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Mitochondrial Membranes of Inositol-Requiring *Saccharomyces carlsbergensis:* Covalent Binding of a Radioactive Marker to the Outer Membrane†

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ABSTRACT: Tritium can be bound covalently and specifically to the outer membrane of intact mitochondria from *Saccharomyces carlsbergensis,* providing a sensitive marker for monitoring separation of mitochondrial membranes. Mitochondria are obtained by osmotic shock from *S. carlsbergensis* spheroplasts. They are labeled with tritium by NaB^3H_4 reduction of Schiff bases, formed between externally added pyridoxal phosphate and free amino groups on the surface of the mitochondrion. The mitochondria rupture when sonicated and yield inner and outer membranes which separate on sucrose density gradients. Outer membrane fractions from the gradient appear at 28–35% sucrose and contain 70% of the monoamine oxidase activity, 90% of the tritium radioactivity, and essentially no cytochrome *c* oxidase activity. Inner membrane fractions from the gradient appear at 54–68% sucrose and contain 90% of the cytochrome *c* oxidase and 27% of the monoamine oxidase activity.

The study of mitochondrial structure, composition, and function is basically dependent upon an adequate separation of the mitochondria from the cells, and upon successful fractionation of the mitochondria into their component parts (inner and outer membranes, and matrix fractions). Mammalian mitochondria are easily accessible and are generally prepared by a variety of differential centrifugation procedures (Parsons *et al.,* 1967; Schnaitman and Greenawalt, 1968; de Duve *et al.,* 1955). Yeast mitochondria are less accessible due to the heavy cell wall. They can be prepared by breaking the cells in a Braun homogenizer (Henson *et al.,* 1968) or by homogenization or osmotic shock of the spheroplasted cells (Hutchison and Hartwell, 1967). The separation of mitochondrial inner and outer membranes can be accomplished by osmotic shock (Bandlow, 1972), digitonin treatment (Schnaitman *et al.,* 1967), or by sonication (Whereat *et al.,* 1969), all usually followed by sucrose density gradient fractionation.

All of the above procedures are monitored for success on the basis of enzymatic activity for marker enzymes of the mitochondrion and its component membranes and compartments. As pointed out by Huber and Morrison (1973), difficulties arise when the exact location of these enzymes is not clear. This is especially true with regard to the outer membrane. In addition, known marker enzymes (such as the monoamine oxidase) may be quite low in activity, or appear in mammalian mitochondria but, perhaps, not in yeast mitochondria (Bandlow, 1972). Problems such as these may be circumvented by the use of nonenzymatic markers, such as a radioactive label. The addition of a tritium label to the outer surface of an intact mitochondrion can be accomplished by reduction of Schiff bases with Na^3BH_4. The Schiff base formation between free amino groups on the mitochondrion and pyridoxal phosphate, and the subsequent gentle reduction with sodium borotritide, proceeds under physiological conditions and apparently does not affect mitochondrial function. This procedure was first described by Cooper and Reich (1972) for labeling isolated proteins and was subsequently used by Rifkin *et al.* (1972) for labeling the outer surface of influenza viruses.

Materials and Methods

Chemicals and Chemical Methods. Ultrapure sucrose for density gradients and tris(hydroxymethyl)aminomethane (Trizma Base) were obtained from Sigma Chemical Co. (St. Louis, Mo.); glusulase, Endo Laboratories (Garden City, N. Y.); and pyridoxal phosphate, Calbiochem (La Jolla, Calif.). [1-^{14}C]Stearic acid (56 Ci/mol) was obtained from Amersham/Searle (Des Plaines, Ill.); benzylamine, Eastman Kodak Chemicals (Rochester, N. Y.); cytochrome *c* (horse heart, crystalline), Boehringer Mannheim Corp. (New York, N. Y.). Sodium boro[^{3}H]hydride (138 Ci/mol) was obtained from

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New England Nuclear (Boston, Mass.). All other chemicals were of reagent grade.

Cytochrome *c* was oxidized with a slight stoichiometric excess of potassium ferricyanide and dialyzed overnight at 4° in a 1:200 ratio against 0.smcnM potassium phosphate, pH 6.4. Cytochrome *c* was reduced catalytically at 24° with hydrogen gas in the presence of platinum asbestos. The reduced cytochrome *c* was filtered, and had an absorbance ratio (500 nm/565 nm) of 6.0.

