent interruptions of sonication. Sonication is continued until cell breakage is complete as judged by phase contrast microscope. Usually, complete breakage requires about 10 min of total sonication time for a 50-ml portion. The crude extract is prepared by centrifugation in a Sorvall SS-34 rotor at 18,000 rpm for 20 min. The supernatant is mixed with 0.05 g of wet BioRex 70 (200–400 mesh, Bio-Rad, Richmond, California) per milliliter preequilibrated with 15 mM KP_i , pH 7.0. The suspension is diluted with constant stirring by addition of 9 ml of cold distilled water per milliliter. Adsorption of cytochrome *c* to the resin is allowed to proceed under constant stirring at 4° for 60 min. The resin is separated from the homogenate by filtration on cellulose filter paper, and resuspended in 0.7 ml of 10 mM KP_i , pH 7.0, per gram of wet resin. The filtrate is incubated for a second time with newly added resin and treated as described before.

The combined slurry of BioRex is transferred into a column of appropriate size (for isolation of cytochrome *c* from 50 g of cells, a column size of 0.9×30 cm is required). The packed column is thoroughly washed with 10 mM KP_i , pH 7.0, at a flow rate of 100 ml/hr per square centimeter (at least 10 column volumes are run through). Cytochrome *c* is eluted from the column with 0.5 M NaCl, 10 mM KP_i , pH 7.0, and the fractions absorbing at 410 nm are collected.

The pooled fractions (about 5 ml) are applied onto a column of Sephadex G-75 (1.6×86 cm) and eluted with 0.2 M NH_4HCO_3 . The eluate is monitored for absorption at both 280 nm and 410 nm. The cytochrome

⁵ B. Hennig, *Eur. J. Biochem.* **55**, 167 (1975).

c-containing fractions separate well from a large 280 nm peak. They are collected and adjusted to pH 6.5 by addition of 5 M HCl.

Then the solution is pumped into a short precolumn (1.6 × 1 cm) of CM-32-cellulose (Whatman, Maidstone, England) equilibrated with 5 mM KP_i , pH 6.5. Stable binding of cytochrome *c* to the CM-cellulose requires 10-fold dilution of the applied sample. To avoid loss of cytochrome *c* due to adsorption on the glassware, this dilution should occur concomitantly with application of the sample to the column by means of a three-way valve. The precolumn is then washed with at least 100 ml of 5 mM KP_i , pH 6.5. Cytochrome *c* is oxidized by an additional wash with 5 ml of 1 mM $K_3Fe(CN)_6$, 20 mM KP_i , pH 6.5, followed by washing with 10 ml of 20 mM KP_i , pH 6.5. The precolumn is connected to the fractionation column (0.9 × 46 cm) of CM-32-cellulose preequilibrated with 20 mM KP_i , pH 6.5. The interconnected columns are eluted with a linear gradient of 0.02 M to 0.3 M KP_i , pH 6.5, at 28 ml/hr per square centimeter. The slope of the gradient is about 0.5 mM KP_i per hour per cubic centimeter of column volume.

Cytochrome *c*-containing fractions are detected by their absorption at 410 nm. They are pooled, lyophilized, dissolved with distilled water to a concentration of 10 mg of cytochrome *c* per milliliter, and dialyzed in Visking cellulose tubing 18/32 (Union Carbide Corporation, Chicago, Illinois) against the desired final buffer (we use either 100 mM NaP_i , pH 7.0, or 0.25 M NH_4HCO_3). Cytochrome *c* thus obtained is pure as judged by its absorption spectrum (A_{410}/A_{280} of the oxidized cytochrome *c* = 4.7), by electrophoresis on SDS-polyacrylamide gels, and by amino acid analysis.

Procedure B. This procedure allows isolation of 20–100 mg of cytochrome *c* by a modified version of a previously described method,⁶ but it is not designed for quantitative recovery of cytochrome *c*. Cells (1 kg, wet weight) are suspended in 3 liters of distilled water and homogenized with a Waring blender (Dynamics Corp., New Hartford, Connecticut) at 4°. The resulting slurry is passed three times through a grind mill⁷ to break the cells. The homogenate is adjusted to pH 9.8 with 25% NH_4OH and stirred for 1 at 4°. It is then centrifuged in a Sorvall GS-3 rotor for 10 min at 8000 rpm. The resulting pellet is discarded. The supernatant is adjusted to pH 7.4 by addition of glacial acetic acid. The mixture is centrifuged as above for 50 min, and the pellet is discarded. The supernatant is diluted with water to 35 liters, and 0.875 g of NaN_3 and 1.75 g of $K_3Fe(CN)_6$ are added and dissolved by stirring. Then 52.5 g of wet BioRex 70 (Bio-Rad, Richmond, California) is added while stirring with a motor-driven rotor blade, and stirring is continued for 12 hr at 4°. The resin is preequilibrated with 20 mM NH_4P_i , pH 7.0.

