In Vitro Biosynthesis of Membrane Proteins in Isolated Mitochondria from *Saccharomyces carlsbergensis**

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ABSTRACT: The products of *in vitro* incorporation of [^{14}C]amino acids into isolated mitochondria from *Saccharomyces carlsbergensis* have been investigated and found to be associated with the membrane fraction. The yeast mitochondrial membrane proteins were fractionated into four major subfractions. The proteins of these subfractions were then solubilized by reduction and carboxymethylation or sulfonation in the presence of sodium dodecyl sulfate and separated by disc gel electrophoresis. The level of incorporation of radioactive amino acids was rather low; however, it was not due to microsomal or bacterial contamination. Actinomycin D, rifamycin, and ethidium bromide had no effect on this *in vitro* incorporation into mitochondria. CS-1, the predominant component from membrane fractionation, has the highest specific radioactivity and accounts for 50–60% of the total radioactivity incorporated into insoluble membrane proteins. The incorporation of radioactive amino acid into CS-1 is strongly inhibited by chloramphenicol. While other membrane components are labeled to a much lesser degree, incorporation into these other components is not particularly sensitive to chloramphenicol inhibition. Comparison of amino acid compositions and fingerprints of peptides obtained from tryptic digestion of the three major membrane components, CS-1, Ci-1, and Ci-3, suggest that all are extremely similar in spite of differences in labeling and in migration in a sodium dodecyl sulfate containing disc gel electrophoresis system.

The ability of isolated mitochondria to incorporate radioactive amino acids into protein was first detected by Siekevitz (1952). Later studies by Roodyn *et al.* (1961), Truman and Korner (1962), Simpson (1962), and Kroon (1963a,b, 1964) have confirmed the original observation; however, solution conditions reported for efficient incorporation were rather conflicting. In recent reports, Wheeldon and Lehninger (1966), Beattie *et al.* (1967), and Lamb *et al.* (1968) have established quite well the requirements for *in vitro* amino acid incorporation. Most of the radioactivity incorporated has been shown to be associated with insoluble membrane proteins (Beattie *et al.*, 1967; Haldar *et al.*, 1966). Haldar *et al.* have demonstrated that several protein species separable on disc gel electrophoresis have incorporated radioactive amino acid; however, no specific protein products of mitochonrial synthesis have been resolved. The present studies provide a reproducible method for separation of membrane components by extraction and electrophoretic procedures, demonstrate that one of the membrane protein fractions contains the major product(s) of protein synthesis in isolated yeast mitochondria, and describe some properties of the protein from various membrane fractions.

Materials and Methods

D-Chloramphenicol, cycloheximide, oligomycin, and bovine pancreatic trypsin (type I) were obtained from Sigma Chemical Co. Rabbit muscle A grade pyruvate kinase and ethidium bromide were purchased from Calbiochem. Rifamycin was purchased from Dow Chemical. Actinomycin D was purchased from Merck Sharp and Dohme. Glusulase was obtained from Endo laboratory. [^{14}C]Protein hydrolysate was purchased from Amersham-Searle Corp. [^{14}C]Leucine, [^{14}C]-valine, [^{14}C]lysine, and [^{3}H]protein hydrolysate were obtained from New England Nuclear Corp. [^{14}C]Tyrosine and [^{14}C]-arginine were purchased from Schwartz BioResearch Inc. Other reagents were reagent grade and used without further purification unless otherwise stated.

Isolation and Solubilization of Mitochondrial Membrane Components. ISOLATION OF MITOCHONDRIA. A double mutant of *Saccharomyces carlsbergensis* requiring both lysine and methionine for growth was employed in these studies. Cells were broken either by mechanical means or by enzymic digestion.

MITOCHONDRIA PREPARED FROM MECHANICALLY BROKEN CELLS. Yeast cells were grown aerobically at 25–28° (room temperature) in 1% peptone–1% yeast extract–1% glucose medium and were harvested in late-log phase when the absorbance at 660 mμ reached 8–9 optical density units and were collected with a Sharples centrifuge. A suspension of 10 g of cells was added to bottles containing 20 g of 0.05–0.45-mm glass beads and homogenized in a B. Braun cell homogenizer at 0–4° for 45 sec. Crude mitochondria were obtained by differential centrifugation at 1000*g* and 17,000*g*. The crude mitochondrial fraction, suspended at 20 mg/ml in STE[1] buffer, was purified in a 20% to 60% (w/w) sucrose density gradient. All sucrose solutions were made in TE buffer. The density

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[1] STE buffer, 0.01 M Tris-Cl–0.25 M sucrose–0.001 M EDTA (pH 7.4); TE buffer, 0.01 M Tris-Cl–0.001 M EDTA (pH 7.4); MET buffer, 0.2 M mercaptoethylamine–0.01 M EDTA–0.1 M Tris-Cl (pH 8.0); CPS buffer, 3.08 mM citric acid–21.6 mM Na_2HPO_4–1 mM EDTA–1.2 M sorbitol (pH 6.8).

gradient was preformed in a Beckman L-4 zonal centrifuge at 4000 rpm at 4°. Equilibrium was attained after centrifuging at 20,000 rpm for 8–12 hr.

The purified mitochondrial fractions were pooled, diluted with an equal volume of TE buffer, and centrifuged at 30,000 rpm in the Sorvall ultracentrifuge for 1–2 hr. The mitochondria thus obtained were used in preparative scale isolation of membrane protein subfractions.

MITOCHONDRIA PREPARED FROM SPHEROPLASTS. Cells were grown and harvested as above. Spheroplasts were prepared using a modification of the method of Duell *et al.* (1964). Each gram of cells was incubated with 2.5 ml of MET buffer at 30° for 30 min. The suspension was centrifuged at 1000*g* for 10 min and the pellet was washed with CPS buffer three times. The final pellet was suspended in CPS buffer (1.7 ml of CPS buffer/g of cells) and incubated with glusulase (20 mg of enzyme/g of cells) at 30° for 1 hr. The suspension was centrifuged at 1000*g*, washed with CPS buffer three times and centrifuged at 3000*g* for 10 min. The spheroplast suspension was homogenized in a Potter-Elvehjem glass homogenizer in the lysis buffer (2 ml of lysis buffer/g of cells) containing 0.4 M sorbitol–20 mM $MgCl_2$–10 mM KCl–5 mM phosphate (pH 6.8). The homogenate was mixed vigorously with Vortex mixer and then incubated 20 min with shaking at 0°. A mitochondrial pellet was obtained by differential centrifugation and then purified by equilibrium sedimentation in linear sucrose density gradients (Criddle and Schatz, 1969). Mitochondria prepared in this way were used in all [^{14}C]amino acid incorporation experiments.