Protein was determined colorimetrically (Lowry *et al.*, 1951). Refractive indices of sucrose gradient fractions were measured with a Bausch and Lomb refractometer (24°). Sucrose concentration and density then were determined from standard reference tables.

Cell Growth, Harvesting, and Fractionation. Sacchromyces carlsbergensis 4228 (ATCC 9080), an inositol-requiring strain, was used in all experiments.

Cells were grown aerobically in 2-l. flasks in a New Brunswick incubator (rotary) shaker set at 27° and 250 rpm. The growth medium was chemically defined (McKibbin, 1959), and was supplemented with 0.5 mg/ml of inositol where indicated; 2% glucose, 2% galactose, or 2% lactate was used as indicated in the text. A 24-hr culture, grown on the appropriate carbon source and supplemented with inositol as desired, was used as the inoculum for each experiment.

Cell growth was monitored at 650 nm in a Bausch and Lomb Spectronic 20, or by colony counts of appropriate dilutions of the cultures on solid medium (2% agar, 1% yeast extract, 1% bactopeptone, 0.1% KH_2PO_4, 0.12% $(NH_4)SO_4$, and 2% glucose or galactose). Cells were harvested by continuous flow centrifugation at 8° and 23,500*g* (R_{max} = 4.25 in.), using the Sorvall Szent-Gyorgi adapter and SS-34 rotor.

The harvested cells were washed three times with glass-distilled H_2O, 4340*g* (R_{max} = 4.75 in.) for 10 min at 4°, and resuspended at one-tenth the growth volume in 0.8 M sorbitol, 30°. Cells were converted to spheroplasts in the presence of 1% glusulase, as described elsewhere (Hutchison and Hartwell, 1967). Spheroplasting was monitored by diluting 0.1 ml of the cells (spheroplasts) in 0.9 ml of 0.25 M sucrose–0.02 M Tris–0.001 M EDTA, pH 7.4 (hereafter called sucrose–Tris–EDTA buffer). Decrease in A_{620nm} due to spheroplast lysis was monitored until maximum spheroplasting or fragility of the cells (60–90%) was attained (15–20 min). The spheroplasts were chilled and sedimented at 4340*g* (R_{max} = 4.25 in.) for 10 min and resuspended with agitation in one-tenth the growth volume of sucrose–Tris–EDTA buffer, 4°. The lysed spheroplasts were centrifuged at 2000*g* for 10 min. The resulting pellet contained cell walls and unlysed cells and was discarded. The supernate was recentrifuged under the same conditions. The second, small pellet was also discarded and the supernate was centrifuged at 15,956*g* (R_{max} = 10.5 cm) for 30 min in a Spinco 30 rotor. The resulting mitochondrial pellet was washed 4–5 times under the same conditions in sucrose–Tris–EDTA buffer. The yield of mitochondrial fraction protein from 1 l. of cells grown, in the presence of inositol, to early- and mid-to-late-log phase of growth was 3–8 and 15–20 mg of protein, respectively. The yield from 1 l. of cells grown, in the absence of inositol, to early- and mid-to-late-log phase was 3–5 and 8–10 mg of protein, respectively.

In a few experiments, washed cells were suspended in sucrose–Tris–EDTA buffer and ruptured mechanically for 10 sec with glass beads in a cell disintegrator (Bronwill Model MSK); 3 g of glass beads (0.45 mm diameter) were used for each gram of yeast cells (wet weight). The yield of mitochondrial fraction protein from 1 l. of cells fractionated in this manner was variable. On an average, it was 6 mg of protein and 7–10 mg of protein in the cases of early- and mid-to-late-log phase of growth, respectively. No cells grown in the absence of inositol were fractionated in this manner.