⁶ J. Heller and E. L. Smith, *J. Biol. Chem.* **241**, 3158 (1966).

⁷ H. Weiss, G. von Jagow, M. Klingenberg, and T. Bücher, *Eur. J. Biochem.* **14**, 75 (1970).

The resin with adsorbed cytochrome *c* is collected by filtration on cellulose filter paper in a Büchner funnel. The filtrate is supplemented with an additional 1.75 g of $K_3Fe(CN)_6$ and 45 g of preequilibrated BioRex 70, and stirring is continued for 4 hr. Again, the resin is collected by filtration, and the two batches of resin are combined. The resin is washed twice with distilled water, once with 50 mM NH_4P_i , pH 7.4, and is collected by filtration after each wash. Then a column (2.6×40 cm) is filled to a height of 5 cm with BioRex 70 preequilibrated with 20 mM NH_4P_i , pH 7.4, and the sample of BioRex containing the bound cytochrome *c* is layered on top; 100 ml of 50 mM NH_4P_i , pH 7.4, are passed through the column, followed by 60 ml of 0.1 M NH_4P_i , pH 7.4. Upon this treatment, the red band of cytochrome *c* starts to migrate down the column. It is eluted with 0.5 M NH_4P_i , pH 7.4, and is recovered in about 10–15 ml of the eluate.

A column of CM 32-cellulose (1.6×5 cm) is equilibrated with 20 mM KP_i , pH 6.5. The sample of cytochrome *c* is diluted with distilled water by 1:25 and is pumped over the column. Cytochrome *c* is retained. Then 5 ml of 1 mM $K_3Fe(CN)_6$ are passed through the column, followed by 20 mM KP_i , pH 6.5, for 12 hr at a rate of 20 ml/hr. A linear gradient of 20 mM to 300 mM KP_i , pH 6.5, is applied, and the eluate is directly passed over a second column of CM-32-cellulose (2.6×20 cm) equilibrated with 20 mM KP_i , pH 6.5. The gradient has a total volume of 500 ml and is run for 16 hr. Fractions of 5 ml are collected.

Cytochrome *c* is detected by its red color and is recovered in about five fractions. These are combined, diluted with distilled water by 1:25 and pumped onto a BioRex 70 column (1.5×1.5 cm) equilibrated with 20 mM KP_i , pH 7.0. Cytochrome *c* binds to the column. It is washed first with 5 ml of 1 mM $K_3Fe(CN)_6$, 10 mM KP_i , pH 7.0, and then with 100 ml of 10 mM KP_i , pH 7.0 (flow rate 10 ml/hr). A gradient of 50 mM KP_i to 300 mM NaCl, 50 mM KP_i , pH 7.0 (500 ml total volume) is applied. The eluate is lead directly to a second column of BioRex 70 (2.6×20 cm) equilibrated with 50 mM KP_i , pH 7.0. The cytochrome *c*-containing fractions are combined and lyophilized. The dry material is dissolved with 5 ml distilled water and dialyzed in Visking cellulose tubing 18/32 against 0.1 M NaP_i, pH 7.0. The total yield is 30–40 mg of cytochrome *c*. It is pure according to its absorption spectrum and gel electrophoresis.

Comments. Cytochrome *c* strongly adsorbs to glass walls, dialysis tubing, gel matrix, etc., at low ionic strength. For quantitative recovery, cytochrome *c* should be kept in buffers of sufficiently high ionic strength (at least $I = 0.1$); 0.25 M NH_4HCO_3 is recommended as a volatile solvent. The methylated and unmethylated forms of cytochrome *c*⁸ can be separated by chromatography on BioRex 70 or Amberlite CG-50 using the gradient described for the resin in procedure B. Visking cellulose tubing, 18/32, was found to be

⁸ W. A. Scott and H. K. Mitchell, *Biochemistry* **8**, 4282 (1969).

particularly suitable for dialysis of cytochrome *c*, since it is completely impermeable for this small protein. The tubing is presoaked in a solution containing 50% (v/v) ethanol, 10 mM EDTA for several hours and washed with distilled water.