FRACTIONATION OF MEMBRANE PROTEINS. Purified mitochondrial protein was fractionated initially into subfractions by the method of Richardson *et al.* (1966). Soluble mitochondrial protein was removed by freeze and thaw treatments followed by washing the mitochondria in 0.4 M KCl solution. The KCl-insoluble membrane protein was solubilized with deoxycholate (2 mg/mg of protein)–cholate (1 mg/mg of protein) solution and precipitated by addition of saturated (at 4°) $(NH_4)_2SO_4$ solution to a final concentration of 15% saturation. Lipid was extracted with 95% acetone at 0° and the "structural protein" subfraction thus obtained was washed with 0.01 M Tris-Cl buffer (pH 8.5) three more times. The product was either washed with 8 M urea (pH 5.5) at 5 mg/ml according to the method of Lenaz and Green (1968) or suspended in 8 M urea–0.1 N NaOH solution and then dialyzed exhaustively against deionized water. All protein fractions were collected and analyzed as described below for level of [^{14}C]amino acid incorporation.

SOLUBILIZATION OF MEMBRANE PROTEIN SUBFRACTIONS BY REDUCTION AND CARBOXYMETHYLATION. Membrane protein subfractions were suspended in 0.01 M Tris-Cl (pH 8.5)–0.5% sodium dodecyl sulfate solution to a final concentration of 2 mg/ml and reduced with 100 × excess (mole/mole of protein of 20,000 molecular weight) of dithiothreitol (20 mM) for 4 hr at room temperature. A tenfold molar excess of iodoacetate (relative to dithiothreitol) was added and the reaction was allowed to proceed for 1 hr in the dark at pH 8.5 and room temperature. The reduced and carboxymethylated protein was dialyzed against three changes of 0.01 M Tris-Cl (pH 8.5) buffer.

SOLUBILIZATION BY SULFONATION OF MEMBRANE PROTEIN SUBFRACTIONS. Sulfonation was carried out essentially as reported by Chen (1968). Membrane proteins were suspended in 1% sodium dodecyl sulfate–0.2 M Tris-Cl (pH 8.4) solution and then incubated with shaking in a medium containing 0.05 M Na_2SO_3, 5×10^{-4} M cysteine, 2×10^{-7} M $CuSO_4$, 0.5% sodium dodecyl sulfate, and 0.1 M Tris-Cl (pH 8.5) at 25° for 1 hr. The solution was then dialyzed against 0.1 M Tris-Cl (pH 8.5) with three changes of buffer.

FRACTIONATION OF COMPONENTS FROM SOLUBILIZED MITOCHONDRIAL SUBFRACTIONS BY DISC GEL ELECTROPHORESIS. The system of preparative disc gel electrophoresis published in the Büchler Instrument Manual (Ornstein and Davis, 1964) modified to contain 0.03% sodium dodecyl sulfate in the upper buffer was used to further fractionate the major subfractions into various components. Preparative disc gel electrophoresis was carried out in 0.5 × 3.375 in. tubes with 7 ml of running gel and 1.2 ml of stacking gel solution. Stacking and running were carried out at 4 and 8 mA per tube, respectively. Protein bands were identified by staining gels in 0.1% Buffalo blue black in 40% ethanol–15% acetic acid solution for 15 min, destaining with 10% acetic acid until gels were almost completely clear and then washing in 40% ethanol–5% acetic acid solution. The gels were scanned in a Schoeffel spectrodensitometer SD 3000 at 540 mμ. Protein bands could also be detected by cooling the gels to 0° for 4 hr. Protein-bound sodium dodecyl sulfate precipitated in the cold and facilitated the identification of protein bands.

ISOLATION OF MEMBRANE COMPONENTS. The major membrane components separated by preparative disc gel electrophoresis were located by cold precipitation of the sodium dodecyl sulfate–protein bands and the gel segments cut out accordingly. The membrane components were pooled and eluted electrophoretically through a coarse sintered glass filter and into attached dialysis tubing. The protein components were dialyzed against three changes of 0.01 N Tris-Cl (pH 8.5) buffer.

Characterization of Various Membrane Components. REMOVAL OF PROTEIN BOUND SODIUM DODECYL SULFATE. Membrane components were treated with 0.5 N KOH and centrifuged repeatedly to remove most of the bound sodium dodecyl sulfate and then dialyzed exhaustively against deionized water prior to any chemical or enzymatic analysis.

MOLECULAR WEIGHT DETERMINATION. Molecular weights of CS-1, Ci-1, and Ci-3 were estimated in sodium dodecyl sulfate gels according to the method of Shapiro *et al.* (1967). The following proteins with known molecular weight: bovine hemoglobin, ovalbumin, myoglobulin, lactate dehydrogenase, β-lactoglobulin, lysozyme, and chymotrypsinogen A were sulfonated or reduced and carboxymethylated at concentrations of 2 mg/ml as described above. Electrophoresis was run in 12% polyacrylamide gel.

AMINO ACID ANALYSIS. Purified membrane components were hydrolyzed under vacuum in sealed vials for 12, 24, 48, 96 or 24, 48, and 72 hr at 110° in constant-boiling HCl (5.7 N). After evaporating to dryness twice to remove hydrochloric acid, the amino acids were dissolved in pH 2.2 citrate buffer. The analyses were carried out on a Phoenix Precision Instrument Co. amino acid analyzer Model K 800 VG at a temperature of 50° using the buffer system of Moore *et al.* (1968).

FINGERPRINTING. The sodium dodecyl sulfate free sulfonated components were suspended in 10^{-2} M NH_4HCO_3–10^{-3} M $CaCl_2$ solution at pH 8.5 to 1–2 mg/ml and trypsin suspended in 10^{-3} M HCl was added at 0.05 protein concentration. The digestion was carried out at 37° for 6 hr and the reaction was stopped by addition of 25 μl of 1 N HCl/ml. The peptides were lyophilized, resuspended in H_2O, lyophilized, suspended

in minimum amount of 10^{-3} N HCl, and spotted on Whatman No. 3MM paper.

Two-dimensional peptide maps of components were prepared using the procedure of Katz *et al.* (1959). Chromatography was carried out at room temperature in freshly prepared butanol–acetic acid–water (4:1:5, v/v) for 21 hr. Electrophoresis was run in pyridine–acetic acid–water buffer (25:1:225, v/v, pH 6.4) for 2 hr in the direction perpendicular to the direction of chromatography. The peptide spots were detected by spraying with 0.3% ninhydrin suspended in 95% ethanol.

Biosynthesis of Mitochondrial Membrane Proteins. INCORPORATION OF [^{14}C]AMINO ACIDS INTO MITOCHONDRIA. Mitochondria prepared from spheroplasts and purified in 20–60% w/w sucrose gradient were used in all incorporation experiments. The incubation medium used for amino acid incorporation into isolated mitochondria was a modification of that described by Lamb *et al.* (1968). The standard incubation mixture contained 40 mM Tris-Cl (pH 7.4), 250 mM sorbitol, 10 mM $MgCl_2$, 100 mM KCl, 5 mM P-enolpyruvate, 1.5 mM ATP, 5 mM PO_4 (pH 7.4), and 0.18 mM cycloheximide. Also, each 1 ml of medium contained [^{14}C]amino acid (0.8–1.2 μCi), 10 μg of oligomycin, and 1–3 mg of mitochondrial of protein. A single [^{14}C]amino acid or several different [^{14}C]amino acids in various combinations were used. In the incubation mixtures containing chloramphenicol, the concentration of chloramphenicol was 5 mM. Where possible solutions used were sterilized and operations were carried out in a sterile hood to minimize bacterial contamination.