Mitochondrial Fractionation. Mitochondria were ruptured in one of two ways. One procedure involved osmotic shock. The mitochondrial fraction was resuspended in 70% sucrose–0.02 M Tris (pH 7.4) such that protein concentration was 10 mg/ml. The suspension was gently stirred at 4° for 15 min. The mitochondrial fraction was diluted rapidly with 0.02 M Tris (pH 7.4) to 10% sucrose (and 1.4 mg/ml protein), and gently homogenized at 4° with four complete up and down strokes in a 5- or 10-ml glass–Teflon homogenizer, depending on the volume of mitochondrial fraction.

A second procedure involved sonication. The mitochondrial fraction was resuspended in sucrose–Tris–EDTA (pH 7.4) at 7–10 mg of protein/ml, 4°. In an ice bath at 4°, the suspension was sonicated for 1 sec and stirred gently for 30 sec. The process of sonication and stirring was repeated six times, with the sonicator (Blackstone Biosonic, regular probe) set for maximum output.

Inner and outer mitochondrial membranes were separated from each other and from intact mitochondria by centrifugation of the disrupted mitochondria on a continuous 20–70% sucrose gradient (containing 0.02 M Tris + 0.001 M EDTA, pH 7.4, hereafter termed a buffered sucrose gradient) (SW 27 or 27.1 rotor, 67,100*g* for 120 min). The gradients were established with a standard gradient maker and polystaltic pump. The gradient fractions were collected from the top with a Buchler Automatic Densiflow apparatus and an LKB Ultrorac fraction collector.

Labeling Procedures. [1-^{14}C]Stearic acid (27 μCi) was added as the sodium salt to each 1000 ml of initial growth medium. Cells from a 24-hr inoculum were added to A_{620nm} = 0.02–0.04 to this same medium.

Tritium incorporation onto the mitochondrial surface was performed as described for virus labeling (Rifkin *et al.*, 1972), with a few modifications. Mitochondria were diluted to 4 mg of protein/ml (instead of 1 mg/ml) in sucrose–Tris–EDTA buffer, and treated with 0.04 μmol of pyridoxal phosphate/mg of mitochondrial protein. With regard to virus labeling, this concentration would provide a 50-fold excess of pyridoxal phosphate as calculated from the number of amino termini per virion (Rifkin *et al.*, 1972). However, it probably would not provide such an excess in the case of mitochondrial protein. As will be shown, however, it did result in successful labeling of at least part of the mitochondrial proteins. A tenfold molar excess of NaB^3H_4 then was added, and incubation proceeded at 4° for 15 min. Residual sodium borotritide was consumed by addition of an excess of pyridoxal phosphate. The mitochondria were sedimented at 35,956*g* (R_{max} = 10.5 cm) for 30 min and dialyzed overnight against three changes of sucrose–Tris–EDTA buffer at 4°.

Radioactivity in the various fractions was determined in a Nuclear Chicago Mark I liquid scintillation counter, set for single or dual label counting as needed. Particulate samples were dissolved overnight in 0.4 ml of Beckman BBS-2 Biosolve prior to counting in 10 ml of counting fluid (60 g of naphthalene, 4 g of 2,5-diphenyloxazole, 0.2 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene, 100 ml of 95% MEOH, 20 ml of ethylene glycol, and 1,4-dioxane to 1 l.).

Enzyme Assays. Oxygen consumption was measured polarographically at 28° with a modified Cark oxygen electrode in a 3.2-ml chamber, fitted for rapid stirring, and recorded on a Sargent Welch SRG recorder. The chamber contained 3.0 ml

TABLE I: Effects of Temperature, Carbon Source, and Inositol on Doubling Time of *S. carlsbergensis*.

Carbon Source	Temp, °C	Doubling Time[a] (min) With Inositol[b]	Doubling Time[a] (min) Without Inositol
Glucose (2%)	31	90	96
	27	102	156
	24	120	168
Galactose (2%)	27	108	156
Lactate (2%)	27	318	378

[a] Cells were grown aerobically as described in Materials and Methods; doubling time was determined from the straight line portion of log increase in culture density and cell number. [b] 0.5 mg/ml inositol.

of buffer (1% ETOH + 0.05 M phosphate buffer, pH 7.4) and 0.2 ml of yeast cell suspension. Measure of oxygen consumption was based on 0.240 μmol of O_2/ml of buffer, and is expressed as nmol of O_2 utilized per min per mg of protein (or Q_{O_2}), or nmol of O_2 utilized per min per ml of cell suspension (units of O_2 consumption).