Preparation of Apocytochrome *c*

The recommended procedure is a modified version of that described for bacterial cytochromes *c*.⁹ After dialysis against 0.25 M NH₄HCO₃, holocytochrome *c* is lyophilized. A solution of 8 M urea and 0.1 M NaCl is prepared and adjusted to pH 2.0 by addition of 5 M HCl; 10 mg of cytochrome *c* and 50 mg of HgCl₂ are dissolved each in 1 ml of this solution. They are mixed and incubated at room temperature for 16 hr in the dark. The mixture is applied on a column of Sephadex G-25 (0.9 × 84 cm) and eluted with 0.25 M NH₄OAc, pH 6.0. Fractions containing the protein are collected, combined, and lyophilized. The dry sample is dissolved in 1 ml of 8 M urea, 0.25 M NH₄HCO₃; 1 mg of solid dithiothreitol is added, and incubation is performed for 30 min at 37° in order to remove protein-bound Hg. The sample is applied on a column of Sephadex G-25 (0.9 × 84) and eluted with 0.25 M NH₄HCO₃. The fractions containing the protein are collected and lyophilized. To renature the protein, it is dissolved in 1 ml of 8 M urea, 0.25 M NH₄HCO₃, 1 mM dithiothreitol and dialyzed in Visking cellulose tubing, 18/32, at 4° against 0.25 M NH₄HCO₃, 0.01% 2-mercaptoethanol for 16 hr. The exact quantity of apocytochrome *c* can be determined by amino acid analysis. The measured value of arginine is a reliable basis for rapid calculation. The protein contains 3 mol of arginine per mole of protein.¹⁰ The yield is higher than 90%. The protein has no absorption at 410 nm, reflecting complete absence of heme.

For the preparation of small amounts of apocytochrome *c*, the following method is more convenient: 100 μg of cytochrome *c* are treated with HgCl₂ as described above, but in a total volume of 20 μl of 8 M urea, 0.1 M NaCl, pH 2.0. After incubation for 16 hr, the mixture is transferred into a 50-μl microdialysis chamber (Schütt, Göttingen, Federal Republic of Germany) and dialyzed for 16 hr against 0.25 M NH₄OAc, pH 6.0, at 4°. Then dialysis is continued against a freshly prepared solution containing 8 M urea, 0.25 M NH₄HCO₃, 1 mM dithiothreitol for 30 min at 37°. Finally, dialysis is carried out against 0.25 M NH₄HCO₃, 0.01% 2-mercaptoethanol for 16 hr at 4°. The yield is about 95%.

Comment. Apocytochrome *c* is easily denatured. This is in contrast to the stability of holocytochrome *c*. Solutions of apocytochrome *c* should be

⁹ R. P. Ambler and M. Wynn, *Biochem. J.* **131**, 485 (1973).

¹⁰ J. Heller and E. L. Smith, *J. Biol. Chem.* **241**, 3165 (1966).

stored in aliquots at -20° . They should be thawed immediately before use. Repeated thawing and freezing must be avoided, since this denatures the protein. Denatured protein can be renatured by dialysis first against 8 *M* urea, 0.25 *M* NH_4HCO_3 , 1 *mM* dithiothreitol and then against urea-free NH_4HCO_3 containing 0.01% 2-mercaptoethanol.

Preparation of Antibodies against Holocytochrome *c* and Apocytochrome *c* and Immunoprecipitation

Holocytochrome *c* or apocytochrome *c* is dialyzed against 0.1 *M* NaP_i , pH 7.0, and adjusted to a concentration of 0.6 mg of protein per milliliter; 2 μl of 25% glutaraldehyde are added per milliliter, and the mixture is kept for 1 hr at room temperature.¹¹ Then 18 mg of solid lysine hydrochloride are added per milliliter, and incubation is continued for 1 hr. The mixture is dialyzed in Visking 18/32 tubing against 0.1 *M* NaP_i , pH 7.0, for 16 hr at 4° . Aliquots containing 0.2 mg of protein in 250 μl of 0.1 *M* NaP_i , pH 7.0, are stored at -20° . Rabbits are injected first with a mixture of 250 μl of cytochrome *c* solution and 250 μl of Freund's complete adjuvant (Behringwerke, Marburg, Federal Republic of Germany) into the neck region at four positions. Injections are repeated 4 times at weekly intervals with 250 μl of cytochrome *c* solution and 250 μl of Freund's incomplete adjuvants. After 6 weeks, 40–60 ml blood are drawn from the ear vein. The rabbits can be boosted after 3–4 weeks by two injections with the same amount of protein mixed with incomplete Freund's adjuvant. Blood is collected after a further week. The rabbit can be boosted repeatedly. Serum is collected from the blood samples after clotting for 1 hr at room temperature. It is centrifuged at 10,000 rpm in a Sorvall SS-34 rotor for 30 min. Aliquots of 1 ml are kept frozen at -20° . Higher titers can be obtained when the protein is coupled to keyhole limpet hemocyanin (Calbiochem, Lahn-Giessen, Federal Republic of Germany). One milligram of cytochrome *c* and 10 mg of hemocyanin in 1 ml of 0.1 *M* NaP_i , pH 7.0, are polymerized with glutaraldehyde as described above; 0.1 mg of cytochrome *c* is applied per injection. Immune response and cross-reactivity are checked by double immunodiffusion, according to Ouchterlony.¹² The titers of the sera are determined by direct immunoprecipitation from ^{35}S -labeled mitochondria for holocytochrome *c*, and by immunoabsorption employing Sepharose-bound protein A (Pharmacia, Uppsala, Sweden) from [^3H]leucine-labeled cell-free translation mixtures (see below) for apocytochrome *c*.