All incubations were carried out at 32° in a metabolic shaker. Mitochondrial suspensions were incubated with protein synthesis inhibitors for 15 min prior to the addition of radioactive amino acids.

Incorporation was stopped by addition of an equal volume of cold buffer (0°) containing 200 × excess of unlabeled amino acids in STE buffer. After washing three times with STE buffer containing unlabeled amino acids, mitochondrial proteins were fractionated according to the simplified scheme of Figure 1. Aliquots of each fraction were used in counting of radioactivity. The proteins were washed according to the method reported by Criddle and Schatz (1968) and radioactivity of the protein solutions was counted in a Nuclear-Chicago scintillation counter using Bray's scintillation fluid. Protein concentrations were determined by the method of Lowry *et al.* (1951).

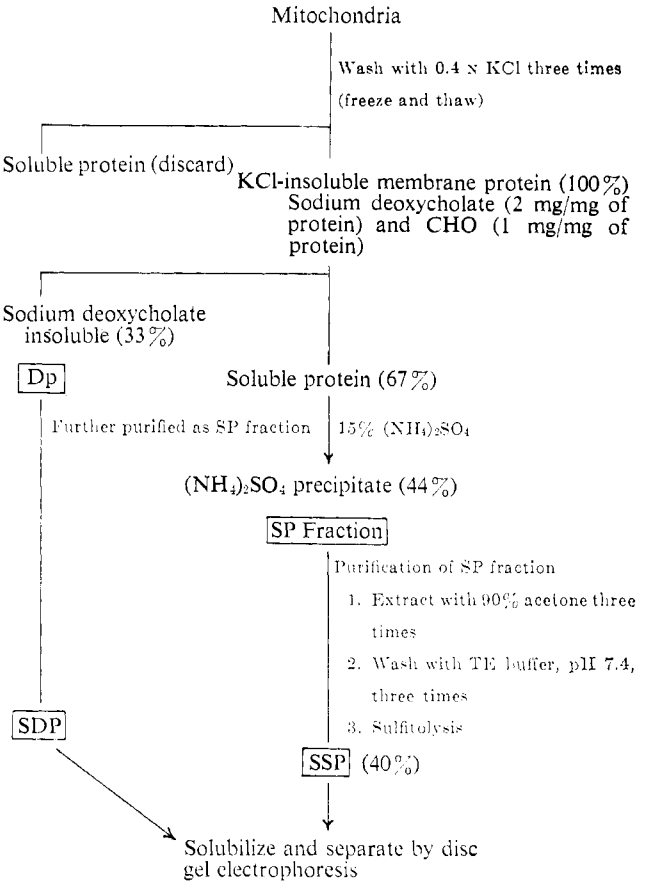

FIGURE 1: Scheme for preparation of mitochondrial protein subfractions. The deoxycholate-cholate-insoluble material was fractionated further using the same conditions as used for structural protein fraction. Abbreviations used: SP = structural protein fraction; Dp = deoxycholate-cholate-insoluble fraction; SSP = sulfonated structural protein fraction; SDp = sulfonated Dp fraction.

Results

Isolation of Mitochondrial Membrane Components. ISOLATION OF MITOCHONDRIA. The yield of mitochondria per cell when prepared from spheroplasts is much higher (five times) than the yield when prepared by mechanical breakage of yeast cells. To obtain a high yield of spheroplasts a higher concentration of mercaptoethylamine and higher pH than described in the procedure of Duell *et al.* (1964) was employed. Lysis of spheroplasts in this case becomes the major limitation in obtaining high yield of mitochondria. Vigorous stirring at 0° facilitates release of mitochondria from the spheroplasts. Mitochondria obtained by this method are more intact than those obtained by mechanical breakage and form a distinct sharp band in sucrose density gradient.

FRACTIONATION OF MEMBRANE PROTEINS. The scheme for fractionation of mitochondrial membrane proteins and yield at each step are shown in Figure 1. The sulfonated membrane protein subfractions are readily soluble at low protein concentration. When the SSP subfraction is subjected to disc gel electrophoresis at pH 8.9 in 0.03% sodium dodecyl sulfate, four distinctly resolved bands (plus trace amounts of other components) are observed (Figure 2). CS-1, the fastest migrating component, is 45% of the total protein of this fraction; the remainder of the material is distributed among the bands Ci-1 (30%), Ci-3 (12%), and Ci-2 plus Ci-4 (10%). Disc gel electrophoresis patterns of the SDp subfraction is also shown in Figure 2. While similarities exist among the major components of these two subfractions, SDp has several additional minor components. The gel patterns of only two subfractions are illustrated in Figure 2; however all major membrane subfractions were collected, sulfonted, and separated by disc gel electrophoresis.

ISOLATION OF MEMBRANE COMPONENTS. Membrane components from the CMSP or SSP subfractions were isolated for further characterization using preparative scale disc gel electrophoresis. CS-1, Ci-1, and Ci-3 obtained under these conditions appear to be pure when protein is reapplied in 8% gel and electrophoresed at pH 8.9 (Figure 3). However, when electro-

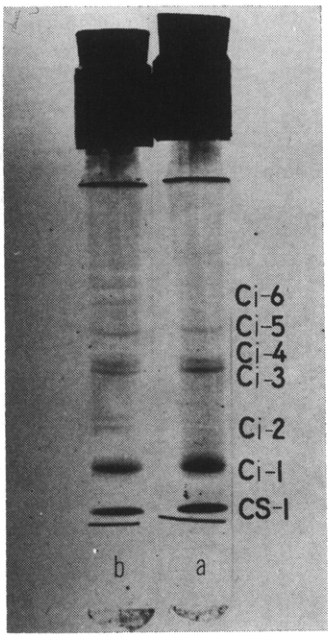

FIGURE 2: Disc gel electrophoresis patterns of SSP (a) and SDp (b) subfractions. SSP and SDp (700 μg) were applied on 8% gel. The front is marked by a copper wire.