Mitochondria were assayed for monoamine oxidase (Schnaitman *et al.*, 1967; EC 1.4.3.4) using benzylamine hydrochloride as substrate. The reaction was monitored at 250 nm in a Cary 15 recording spectrophotometer; the reference cuvet contained all the components of the reaction mixture except benzylamine hydrochloride.

Cytochrome *c* oxidase (Wharton and Tzagoloff, 1967, EC 1.9.3.1) and succinate-cytochrome *c* oxidoreductase (Mackler *et al.*, 1962; Polakis *et al.*, 1965; EC 1.3.99.1) were monitored at 545 nm in the Cary 15. One unit of monoamine oxidase, cytochrome *c* oxidase, or succinate-cytochrome *c* oxidoreductase activity equals a change in optical density (at the appropriate wavelength) of 0.001 per min per 0.1 ml of enzyme of mitochondrial fraction. Kynurenine hydroxylase (EC i.14.13.9) and malate dehydrogenase (EC 1.1.1.37) were assayed according to Bandlow (1972).

Results

Saccharomyces carlsbergensis strain 4228 (ATCC 9080) requires inositol for optimum growth (Table I). Cells growing aerobically on 2% glucose at 27° in the presence or absence of inositol (final concentration, 0.5 mg/ml) exhibit doubling times of 102 and 156 min, respectively, during exponential growth. Cells grown aerobically on 2% galactose at 27°, in the presence or absence of inositol, exhibit doubling times of 108 and 156 min, respectively. *S. carlsbergensis* cells grow very slowly on 2% lactate either in the presence or absence of inositol. In addition, as shown in Table II, Q_{O_2} for cells grown on 2% glucose in the presence of 0.5 mg/ml of inositol was higher, at all points in the growth curve (only early- and late-log phase values are shown), than the Q_{O_2} for cells grown on osucose minus inositol. Initol supplementation did not affect Q_{O_2} of galactose grown cells until the mid-log phase of growth. At the late-log state, the effect of inositol supplementation on Q_{O_2} was pronounced.

In all cases, growth in the presence of inositol yields a shorter lag phase and doubling time, and also a higher Q_{O_2} during the log phase of growth. Although galactose grown cells have a longer doubling time (Table I) than glucose grown cells, 2% galactose was used in all subsequent experiments. The Q_{O_2} for galactose grown cells is consistently higher than for cells grown on glucose (Table II). Since Q_{O_2} values may be partially an indication of mitochondrial function, it appeared that galactose provided less "glucose or catabolite repression" of mitochondrial development (Polakis *et al.*, 1965; Lukins *et al.*, 1968).

Saccharomyces carlsbergensis cells were broken and fractionated by the procedures described in Materials and Methods. Cells broken by homogenization with glass beads in the Bronwill homogenizer yielded mitochondria which were damaged as viewed by electron microscopy. When the mitochondria thus obtained were layered on a 20–70% buffered sucrose gradient and centrifuged at 67,100*g* (R_{av} = 11.7 cm) for 120 min, there was no single distinct band for mitochondrial cytochrome *c* oxidase, succinate-cytochrome *c* oxidoreductase, or monoamine oxidase activities. These enzymes were spread across the gradient in broad bands. Recentrifugation, under the conditions described above, of the pooled fractions represented by these bands resulted in no resolution into finer peaks.

In contrast, *S. carlsbergensis* cells broken by glusulase treatment yielded more intact mitochondria, confirmed by electron microscopy. When these mitochondria were sedimented as described above, they yielded a sharper band of mitochondrial enzyme activity (cytochrome *c* oxidase) (Figure 1). Some damaged mitochondria were present, as is shown by the broad base of the peak. The peak of mitochondrial enzyme activity is at 50% sucrose density (ρ = 1.18–1.19 g/ml), which is intermediate to the results reported for wild type *S. cerevisiae* and *carls-*

TABLE II: Absorbance and Q_{O_2} during Two Stages in the Growth Cycle of *S. carlsbergensis* Cells Grown under Various Conditions.