Specific antibodies directed against holocytochrome *c* or apocytochrome *c* are isolated by affinity chromatography on Sepharose containing the

¹¹ M. Reichlin, A. Nisonoff, and E. Margoliash, *J. Biol. Chem.* **245**, 947 (1970).

¹² O. Ouchterlony, *Acta Pathol. Microbiol. Scand.* **32**, 231 (1953).

covalently bound antigens. The affinity matrix is prepared by coupling 1 mg of pure protein to 0.2 g (dry weight) of preswollen CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). The coupling reaction is carried out following the instructions of the manufacturer. The affinity matrix is transferred into a column (0.6×5 cm) and washed with 50 mM Tris-HCl, pH 7.5 (adjusted at 20°), 0.3 M KCl. Thirty milliliters of serum are thawed, subjected to a clarifying spin (20 min at 10,000 rpm, Sorvall SS-34 rotor), and pumped over the column with a flow rate of 10 ml/hr at 4°. Then the column is washed with 20 ml of the same buffer used for prewashing. Elution is performed with 0.2 M acetic acid, 0.5 M KCl (pH 2.8), and the eluate (3 ml) is adjusted immediately to pH 7 with 6 M KOH. Aliquots of 100 μ l are kept frozen at -20°.

Immunoprecipitation of holocytochrome *c* from mitochondria is carried out by dissolving mitochondria (1 mg of mitochondrial protein) in 1 ml of "lysis buffer" (1% Triton X-100, 0.3 M KCl, 10 mM Tris-HCl, pH 7.5, adjusted at 20°) and adding about 0.25 ml of serum or 20 μ l of specific antibodies (according to the measured titer). The mixture is incubated in Eppendorf tubes for 15 hr at 4°. The immunoprecipitate is collected by centrifugation in a Sorvall SS-34 rotor using plastic adaptors for 10 min at 10,000 rpm. The pellet is washed twice with 1.5 ml of lysis buffer and three times with 0.01 M Tris-HCl, pH 7.5 (adjusted at 20°). Then the pellet is dissolved in 40 μ l of 2% SDS, 10 mM Tris-HCl, pH 7.5, and 0.35 M 2-mercaptoethanol and heated to 95° for 5 min.

For immunoprecipitation of apocytochrome *c* from mitochondria, pellets are lysed with "lysis buffer." For immunoprecipitation from postribosomal supernatant fraction, 110 μ l of 3 M KCl, 10 mM Tris-HCl, pH 7.5, and 60 μ l of 20% Triton X-100 are added to 1-ml samples. Then about 75 μ l of antiserum or 10 μ l of specific antibodies are added (according to the measured titers), and the samples are incubated for 20 min at 4°. Ten milligrams of protein A-Sepharose (Pharmacia, Uppsala, Sweden) or protein A-Agarose (Sigma Chem. Corp., St. Louis, Missouri) are swollen for 15 min in 100 μ l of 10 mM Tris-HCl, pH 7.5, and then added to the samples. They are shaken for 20 min at 4°. The beads are collected by centrifugation in an Eppendorf centrifuge and washed as described above for the direct immunoprecipitation of holocytochrome *c*. Finally, the beads are suspended in 50 μ l of "dissociation buffer" (2% SDS, 10 mM Tris-HCl, pH 7.5, 0.35 M 2-mercaptoethanol). The suspension is heated for 5 min at 95°; after centrifugation for 3 min in an Eppendorf centrifuge, the supernatant is collected.

The dissolved immunoprecipitates are subjected to SDS-gel electrophoresis either on horizontal slab gels¹³ or on vertical slab gels.¹⁴

¹³ H. Korb and W. Neupert, *Eur. J. Biochem.* **91**, 609 (1978).

¹⁴ U. K. Laemmli, *Nature (London)* **227**, 680 (1970).

Comments. The antibodies against holocytochrome *c* and apocytochrome *c* prepared by these procedures did not cross-react either on Ouchterlony plates or upon direct or indirect immunoprecipitation (Fig. 2). Heating of apocytochrome *c* before immunoprecipitation, however, should be avoided, since under this condition cross-reaction with holocytochrome *c* antibodies may occur. Small amounts of unlabeled cytochrome *c* can be analyzed by blotting the gel to nitrocellulose membranes and subsequent treatment with the specific antibodies and radioactive protein A.¹⁵

Synthesis of Apocytochrome *c* in Cell-Free Systems

Synthesis of apocytochrome *c* can be performed *in vitro* with similar efficiency either in a homologous cell-free translation system prepared from *Neurospora* or in the heterologous rabbit reticulocyte lysate system. The homologous system uses endogenous mRNA; the heterologous system requires isolation of *Neurospora* mRNA.