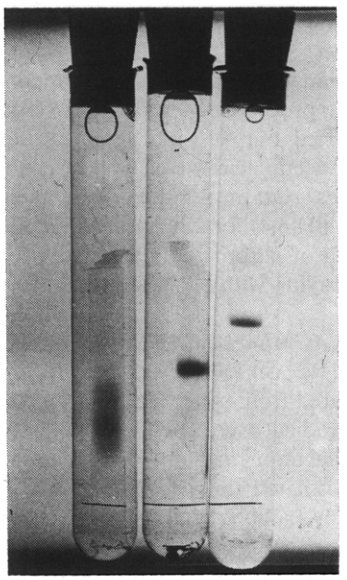

FIGURE 4: Disc gel electrophoresis pattern of CS-1, Ci-1, and Ci-3 (left to right) obtained from SSP subfraction. Electrophoresis was carried out at 12% gel concentration. The dye front is apparent from marker wire; 80 μg of protein was applied per sample.

phoresis is carried out at 12% gel concentration, CS-1 separates into four poorly resolved bands termed CS-1 a, b, c, and d, while Ci-1 and Ci-3 still migrate as single components (Figure 4). Ci-1 and Ci-3 remain as single gel components at 10–16% gel concentrations (Figure 5). The multiplicity of CS-1 bands seems to indicate either heterogeneity or a process of association and dissociation in the solvent system employed. Disc gel electrophoretic separation at other pH's, *i.e.*, 7.5, 8.3, 11.3, have been attempted, but have not been successful because of protein precipitation or lack of resolution.

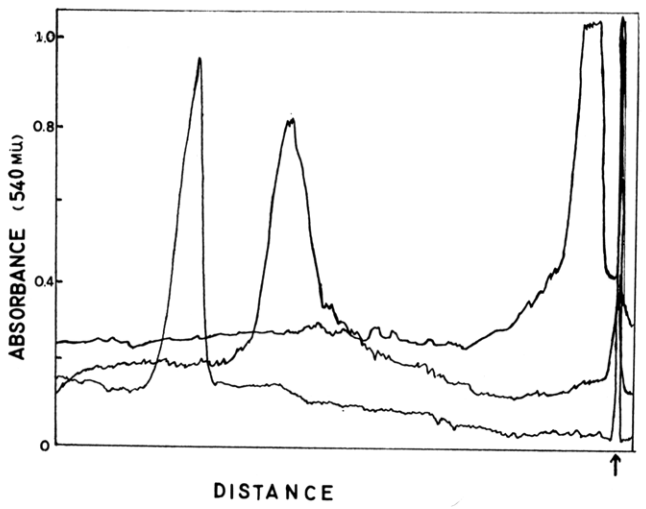

FIGURE 3: Densitometer tracings of disc gel electrophoresis patterns of CS-1, Ci-1, and Ci-3 obtained from SSP subfraction. Tracing with peak at left is Ci-3, center Ci-1, and right CS-1. Electrophoresis was carried out at 8% gel concentration. The dye front is marked by an arrow and the direction of migration is left to right; 80 μg of each protein was applied.

Characterization of Membrane Components. MOLECULAR WEIGHT DETERMINATION. The relative mobilities of standard proteins of known molecular weight as well as of CS-1, Ci-1, and Ci-3 were measured and plotted as relative mobility *vs.* the logarithmic value of the molecular weight. A best-fitted straight line is obtained from this plot. With the exception of the standards lysozyme and lactate dehydrogenase, which show deviation, the other protein molecular weights fall within $\pm 5\%$ of a straight-line function. Molecular weights of CS-1a, CS-1b, CS-1c, CS-1d, Ci-1, and Ci-3 determined according to this method are 15,000, 20,000, 26,500, 40,000, 39,000, and 56,000, respectively.

AMINO ACID ANALYSIS. Acid hydrolyses of CS-1, Ci-1, and Ci-3 were run using three to four different hydrolysis times for each protein. Serine and threonine concentrations shown in Table I were determined by extrapolation to zero-time hydrolysis. The amounts of the other amino acids reported were determined at hydrolysis time which ensured complete hydrolysis of the protein.

In order to facilitate the comparison of the amino acid compositions in each protein component, results are presented on the basis of an assumed molecular weight of 20,000 and the number of residues of each amino acid in the various components is normalized accordingly. It is apparent that the amino acid composition of all three components, shown in Table I, are extremely similar. On close examination, one notices that Ci-3 has three to four more residues of Glu, one to two residues of fewer Leu, one residue less of Tyr, one to two fewer residues of Lys, and two fewer met residues than either Ci-1 or CS-1. The differences between CS-1 and Ci-1 are indeed minute and can only be detected in Ser and Lys. The number of residues of cysteine and tryptophan of these components have not been determined.

FINGERPRINTING. A composite peptide map of the SSP subfractions is shown in Figure 6. The number of peptides corresponds fairly well with the number of lysine and arginine groups

TABLE I: Amino Acid Composition of Membrane Components.[a]

	CS-1	Ci-1	Ci-3
Asp	15	15	15
Thr	10.8	10.8	10.8
Ser	12.5	16.3	14.5
Glu	16.5	15.0	19.8
Pro	8.75	8.5	6.5
Gly	14.5	16.8	15.5
Ala	15.0	16	15.6
Val	11.7	11.4	12.5
Met	3.35	3.6	1.5
Ile	10.6	10.1	9.5
Leu	18.6	18.0	16.7
Tyr	5.4	5.5	4.4
Phe	8.8	8.9	6.5
Lys	13.8	12.5	11.7
His	2.8	2	2.6
Arg	7.2	6.6	7.7
NH_3	4.73	20.63	20.65

[a] The number of residues of all amino acids are normalized in such a way that the molecular weight of all three components is set at 20,000. Data represent values calculated from 18, 7, and 7 amino acid analyses of CS-1, Ci-1, and Ci-3, respectively.

in the protein obtained from amino acid analysis assuming the molecular weight of the protein to be 20,000. The number of peptides in Ci-1 and Ci-3 is somewhat smaller than in CS-1 as seen in Figure 6. Ten peptides are common to all the membrane components studied.

In Vitro Biosynthesis of Mitochondrial Membrane Proteins. Mitochondria prepared from spheroplasts were used immediately after preparation in all studies of *in vitro* biosynthesis. The radioactivity from ^{14}C-labeled amino acid mixture incorporated into mitochondrial protein as a function of time is shown in Figure 7. Radioactivity is incorporated most rapidly in the first 5 min, and the rate of incorporation levels out slowly and approaches zero after a period of 1 hr.

At various time periods during the incubation, 0.1 ml of incubation mixture was plated on enriched agar plates. The number of bacteria in these plates was subsequently counted after incubating the plates at 37° for 48 hr. The number of bacteria counted was not proportional to the duration of incubation with ^{14}C-radioactive amino acids and the maximum number of bacteria detected per milliliter of solution was never greater than 10, thus eliminating bacterial contamination as a major contributing source of incorporation of [^{14}C]amino acids into mitochondria in these experiments. Studies with protein synthesis inhibitors verify this conclusion.