Growth Conditions[a]	Early-Log Phase A_{620nm}	Early-Log Phase Cells/ml	Early-Log Phase Q_{O_2}[b]	Late-Log Phase A_{620nm}	Late-Log Phase Cells/ml	Late-Log Phase Q_{O_2}
With inositol						
Glucose	0.23	5.5×10^6	47	1.40	3.6×10^7	102
Galactose	0.25	6.0×10^6	75	1.40	3.5×10^7	401
Without inositol						
Glucose	0.19	4.5×10^6	14	0.75	1.9×10^7	40
Galactose	0.19	4.6×10^6	74	1.10	2.9×10^7	241

[a] Cells were grown as described in Materials and Methods on 2% hexose plus 0.5 mg/ml of inositol as indicated. [b] Q_{O_2} is expressed in nmoles of O_2 utilized per min per mg of protein at 25°. Assay is described in Materials and Methods.

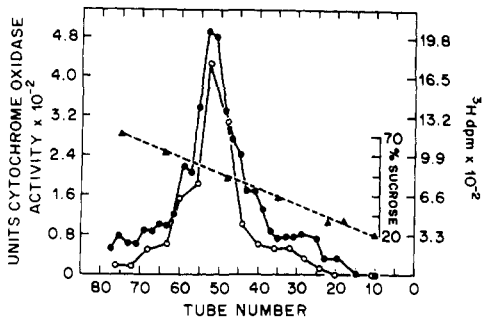

FIGURE 1: Sucrose density gradient profile of whole mitochondria derived from cells grown to A_{620nm} = 0.5 on 2% glucose medium containing 0.5 mg/ml of inositol. Cells were treated with glusulase and the mitochondria labeled with tritium according to Materials and Methods. Fractions of 0.5 were collected. Aliquots (0.1 ml) of indicated fractions ml were used for measuring cytochrome oxidase and ^3H activity. (□) Cytochrome *c* oxidase activity (all units are defined in Materials and Methods); (●) tritium decay (^3H dpm/0.1 ml gradient fraction); (▲) sucrose concentration (determined from refractive index of 50 μl of the designated fractions).

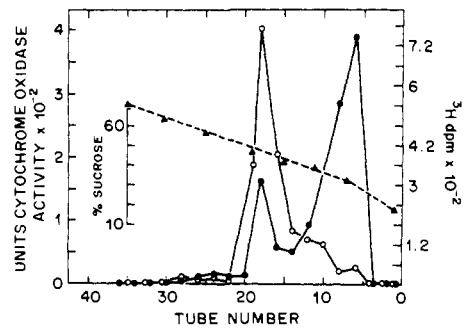

FIGURE 2: Sucrose density gradient profile of ^3H-labeled mitochondria of Figure 1, ruptured by sonication. Fractions of 0.4 ml were collected. (○) Cytochrome oxidase activity; (●) ^3H decay; (▲) sucrose concentration.

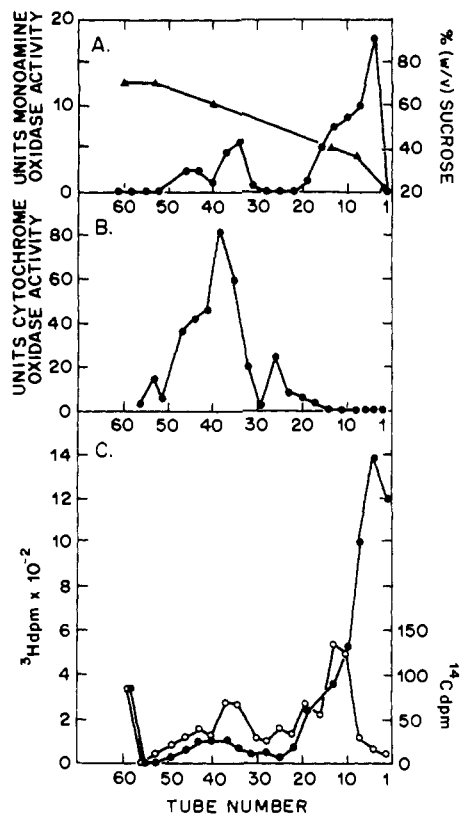

FIGURE 3: Sucrose density gradient profile of ^3H-labeled, sonicated mitochondria obtained from cells grown to A_{620nm} = 0.35 on 2% galactose medium containing inositol (0.5 mg/ml) and sodium [1-^{14}C]stearate. Cells were treated with glusulase and the mitochondria labeled with tritium and sonicated according to Materials and Methods. Fractions of 0.3 ml were collected. Aliquots (0.1 ml) of indicated fractions were used for measuring enzyme activity and radioactivity. (A) ●, monoamine oxidase activity; ▲, sucrose concentration. (B) ●, cytochrome oxidase activity. (C) ●, ^3H decay; ○, ^{14}C decay.