Homologous System. *Neurospora* cells (WT 74 A) are grown to mid log phase. Cells labeled *in vivo* with [³⁵S]sulfate are grown in 1 liter of Vogel's medium with unlabeled sulfate reduced to 0.08 mM in the presence of 250 μCi of [³⁵S]sulfate. Growth is stopped by pouring a 1-liter culture into 1 liter of ice-cold wash buffer (100 g crushed ice suspended in 10 mM MgCl₂, 30 mM sucrose, pH 7.4, adjusted with triethylamine). Cells are collected by suction on cellulose filter paper, resuspended in 500 ml of ice-cold wash buffer, and filtered again. Four grams of the resulting cell pad are mixed in a sterile mortar with 6 g of washed quartz sand (Riedel de Haen, Hannover), which has been dried and sterilized at 200°. The mixture is ground for 5 min at 4° with four successive additions of 2 ml of medium A. Medium A contains 0.3 M sucrose, 30 mM KCl, 60 mM triethanolamine-HCl, 30 mM triethanolamine, 2 mM EDTA, 5 mM MgCl₂, 20 mM KH₂PO₄, 0.25 mM each of the L-amino acids except the labeled one, 0.007% 2-mercaptoethanol, adjusted to pH 7.4, with 5 M KOH. It can be stored at -20°.

The homogenate is centrifuged twice in a Sorvall SS-34 rotor for 5 min at 4000 rpm. The pellets are discarded, and the supernatant is centrifuged in an SS-34 rotor for 12 min at 12,000 rpm to sediment the mitochondria. The supernatant is centrifuged once more in the SS-34 rotor for 12 min at 12,000 rpm to obtain the postmitochondrial supernatant. This is used for cell-free translation, and the following additions are made: 4 mM ATP (20 μl of 200 mM stock solution per milliliter), 1 mM GTP (20 μl of 50 mM stock solution per milliliter), 8 mM creatine phosphate (3.6 mg/ml), 50 μg of creatine kinase per milliliter (10 μl of 0.5% stock solution in 50% glycerol per milliliter). Usually labeling is carried out with [³H]leucine. Then

¹⁵ W. N. Burnette, *Anal. Biochem.* **112**, 195 (1981).