Cycloheximide, a specific cytoplasmic protein synthesis inhibitor for eucaryotic cell, was shown to have no effect on [^{14}C]amino acid incorporation into mitochondria. Even though ^{14}C incorporation experiments were routinely run with added cycloheximide, the possibility of incorporation being due to microsomal contamination was investigated by adding

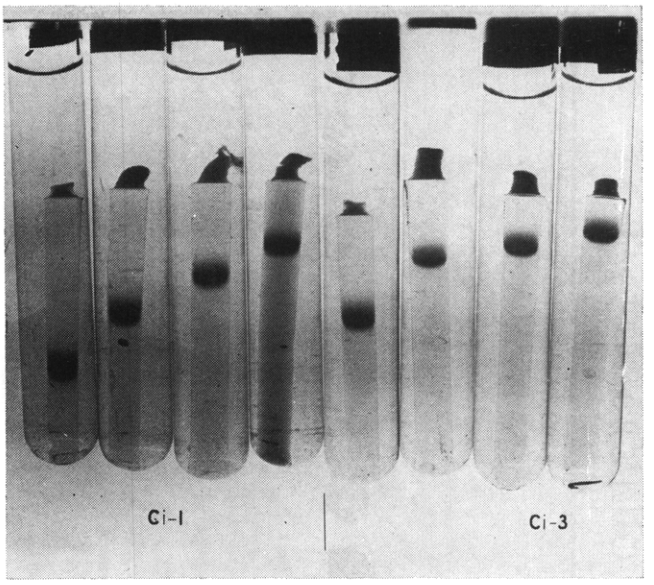

FIGURE 5: Disc gel electrophoresis patterns of Ci-1 and Ci-3 isolated from SSP subfractions. Electrophoresis was carried out at 10, 12, 14, and 16% acrylamide gel concentrations (left to right). The dye front is marked by wire; 100 μg of protein was applied on each gel.

different concentrations of microsomal material to the incubation mixture. The specific radioactivity incorporated decreased as the concentration of microsomal fraction increased, possibly as a result of nuclease activity. This indicates clearly that the incorporation cannot be accounted for microsomal contamination.

The incorporation of [^{14}C]amino acid into mitochondria is sensitive to osmolality. At 200 mM sorbitol and 32 mM Tris-Cl concentration, the protein-synthesizing system has maximal activity.

TABLE II: Effect of Antibiotics on Amino Acid Incorporation into Isolated Mitochondria.

Added Antibiotics	cpm/mg
None[a]	248
Ethanol (50 μg) (1.67%)	303
Actinomycin D (10 μg/ml)	285
Actinomycin D (40 μg/ml)	363
Actinomycin D (80 μg/ml)	342
Rifamycin (5 μg/ml)	219
Rifamycin (20 μg/ml)	254
None[b]	141
Ethidium bromide (10 μM)	151
Ethidium bromide (20 μMl	146
Blank	19

[a] Each 1 ml of reaction mixture contained 1.0 μCi of [^{14}C]amino acid mixture. The mitochondrial protein concentration was 0.8 mg/ml. [b] Each 1 ml of reaction mixture contained 0.8 μCi of [^{14}C]amino acid mixture. The mitochondrial protein concentration was 0.7 mg/ml.

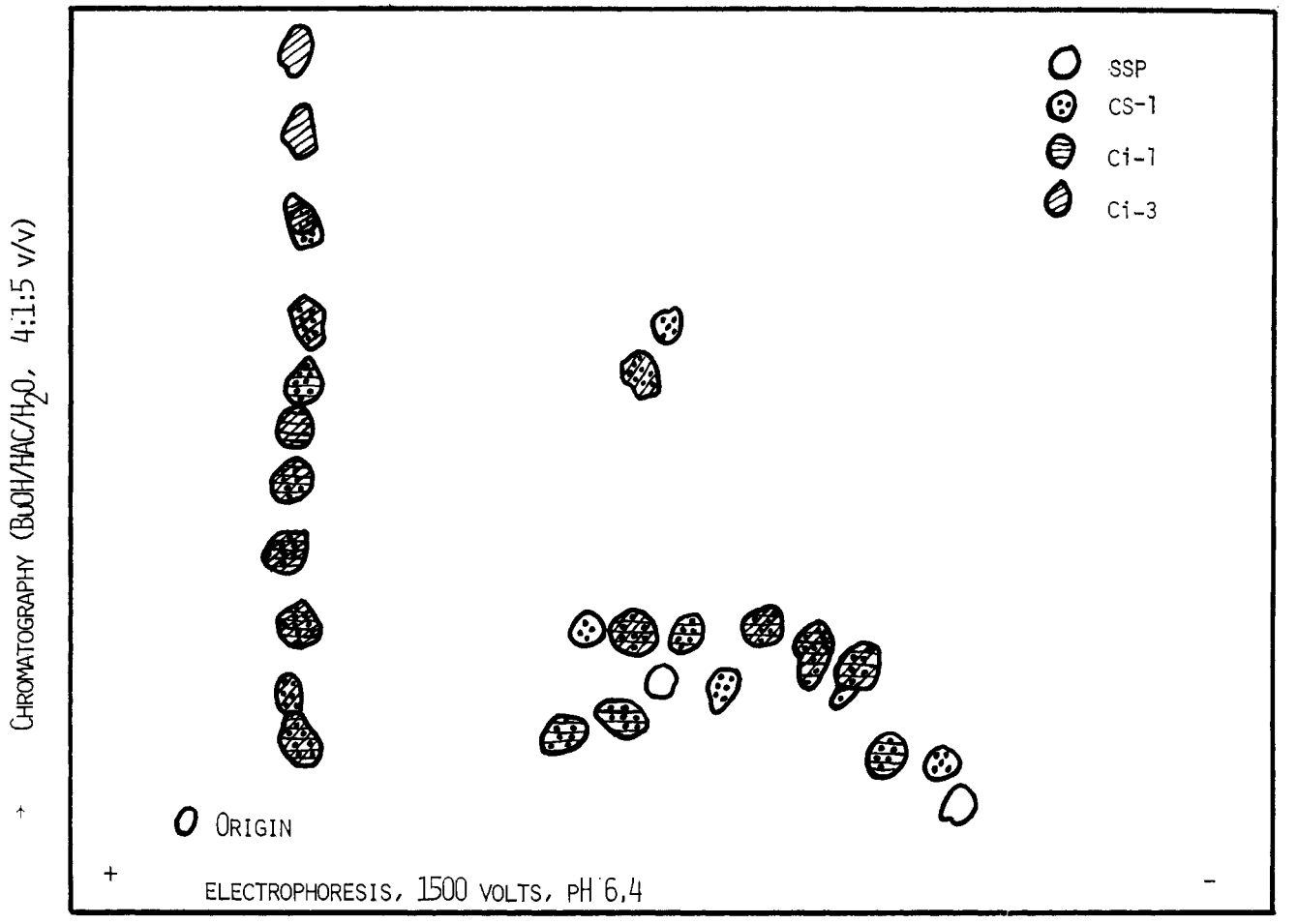

FIGURE 6: A representative tracing of "fingerprints" of the tryptic digested membrane components. Conditions for analysis are described in Materials and Methods.