bergensis mitochondria harvested at late-log phase and maximum stationary phases of growth (Tuppy *et al.*, 1968; Cartledge and Lloyd, 1972; Schatz, 1963).

Prior to centrifugation, the outer surface of the mitochondria of Figure 2 were labeled with NaB^3H_4, as described in Materials and Methods. This technique was described by Cooper (Cooper and Reich, 1972) and applied and studied in detail by Rifkin using virus particles (Rifkin *et al.*, 1972). Tritium label is applied to the outer surface only of an intact cell, or in this case, organelle by reduction of Schiff bases formed at the surface between pyridoxal phosphate and any free amino groups present in the membrane protein. Such groups could be ε-amino groups of lysine or N-termini of polypeptides (Rifkin *et al.*, 1972). The tritium label should not appear on any inner membrane proteins if the mitochondrion is intact, since the pyridoxal phosphate (a phosphomonoester) should not pass through a biological membrane (Rifkin *et al.*, 1972). Therefore, the only Schiff bases formed should be on the outer membrane (and in fact, on the outside of the outer membrane), and reduction of these bases with NaB^3H_4 should leave the tritium label only on the outer membrane. As can be seen in Figure 2, this radioactive marker follows closely the marker for enzyme activity of these intact mitochondria.

Schiff base formation and subsequent reduction proceed at physiological pH (7.4) and temperature (37°). In a test experiment, cytochrome *c* oxidase and monoamine oxidase activities were measured before, during, and after treatment of intact mitochondria with pyridoxal phosphate and NaB^3H_4. The activities (expressed in terms of change in optical density of 0.001 per min per mg of protein at 24°) were as follows: (1) before treatment, 390 (cytochrome *c* oxidase) and 9.5 (monoamine oxidase), (2) during treatment, 131 and 1.5, (3) after dialysis for 3 hr, 405 and 10.1. Thus, the introduction of the ^3H label (and the procedure for doing so) does not affect permanently, at least, these mitochondrial functions.

The mitochondria labeled with ^3H in this manner were ruptured by sonication, or by osmotic shock and homogenization (see Materials and Methods), and were centrifuged through a 20–70% buffered sucrose gradient at 67,100*g* for 2 hr. Figure 2 shows the result of this procedure using sonication as a means of mitochondrial disruption. Cytochrome oxidase, a specific marker for mitochondrial inner membrane, is present as one major peak at 46% sucrose (ρ = 1.174), a slightly lower density than that observed for whole mitochondria (Figure 2). The tritium activity in Figure 2 is present as a major new peak at 30% sucrose (ρ = 1.115). A small but significant peak of ^3H activity remains at 46% sucrose (see below).

Data in Figure 3 show that the tritium actitvity follows a functional marker for mitochondrial outer membrane. Monoamine oxidase activity is present in the outer membrane of mammalian mitochondria (Schnaitman *et al.*, 1967). One investigator has reported its absence in a strain of *S. cerevisiae* (Bandlow, 1972); however, in our experiments, it could be measured in mitochondria from inositol supplemented *S. carlsbergensis* cells. It could not be measured in mitochondria from unsupplemented cells.