A

6

5

1

4

2

3

B

6

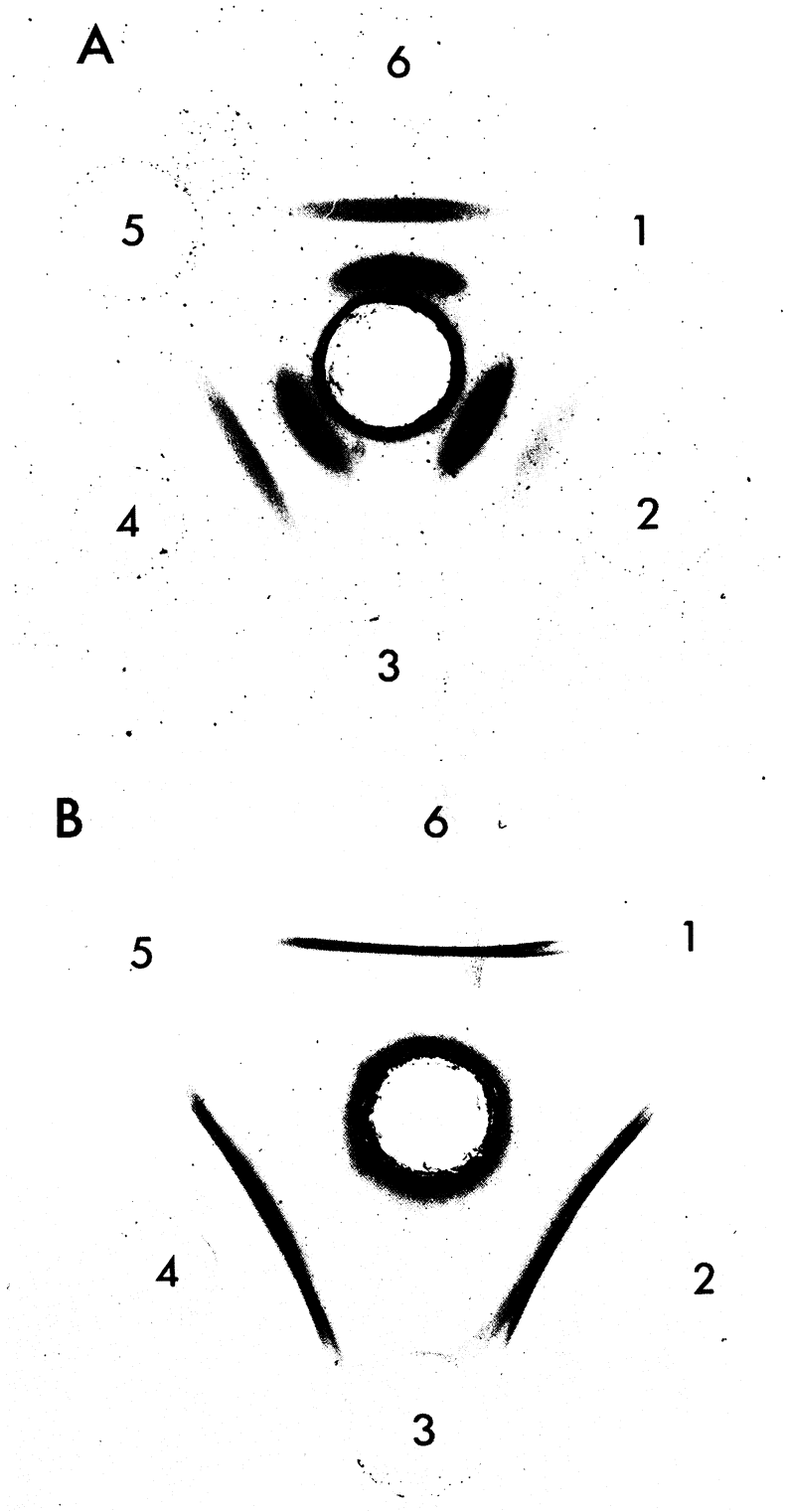
5

1

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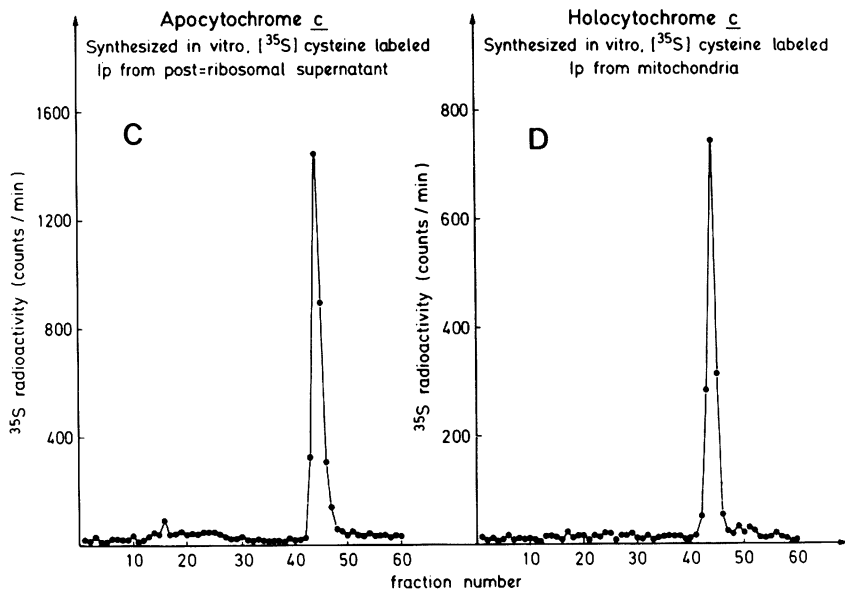


FIG. 2. Specificity of immunoprecipitation by antibodies directed against apocytochrome *c* and holocytochrome *c*. (A) and (B) Immunodiffusion according to Ouchterlony. Center well in (A): antiserum against apocytochrome *c* (20 μ l); outer wells: 2, 4, 6, apocytochrome *c* (0.5 μ g), and 1, 3, 4, holocytochrome *c* (0.5, 1.2 μ g, respectively). Center well in (B): antiserum against holocytochrome *c* (20 μ l); outer well: 2, 4, 6, holocytochrome *c* (0.5 μ g), and 1, 3, 5, apocytochrome *c* (0.5, 1, 5 μ g, respectively). (C) and (D) SDS-PAGE analysis of radiolabeled immunoprecipitates. (C) ³⁵S-labeled apocytochrome *c* was synthesized *in vitro* and immunoprecipitated with antibodies directed against apocytochrome *c*. (D) ³⁵S-labeled holocytochrome *c* labeled *in vitro* immunoprecipitated with antibodies directed against holocytochrome *c*.

0.5 mM [³H]leucine (specific radioactivity 50 Ci/mmol, New England Nuclear Corp. (NEN), Boston, Massachusetts) in 0.5 ml of 2 mM KCl (adjusted to pH 7.4) is added per milliliter of incubation mixture. When radioactive cysteine is to be incorporated, 0.5 mCi of [³⁵S]cysteine (1000 Ci/mmol, NEN, Boston, Massachusetts) in 0.5 ml of 2 mM KCl is added per milliliter of incubation mixture. Incorporation of the radioactive amino acids is performed for 10 min at 25°. Cycloheximide (0.1 mg/ml) is added to the incubation mixture after 10 min, and the sample is cooled to 0°. Centrifugation in Eppendorf tubes is carried out in a Beckman 50 Ti rotor using plastic adaptors for 60 min at 50,000 rpm. The postribosomal supernatant contains the labeled apocytochrome *c*. The average incorporation into total protein is 20 to 30 $\times 10^6$ dpm per milliliter of incubation mixture. Incorporation into apocytochrome *c* is roughly 60 $\times 10^3$ dpm per milliliter of incubation mixture.