Actinomycin D has no measurable effect on the incorporation of [^{14}C]amino acid mixture into intact isolated mitochondria or osmotically shocked mitochondria (Table II). Aside from the slight stimulating effect of low concentrations of ethanol added with the actinomycin D, the radioactivity incorporated in the presence of actinomycin D is essentially the same as the control. Similarly rifamycin, a bacterial RNA polymerase inhibitor, and ethidium bromide, a DNA-polymerase inhibitor, exerted no effect on the incorporation.

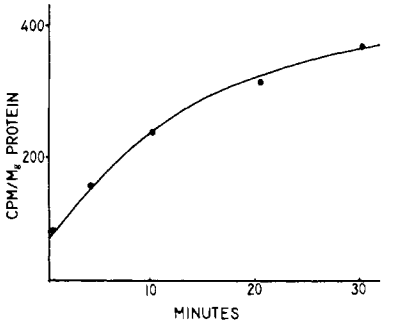

FIGURE 7: Time course of [^{14}C]amino acid incorporation into mitochondria. Each 1 ml of reaction mixture contained 1 μCi of [^{14}C]-amino acid mixture, 10 μg oligomycin, and 2.0 mg of mitochondrial protein. At each time interval noted, 2-ml aliquots were removed and the reaction was stopped immediately with 2 ml of cold 10% trichloroacetic acid.

Iu order to clarify whether lack of inhibition by actinomycin D is due to inability of the antibiotic to bind to mitochondrial DNA or whether simultaneous synthesis of mRNA is not required for the protein synthesis in isolated mitochondria under the present experimental conditions, the incorporation of tritiated uridine into shocked mitochondria was investigated in parallel with *in vitro* protein biosynthesis. The results shown in Figure 8 indicate that the incorporation of [^{3}H]uridine into RNA is 90% inhibited by actinomycin D at concentrations as low as 10 μg/ml. Simultaneous incorporation of [^{14}C]amino acids is totally unaffected (Figure 9). Rifamycin does not inhibit the initial incorporation of [^{3}H]-uridine into RNA.

INCORPORATION OF LABELED AMINO ACIDS INTO MEMBRANE PROTEINS. The uniformity of tritium label in all proteins from cells grown in the presence of [^{3}H]amino acids serves as an internal measure of protein concentration and thus allows accurate evaluation of specific radioactivity in different membrane components by determination of ^{14}C:^{3}H ratios. The results of radioactivity incorporated into major mitochondrial subfractions are shown in Table III. The specific radioactivity increases as the soluble enzymes are removed and the mem-

TABLE III: Distribution of Radioactivity Incorporated into Major Mitochondrial Subfractions.[a]

	Cycloheximide			Chloramphenicol + Cycloheximide			
	^3H	^{14}C	^{14}C/mg	^3H	^{14}C	^{14}C/mg	% Inhibn
Whole mitochondria	2721	685	202	3321	281	68	66.4
KCl insoluble	1378	355	207	327	277	68	67.2
Deoxycholate insoluble	2300	584	204	2469	292	95	53.4
Ammonium sulfate pellet	731	240	264	807	24	24	90.8
Sulfonated SP fraction	2878	1394	389	2314	175	61	84.4
Sulfonated Dp fraction	2532	878	279	4046	901	180	35.5

[a] [^3H]Amino acid mixture (50 μCi) was added to 1 l. of growing culture of *S. carlsbergensis* 2.5 generations prior to harvesting the cells. [^{14}C]Amino acid was then incubated with isolated mitochondria. For each 1-ml incubation mixture containing 1.67 μCi of [^{14}C]amino acid mixture and 0.1 μCi of [^{14}C]Leu. The mitochondrial protein concentration was 3 mg/ml. Data represent measured counts per minute of ^3H and ^{14}C. Ratios of ^{14}C/mg of protein were determined from independent measurement of ^3H/mg of protein; 823 cpm of ^3H was found per mg of protein.

brane proteins are purified in the fractionation process. SSP and SDP subfractions contained the highest specific activity.

This incorporation is sensitive to inhibition by chloramphenicol but as shown, not by cycloheximide. While chloramphenicol inhibition noted with this yeast strain is somewhat less than reported with other mitochondrial systems, it is apparent that inhibition of all subfractions occurs and that inhibition is greatest in the SP subfraction.

The sulfonated SP and Dp subfractions were subjected to disc gel electrophoresis in 12% gel solutions to determine the specific activity of the separated CS-1 subbands. The amounts of radioactive labels in each of the proteins of the SSP subfractions are shown in Table IV. The specific activities of CS-1 components are higher than the Ci components. The inhibition of CS-1 by chloramphenicol is also greater than that of the Ci components.

The distribution of the radioactivity in different components of the sulfonated Dp subfraction is shown in Table V. The specific activities of membrane components in this subfraction are generally somewhat lower than the SP subfraction and the inhibition by chloramphenicol is not as pronounced. However, the CS-1 components are again labeled to a greater extent and their synthesis is affected by chloramphenicol more severely than the Ci components.

In vitro BIOSYNTHESIS OF MEMBRANE PROTEINS IN ISOLATED MITOCHONDRIA OF GLUCOSE-REPRESSED CELLS. The yield of cells of *S. carlsbergensis* grown in 5% glucose medium is higher than those grown in 1% glucose; however, the yield of functional mitochondria is greatly reduced. Mitochondria isolated from these repressed cells have the same buoyant density as the functional mitochondria even though the enzymic composition is drastically different. The white color of mitochondria obtained is indicative of the low content of cytochromes.

The kinetic profile for the incorporation of a [^{14}C]amino

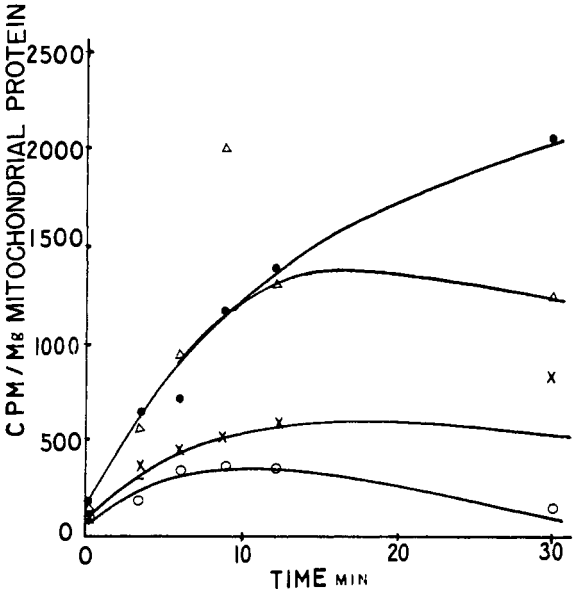

FIGURE 8: Time course of [^3H]uridine into osmotically shocked mitochondria in the presence or in the absence of inhibitors. Each 1 ml of reaction mixture contained 1.85 μCi of [^3H]uridine or 0.8 μCi[^{14}C]amino acid mixture, 10 μg of oligomycin, and 1.5 mg of mitochondrial protein. At each time interval noted, 1.5-ml aliquots were removed and the reaction was stopped immediately with 1.5 ml of cold 10% trichloroacetic acid. (●—●) Control; (Δ—Δ—Δ) rifamycin, 10 μg/ml; (×—×—×) actinomycin D, 1 μg/ml; (○—○) actinomycin D 10, μg/ml.