For the experiment shown in Figure 3, mitochondria were obtained from cells grown in the presence of 2.7 μCi of sodium [1-^{14}C]stearate/100 ml of media. This resulted in the in corporation of ^{14}C into the cellular membranes (Johnston and Paltauf, 1970; Paltauf and Johnston, 1970). One-half of the mitochondria were treated with pyridoxal phosphate and NaB^3H_4 and the other half were left unlabeled. Both mitochondrial fractions were ruptured by sonication, and centrifuged through a 20–70% buffered sucrose gradient; the fractions were collected and analyzed for radioactivity, enzyme activity, and protein as described in Materials and Methods. It was necessary to leave a portion of the mitochondria unlabeled, since the washing and overnight dialysis involved in labeling the mitochondria resulted in a significant loss of monoamine oxidase activity, although cytochrome oxidase activity was not affected. Mere storage of unlabeled mitochondria at 4 or −20° overnight caused an identical loss in monoamine oxidase activity.

Monoamine oxidase activity is present in one major band (containing approximately 70% of the total monoamine oxidase activity units) with a major peak at 28% and shoulder at 35%, and two smaller bands (containing approximately 27% of the total monoamine oxidase activity units) with peaks at 56 and 64% sucrose (Figure 3A). In a separate experiment, kynurenine hydroxylase activity (Bandlow, 1972) showed a similar distribution pattern to monoamine oxidase activity. Essentially all of the cytochrome *c* oxidase activity is present in a broad band from 54–68% sucrose (Figure 3B). At least 90% of the tritium marker is present in the band representing the major monoamine oxidase peak (Figure 3C). Significantly, little or no cytochrome *c* oxidase activity is present in the fractions containing the major tritium activity and monoamine oxidase, thus demonstrating separation of the monoamine oxidase from the cytochrome *c* oxidase. ^{14}C activity was present in all fractions corresponding to major enzyme peaks, demonstrating ^{14}C incorporation into both inner and outer membranes. The ^{14}C is most likely incorporated directly as sodium [1-^{14}C]stearate, based on observations of direct acyl chain incorporation into yeast cellular lipids (Johnston and Paltauf, 1970), and observations, in our laboratory, that less than 0.5% of the added labeled fatty acid is converted to $^{14}CO_2$ whether the cells are grown on glucose or galactose. Again, a small but significant amount of tritium activity (and monoamine oxidase activity) was detectable in the broad band containing mitochondrial cytochrome *c* oxidase. This could be accounted for in several ways.

First, the brief sonication time could leave some mitochondria intact. Indeed, data from a similar experiment (data not shown) in which the mitochondria were ruptured by the milder procedure of osmotic shock and homogenization did not completely lyse the mitochondria. The results were similar to those presented for sonicated mitochondria, except for the distribution of monoamine oxidase in the sucrose gradient; 28% of the total monoamine oxidase activity units appeared in a band from 28 to 40% sucrose, in contrast to 70% of the total activity in the case of sonically disrupted mitochondria. The remaining 72% of the activity units appeared in a band at 52–64% sucrose, corresponding to the major peak for cytochrome *c* oxidase activity. It is also possible that some of the tritium label appeared on the inner membrane due to the presence of some broken mitochondria at the time the label was introduced. This cannot be ruled out, but the presence of monoamine oxidase activity along with tritium activity in the major peak for cytochrome *c* oxidase suggests the presence of a small amount of outer membrane. Another explanation could be that outer membrane was entrained in the inner membrane upon sonication. This could be fortuitous, due to vesicularization of the membrane upon sonication, or it could be due to the reported presence of points of intimate contact between inner and outer membrane in the intact mitochondrion (Hackenbrock, 1968). These areas of contact may remain with the inner membrane fractions upon sonication and gradient centrifugation.

Malate dehydrogenase, a matrix associated enzyme (Bandlow, 1972), appeared in the fractions containing cytochrome oxidase activity (54–68% sucrose). This suggests the inner membrane fractions are not "empty membrane fractions. Malate dehydrogenase activity in the outer membrane fractions was less than 5% of the activity in the inner membrane fractions.

Discussion

The covalently bond tritium marker has been recently introduced for identifying cell surface membranes (Rifkin *et al.*, 1972), but it has not been applied to subcellular organelles. Tritium incorporation is achieved by sodium borotritide reduction of Schiff bases formed between pyridoxal phosphate and protein amino groups on the membrane (Churchich, 1965). Membrane surfaces are preferentially labeled due to the inability of phosphomonoesters, including pyridoxal phosphate, to penetrate the membranes (Rifkin *et al.*, 1972). The reaction proceeds smoothly at physiological pH, temperature, and ionic strength, and leads to the incorporation of tritium at high specific radioactivity without physiological damage.