Heterologous System. Preparation of reticulocyte lysates and cell-free

protein synthesis directed by *Neurospora* RNA is performed as described.¹⁶ After protein synthesis for 60 min, a postribosomal supernatant is prepared by centrifugation for 60 min at 50,000 rpm in a Beckman 50 Ti rotor. This contains about 0.1 pmol of labeled apocytochrome *c* per milliliter.

Preparation of Labeled Apocytochrome *c* by Reductive Methylation

Apocytochrome *c*, 600 μg , is dialyzed in Visking 18/32 cellulose tubing against 0.1 *M* KPi , pH 7.0, and adjusted to a volume of 120 μl . Then at a temperature of 25°, 50 μl of 0.1 *M* KPi , pH 7.0, containing 125 μCi of [¹⁴C]formaldehyde (specific activity 40–60 Ci/mmol; NEN, Boston, Massachusetts) are added, followed by 60 μl of 0.1 *M* KPi , pH 7.0, containing 6 mg of NaBH_3CN per milliliter (Serva, Heidelberg, FRG).¹⁷ The mixture is kept for 1 hr at 25°. It is dialyzed first against 0.1 *M* KPi , pH 7.0, at 4°, then against 0.25 *M* NH_4HCO_3 . Specific activity of ¹⁴C-methylated apocytochrome *c* is about 2.0×10^6 dpm/nmol as determined by amino acid analysis.

Comment. Labeling of holocytochrome *c* with this procedure followed by the removal of the heme group yields apocytochrome *c* that does not bind to mitochondria and is not converted to holocytochrome *c*.

Transfer of Apocytochrome *c* into Mitochondria and Conversion to Holocytochrome *c*

To study import of apocytochrome *c* into mitochondria *in vitro*, mitochondria are incubated either with the postribosomal supernatant of the cell-free system containing labeled apocytochrome *c* or with appropriate buffers containing apocytochrome *c* labeled by reductive methylation. Mitochondria are isolated by grinding 1 g (wet weight) of *Neurospora* cells harvested at mid log phase with 1.5 g of quartz sand and 2 ml of “sucrose medium” containing 0.3 *M* sucrose, 2 *mM* EDTA, 30 *mM* Tris-HCl, pH 7.2 (adjusted at 20°). Sand and cell membranes are removed by centrifugation in a Sorvall SS-34 rotor for 5 min at 5000 rpm. Mitochondria are sedimented by centrifugation in the SS-34 rotor for 12 min at 12,000 rpm. Mitochondria are then washed twice with “sucrose medium,” or further purified by density gradient centrifugation.

For further purification, a mitochondrial suspension is layered on top of a linear sucrose gradient (0.96 *M* to 1.8 *M* sucrose in 10 *mM* Tris-HCl, 2 *mM* EDTA, pH 7.2, and centrifuged in a Beckman SW 60 rotor for 60 min at 60,000 rpm.¹⁸ The mitochondrial fraction bands in the lower half

¹⁶ H. R. B. Pelham and R. J. Jackson, *Eur. J. Biochem.* **67**, 247 (1976).

¹⁷ D. Dottavio-Martin and J. M. Ravel, *Anal. Biochem.* **87**, 562 (1978).

¹⁸ D. J. L. Luck, this series, Vol. 12A, p. 465.

of the gradient. It is recovered by use of a Pasteur pipette, diluted with 0.2 *M* sucrose, 10 *mM* Tris-HCl, 2 *mM* EDTA, pH 7.2, and centrifuged in an SS-34 rotor for 12 min at 12,000 rpm. The yield of mitochondria is about 1 mg of mitochondrial protein per milliliter of original cell homogenate. The isolated mitochondria are resuspended in the postribosomal supernatant of the cell-free translation system at a concentration of about 1 mg of protein per milliliter. Incubation is performed at 25°. Transfer of apocytochrome *c* into mitochondria is practically complete after 15–20 min (Fig. 3). Mitochondria are sedimented by centrifugation in an SS-34 rotor for 12 min at 12,000 rpm and washed once with 1 ml of “sucrose medium.” Apo- and holocytochrome *c* are immunoprecipitated from the lysed mitochondria and from the first postmitochondrial supernatant. About 80–90% of the radioactive apocytochrome *c* originally present is recovered as holocytochrome *c* in the mitochondrial fraction. Conversion of apocytochrome *c* to holocytochrome *c* in this system can be inhibited by addition of deuteroheme; 10 nmol of deuteroheme per milliliter inhibits conversion by about 70%, and 100 nmol/ml by more than 95%. (For details see the next section.)