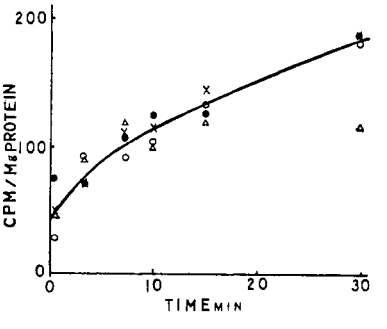

FIGURE 9: Incorporation of [^{14}C]amino acids into mitochondria in the presence of inhibitors of RNA production. Reaction conditions and definition of points on curve are described in Figure 8.

TABLE IV: Distribution of Radioactive Label in Proteins of Sulfonated Structural Protein Subfraction.[a]

	Cycloheximide			Chloramphenicol + Cycloheximide			
	^{14}C	^{3}H	^{14}C/mg	^{14}C	^{3}H	^{14}C/mg	% Inhibn
CS-1A	597	1032	477	26	1101	20	95.8
CS-1B	406	825	407	32	887	30	92.7
CS-1C	553	557	823	26	619	35	95.8
CS-1D	265	554	396	5	533	7	98
Ci-1	81	1505	44	4	1859	2	95
Ci-2	218	1045	172	47	1045	37	78.5
Ci-3	267	2740	74	33	2284	12	83
Ci-4	227	1570	120	44	1301	28	76.6
Ci-5	164	1355	100	32	1201	22	78

[a] Data represents measured cps for ^{3}H and ^{14}C. Determination of ^{14}C/mg was as in Table III.

TABLE V: Distribution of Radioactive Label in Proteins of Sulfonated Deoxycholate-Insoluble Subfraction.[a]

	Cycloheximide			Cycloheximide + Chloramphenicol			
	^{14}C	^{3}H	^{14}C/mg	^{14}C	^{3}H	^{14}C/mg	% Inhibn
CS-1A	231	471	405	39	548	59	85.4
CS-1B	247	672	304	54	694	65	78.6
CS-1C	332	762	360	16	597	22	94
CS-1D	274	656	344	35	630	46	87
Ci-1	317	1763	148	216	2017	86	42
Ci-2	211	1320	132	82	1058	65	51
Ci-3	99	1315	62	76	1483	42	32
Ci-4	166	1137	121	67	1079	51	58
Ci-5	66	1226	45	89	1568	47	0

[a] Data represents measured cps for ^{3}H and ^{14}C. Determination of ^{14}C/mg was as in Table III.

acid mixture into protein resembles those of functional mitochondria with its rate decreasing to zero in 30 min; however the level of the incorporation is only 10% of the incorporation into normal mitochondria.

The quantitative distribution of proteins fractionated into various membrane components is also different from the normal mitochondria. Approximately 70% of the membrane protein is not solubilized by deoxycholate and cholate solutions. Therefore, the amount of the SP membrane subfraction is greatly reduced. When the sulfonated SP and Dp subfractions were subjected to electrophoresis in this case, 30% of the total protein remained at the interface between the running gel and stacking gels. Also, the distribution of the various components is different from that observed in functional mitochondria. CS-1 accounts for only 20% of the protein in the SSP or in the SDp subfractions.

The distributions of [^{14}C]amino acids incorporated into various components of the repressed mitochondria is also altered. It is noted that a gel component migrating at the position of Ci-21 has the highest specific activity and its synthesis is inhibited most by chloramphenicol. Since the incorporation level is low, the absolute magnitude of the incorporation is not certain; however, it is clear the repressed mitochondria behave differently from the normal mitochondria in their ability to synthesize membrane protein components.

Discussion

The insolubility of membrane proteins has long been the major obstacle in characterizing them chemically as well as enzymatically.

Reduction and carboxymethylation or sulfonation of the membrane subfractions in the presence of sodium dodecyl sulfate by techniques described above provides a means for solubilizing the subfractions so that well-defined membrane components can be separated and quantitatively recovered by disc gel electrophoresis. Of all the systems tried, the modified Büchler system for disc gel electrophoresis employed here gives the best resolution of the membrane components. In contrast to previously reported systems (Lenaz and Green, 1968; Takayama *et al.*, 1966; Lejsek and Lusena, 1969), 100% of the protein preparation enters the gel and its distribution can be quantitatively evaluated. When lower pH buffer systems are used, a high percentage of protein does not penetrate the gel, but instead remains at the interface of the large pore size gel. With the higher pH systems employed, a fast-migrating band is observed but other bands are not well resolved.

Of all the membrane components isolated, CS-1 is the major membrane component in all subfractions. Although CS-1 is present in all the membrane subfractions, it is most readily isolated from the sulfonated structural protein subfraction where it constitutes 40% of the total protein. The available evidence suggests that CS-1 is the same from all subfractions. This is based on observations that this component is the only one highly labeled with [^{14}C]amino acids, specific activity of lable in CS-1 from all subfractions is the same in a given experiment, and the amount of chloramphenicol inhibition is also the same for CS-1 from each source. Based on tritium labeling of intact cells, nearly 30% of the total membrane protein is CS-1. When CS-1 is subjected to disc gel electrophoresis in 12% gel, it gives rise to four diffuse bands with molecular weights ranging from 15,000 to 42,000. The multiplicity of CS-1 subfractions suggests either interacting subunits or heterogeneity of the components. Sedimentation equilibrium molecular weight determinations of this component dissolved in 0.1 N NaOH–0.1 N NaCl solution indicate aggregation with a minimum molecular weight of 20,000 and maximum of 70,000. Another two major membrane components, Ci-1 and Ci-3, appear to be homogeneous under different gel concentrations with molecular weight of 39,000 and 60,000, respectively.

The peptide maps of each component are very similar with at least ten peptides common to all Ci and CS components investigated. This suggests that a rather large segment of the peptide within these components may be identical and possibly originate from the same genetic message or that they are homologous proteins with common ancestral gene. The homol-

ogy noted is even more striking when it observed that the unfractionated SSP peptide map shows the same common peptides as noted for the individual components. It is apparent that those peptides predominate in the mixture to such an extent that those nonhomologous peptides simply fail to be detected due to their relatively low concentrations.

The results of the amino acid analysis again showed extreme similarity of the components. Thus minor differences existing among them cannot be identified without detailed examination. The high concentration of NH_3 detected in hydrolysates of the components lead one to suspect that most of the glutamic acid and aspartic acid residues may exist as glutamine and asparagine in the native components. This interpretation may explain the absence of acidic peptides in the fingerprinting of the components.