This now appears particularly useful in mitochondrial systems, where activity is low for enzymes used as markers for outer membrane identification. These enzymes traditionally have been monoamine oxidase (Ernster and Kuylenstierna, 1970; Schnaitman *et al.*, 1967; Schnaitman and Greenawalt, 1968), and kynurenine hydroxylase (Schott *et al.*, 1970; Okamoto *et al.*, 1967). It is additionally helpful in our work involving *S. carlsbergensis* 4228 (ATCC 9080) since this strain, when grown in the absence of inositol, exhibits no monoamine oxidase activity measurable by our techniques. Our results from use of a tritium label to mark the mitochondrial surface show that the radioactive label follows monoamine oxidase activity upon mitochondrial disruption. It therefore can be considered to be associated largely with the outer mitochondrial membrane and provides independent information relative to identification of individually enriched inner and outer membrane preparations.

Another method of membrane labeling is an iodination catalyzed by lactoperoxidase (Phillips and Morrison, 1971; Morrison *et al.*, 1970; Huber and Morrison, 1973). This procedure is also based on the impermeability of the biological membranes to the labeling agent (lactoperoxidase). The effect of this procedure on membrane associated enzyme activity is not clear from published reports using this method on mitochondrial membranes (Huber and Morrison, 1973; Astle and Cooper, 1974). The tritium labeling technique, however, proceeds smoothly without apparent damage to mitochondrial function. Rifkin *et al.* (1972) also report no marked damage of the function of influenza viruses labeled with tritium by this technique.

In preparation of mitochondria for tritium labeling, we found that mitochondria obtained from osmotically shocked *S. carlsbergensis* spheroplasts are the most useful for subsequent separation of inner and outer mitochondrial membrane. The use of glusulase to produce spheroplasts was limited, however, to yeast cells in early-, mid-, and possibly late-log phase. Spheroplasting becomes more difficult as cells age and the cell wall thickens, requiring prolonged periods of glusulase treatment, during which time cellular (and mitochondrial) changes

occur (Hutchison and Hartwell, 1967). Thus, studies were limited to cells in early-to-mid-log growth.

The separation of bands of mitochondrial enzyme activity (cytochrome *c* oxidase and monoamine oxidase) on a sucrose gradient after mitochondrial disruption suggests the separation of functionally intact inner and outer mitochondrial membranes. Mitochondrial disruption by gentlye means such as osmotic shock results in only partial membrane separation following sucrose density centrifugation. Disruption by sonication is successful, although some outer membrane remains with the inner membrane, as shown by the presence of some ^3H (after tritium labeling) and monoamine oxidase activity in the areas of cytochrome oxidase activity. This is probably either due to entrainment of some outer membrane pieces with inner membrane, or to the presence of some intact mitochondria (as discussed in Results). Present studies are underway to show the nature of these fractions by electron microscopy.

In contrast to another report (Bandlow, 1972), our experiments show that protein containing cytochrome *c* oxidase activity from sonicated mitochondria bands at a density of ρ = 1.189 g/ml. Recognizing that this sonication procedure may have some mitochondria intact as mentioned above, the shoulder of cytochrome *c* oxidase activity in Figure 3B on the higher density side of the peak of activity may represent the enzyme present in intact mitochondria, with the major peak of activity corresponding to submitochondrial inner membrane fractions.

Other investigators (Schnaitman *et al.*, 1967; Bandlow, 1972) have shown inner membrane to band at densities of ρ = 1.199 g/ml (liver) and ρ = 1.190 g/ml (yeast). No resolution of the difference between the latter and our results can be given at this time; however, the data reported in this manuscript were obtained from yeast that were just recovering from catabolite repression. This could explain the density differences in these membranes as compared to the inner membrane of older cells since changes in mitochondrial density do occur during derepression (Cartledge and Lloyd, 1972; Schatz, 1963). Further studies on these separated mitochondrial membranes are under way, dealing with compositional changes in the mitochondria from *S. carlsbergensis* 4228 caused by altered growth conditions and by conditions of derepression and age.

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