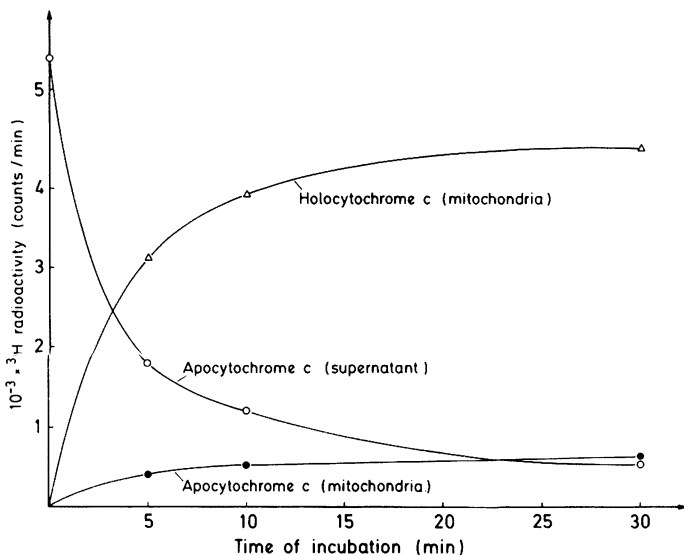


FIG. 3. Kinetics of conversion of apocytochrome *c* to holocytochrome *c* *in vitro*. ³H-labeled apocytochrome *c* was synthesized in a cell-free homologous system in the presence of [³H]leucine. The postribosomal supernatant was prepared and mixed with mitochondria. The mixture was incubated at 25° for the indicated periods of time. Postmitochondrial supernatant and mitochondria were separated by centrifugation. Apocytochrome *c* and holocytochrome *c* were precipitated from the supernatant and from mitochondria employing specific antibodies. The immunoprecipitates were separated by electrophoresis on SDS gels. The radioactivities in the cytochrome *c* peaks were determined.

Comments. The quality of mitochondria is important for binding and import of apocytochrome *c*. Swollen or sonicated mitochondria are unable to convert apocytochrome *c* to holocytochrome *c*. Rapid purification of small amounts of mitochondria can be performed in Eppendorf tubes with density gradients using Percoll (Pharmacia, Uppsala) according to the described procedure.¹⁹ The amount and stability of mitochondria can be controlled when mitochondria are used that are labeled *in vivo* with [³⁵S]sulfate.

Binding of Apocytochrome *c* to Mitochondrial Receptor Sites

Mitochondria (about 1 mg protein) are suspended in 100 μ l of Sucrose-MOPS buffer (250 mM sucrose, 10 mM MOPS, 1 mM EDTA, 0.1% bovine serum albumin, pH 7.2) containing 10–100 nmol of deuterohemin (Porphyrin Products, Logan, Utah).²⁰ Deuterohemin is added from a stock solution prepared as follows. One milligram of deuterohemin is dissolved in 10 μ l in 1 M KOH, then 1 ml of Sucrose-MOPS buffer is added, and the pH is adjusted to 7.2 by addition of 1 M HCl. The mixture is incubated for 5 min at 25°, then 1 ml of postribosomal supernatant containing [³H]leucine-labeled apocytochrome *c* is added; incubation is performed for further 15 min. Mitochondria and supernatant are separated by centrifugation in a Sorvall SS-34 rotor for 12 min at 12,000 rpm.

For binding of isolated apocytochrome *c* labeled by reductive methylation, mitochondria (about 1 mg of protein) are suspended in 50 μ l of Sucrose-MOPS buffer containing 10–100 nmol of deuteroheme. The mixture is incubated for 5 min at 25°. Then 1 ml of additional Sucrose-MOPS buffer is added, and aliquots of 50 μ l are transferred into glass minivials (300 μ l). Appropriate amounts of ¹⁴C-labeled apocytochrome *c* are added, and binding is allowed to occur for 15 min at 25°. Then mitochondria are spun down and washed twice with 200 μ l of Sucrose-MOPS buffer. Radioactivity is determined after dissolving mitochondria in 100 μ l of 0.1% SDS.

Comments. Results obtained with binding of ¹⁴C-labeled apocytochrome *c* labeled by reductive methylation allow a more accurate determination of the number and affinity of binding sites. Conditions and requirements of binding can be studied better in this simple system.

¹⁹ J. R. Mickelson, M. L. Graeser, and B. B. Marsh, *Anal. Biochem.* **109**, 255 (1980).

²⁰ B. Hennig and W. Neupert, *Eur. J. Biochem.* **121**, 203 (1981).