The present studies clearly show that isolated yeast mitochondria are capable of incorporating [^{14}C]amino acids into their membrane protein fractions. The possibility of a bacterial or microsomal contamination giving rise to this synthesis has been ruled out by antibiotic studies. The insensitivity of this protein synthesis to high concentrations of cycloheximide is an indication that microsomal contaimination cannot be the prime origin of this synthesis. Furthermore, the addition of microsomal fraction retards the incorporation rather than enhances it, probably due to increasing concentrations of nuclease associated with the microsomal fraction.

Neither ethidium bromide nor actinomycin D exert any effect on the incorporation of amino acids into the proteins of isolated yeast mitochondria. Actinomycin D does inhibit the simultaneous incorporation of uridine into RNA, however. These two observations rule out a dependence of amino acid incorporation on simultaneous synthesis of mRNA and suggest the presence of stable mRNA within mitochondria.

As is consistent with the above findings, rifamycin, an effective bacterial DNA-dependent RNA polymerase inhibitor, slso has no effect on this synthesis (Wehrli *et al.*, 1968, 1969). It is somewhat more surprising, however, that rifamycin had no observable effect on nucleotide incorporation into RNA by the isolated mitochondria in contrast to the report that liver mitochondrial RNA polymerase is sensitive to this inhibitor (Shmerling, 1969). Since mitochondria were shocked prior to incubation with this antibiotic, transport of rifamycin through the mitochondrial membrane should not be a problem. However, an apparent inability of rifamycin to form a complex with the RNA–polymerase could result from prior association of enzyme with DNA. Association also depends upon the macrocyclic ring portion in the molecule and oxygenation or hydrogenation of this molecule may alter the steric configuration and render it inactive. Other observations in our laboratory, however, indicate that solubilized yeast mRNA polymerase is not inhibited by rifamycin (M. J. Tsai, 1969, unpublished observations).

Based on specific radioactivity, total radioactivity and its inhibition by chloramphenicol, one may conclude that CS-1 contains the major protein component(s), synthesized by isolated mitochondria. In addition this fraction is most sensitive to chloramphenicol inhibition. The specific radioactivity of each of the four bands of CS-1 separated at high gel concentrations is very similar, and all show similar sensitivity to chloramphenicol. Whether these four subcomponents are distinct proteins or not cannot be concluded from this experiment.

The incorporation of [^{14}C]amino acids into other mitochondrial membrane components, though significantly lower than that of CS-1, cannot be neglected. This background incorporation is consistently present and is not as sensitive to chloramphenicol. The nature of the systems which synthesize these components is not clear, but must differ from the synthesis of the chloramphenicol-sensitive CS-1 component.

If CS-1 is a membrane component specific to the inner mitochondrial membrane, it would be expected that mitochondria from repressed cells would have only a limited capacity for synthesizing this component. This is indeed observed to be the case. Promitochondria obtained from glucose repressed cells have a rather low efficiency in incorporating [^{14}C]amino acids. The specific radioactivity incorporated into various components is in general only one-tenth of those of functional mitochondria. The distribution of the radioactivity incorporated into all of the various components appears to be similar within experimental error and the incorporation has very limited sensitivity to chloramphenicol inhibition. It is very likely that this low level of incorporation originates from the same system which gives rise to the chloramphenicol-insensitive [^{14}C]amino acid incorporation in the mitochondira isolated from derepressed cells.

The distribution of the various mitochondrial membrane components in the repressed cells is rather different from the functional mitochondria. The high percentage of deoxycholate-insoluble material in the repressed cells may arise from differences in lipid composition in the organelles or from different organization of the membrane. The lower content of CS-1 seems to suggest that this protein(s) is one of the major protein components synthesized during mitochondrial biogenesis. However, it is unlikely that this protein is the obligatory "priori" for triggering mitochondria genesis since it is still one of the major membrane components in the repressed promitochondria.

A comparison of the chemical and structural properties of the various membrane proteins with the results of [^{14}C]-amino acid incorporation studies leaves some major unresolved questions. The amino acid and peptide mapping data clearly show major homologies among CS-1 and the various Ci components, particularly Ci-1. In contrast, incorporation studies show distinct differences do exist in either the mechanism of synthesis of these components or in some fashion related to their time course of appearance within the mitochondrial membrane. Differential sensitivity to chloramphenicol suggests that real differences in mode of [^{14}C]amino acid incorporation do exist.

Although it is clear in the experiments reported here that the site of assembly of amino acids into protein is in the mitochondrion, it is not possible to conclude anything regarding the site at which the RNA involved in this incorporation was produced. Since no requirement exists for continued RNA synthesis, a stable messenger either of nuclear or mitochondrial origin must exist. While many mechanisms based on production of RNA at either or both of these sites can be proposed, it is evident that further work must be done to resolve these questions.

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Dependence of Sterol Ester Hydrolase Activity on the Position of Ethylenic Bond in Cholesteryl *cis*-Octadecenoates*

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ABSTRACT: The cholesterol esters of the 16 positional isomers of *cis*-octadecenoic acid were synthesized and their rate of hydrolysis with rat liver cholesterol hydrolase (EC 3.1.1.13) was studied. The results showed that the enzyme exhibited a distinct preference for the 9-octadecenoate. With the ethylenic bond moving to either end of the carbon chain the activity gradually decreased. The pattern of substrate preference did not change during purification of the enzyme. When the enzyme preparation was allowed to age, the activity was lost at uniform rates for each isomer and similar patterns of activities were observed. Comparison of the rates of cholesteryl 9,10-methyleneoctadecanoate with cholesteryl *cis*- and *trans*-9,10-octadecenoates showed that the presence of π electrons although perceived by the enzyme was not as important as the configuration of the acyl moiety.

Deykin and Goodman (1962) have presented an extensive study of liver cholesterol ester hydrolytic activity (cholesterol esterase, an enzyme belonging to EC 3.1.1.13 group). The subcellular distribution, properties, and relative activity of the enzyme against commonly occurring cholesterol esters were included in that report.

More recently the specificity of rat liver cholesterol ester hydrolase with regard to the hydrolysis of *cis*- and *trans*-fatty acid cholesterol esters was investigated (Sgoutas, 1968). It was found that *trans*-fatty acid cholesterol esters were hydrolyzed to a lesser degree than *cis* esters. Furthermore, a preference for the hydrolysis of the 9-*cis*-unsaturated octadecenoate was indicated, suggesting a selectivity of the enzyme in response not only to the configuration but also to the location of the *cis* functional group along the acyl chain.

This led us to investigate the cholesterol ester hydrolase activity further by using cholesterol esters of the complete series of *cis*-octadecenoic acids. The results showed that a remarkable selectivity of the enzyme with regard to the position of the *cis* double bond in the fatty acid chain exists.

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