Subcellular Fractionation by Zonal Centrifugation of Glucose-Repressed Anaerobically Grown *Saccharomyces carlsbergensis*

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1. Homogenates were prepared from sphaeroplasts of anaerobically grown, glucose-repressed *Saccharomyces carlsbergensis*, and the distributions of marker enzymes investigated after zonal centrifugation on sucrose gradients containing 2mM-MgCl₂.
2. These homogenates contained no detectable cytochrome *c* oxidase, succinate-cytochrome *c* oxidoreductase, succinate-ferricyanide oxidoreductase, L(+)-lactate-cytochrome *c* oxidoreductase or catalase. Cytochromes *a*+*a*₃ and *c* were not detected.
3. Zonal centrifugation of whole homogenates indicated complex density distributions of the sedimentable portions of NADH- and NADPH-cytochrome *c* oxidoreductases, adenosine triphosphatases (ATPases), adenosine pyrophosphatase (ADPase), pyrophosphatase and acid *p*-nitrophenyl phosphatase. Several different ATPases were distinguished on the basis of their sensitivities to oligomycin and ouabain.
4. Differential centrifugation of whole homogenates at 10⁵g-min left 80–90% of the protein, dithionite-reducible cytochrome *b*, acid hydrolases and pyrophosphatase in a supernatant (S₁) together with 65 and 56% of the NADH- and NADPH-cytochrome *c* oxidoreductases respectively, 25% of the ATPases and 71% of the adenosine monophosphatase.
5. Further analysis of supernatant S₁ revealed the presence of a class of small particles containing NADPH-cytochrome *c* oxidoreductases and ATPases.
6. At least four different populations of large particles were distinguished.
7. Electron microscopy indicated that one of these corresponded to 'promitochondria' as described by other workers.

Despite many earlier conflicting reports (for reviews see Marchant & Smith, 1968; Linnane & Haslam, 1970) it is clear from the work of Plattner & Schatz (1969), Damsky *et al.* (1969) and Watson *et al.* (1970) that the cytology of anaerobically grown *Saccharomyces cerevisiae* is more complex than was previously thought, and depends not only on the extent of glucose repression but also on supplementation of the growth medium by unsaturated fatty acids and sterols.

There have been few attempts at subcellular fractionation of anaerobically grown yeast. Schatz (1965) first described a multiplicity of sedimentable particles in density gradients of Urografin or sucrose, and proposed the existence of organelles ('promitochondria') that possessed low activities of some of the enzymes typically associated with the mitochondria of aerobically grown cells. This concept was later developed in a series of publications which reported the characterization, not only of the enzymic contents of the particles, but also their lipid composition and morphology (Criddle & Schatz, 1969; Paltauf & Schatz, 1969; Plattner & Schatz, 1969). However, these investigators did not present a complete survey of all fractions obtained from the whole

homogenates, but rather concentrated on elucidating the properties of particles taken from a single band of the density gradient.

In preliminary experiments with *Saccharomyces carlsbergensis* (Cartledge *et al.*, 1969, 1971) we applied the sphaeroplast method of breakage rather than the more intense mechanical methods previously employed for *S. cerevisiae*, but used similar fractionation conditions in that the preparation buffer and gradient contained 2mM-EDTA. Zonal centrifugation confirmed the presence of more than one population of sedimentable particles containing ATPases* and cytochrome *c* oxidoreductases. More recently we have found that the replacement of EDTA by MgCl₂ is essential for preservation of the integrity of many membrane systems isolated from aerobically grown *S. carlsbergensis* (as judged both by enzyme distributions and electron micrographs of isolated fractions; Cartledge & Lloyd, 1972a).

In the present paper we apply this modified technique to the fractionation of homogenates from anaerobically grown yeast and present a complete

* Abbreviations: ATPase, adenosine triphosphatase; ADPase, adenosine pyrophosphatase; IDPase, inosine pyrophosphatase; AMP, adenosine phosphatase.

survey of particle populations isolated under these conditions.

Materials and Methods

Maintenance and growth

Saccharomyces carlsbergensis (N.C.Y.C. 74S), was maintained on 10% (w/v) glucose-malt extract-agar slopes (Wallace *et al.*, 1968).

The complex growth medium was a modification of that used by Wallace *et al.* (1968), and contained the following (% w/v): glucose (10), yeast extract (0.05), $(\text{NH}_4)_2\text{SO}_4$ (0.12), CaCl_2 (0.01), NaCl (0.05), $\text{MgCl}_2 \cdot 5\text{H}_2\text{O}$ (0.07), KH_2PO_4 (0.1), Tween 80 (0.5), ergosterol (0.002) and FeCl_3 (0.0005). The pH of the medium was adjusted to 4.5 with 1.0M-HCl. Glucose, MgSO_4 and $(\text{NH}_4)_2\text{SO}_4$ were each sterilized separately and added to the cooled medium.

Starter cultures were grown as previously described (Cartledge & Lloyd, 1972a). Batch cultures (10 litres) were grown in a 14-litre Microferm Laboratory Fermentor (New Brunswick Scientific Co., N.J., U.S.A.); the temperature of growth was 30°C and the stirring rate 400 rev./min.

Residual traces of oxygen were removed from high-purity nitrogen (Air Products Ltd., Cardiff, U.K.) as follows: (1) passage through 4 litres of a thick suspension of yeast cells, (2) through 500 ml of Fiesers solution and (3) through 500 ml of water. Sparging (150 ml of N/min per litre of medium) was for 12 h before inoculation and during growth. Organisms were counted after suitable dilution and gentle hand homogenization (to separate clumps) in a Thoma haemocytometer cell (Hawksley, Lancing, nr. Brighton, Sussex, U.K.). The initial inoculum was 2×10^8 organisms. The culture was harvested 4 h after the onset of stationary phase when the population was between 2.0×10^8 and 2.1×10^8 organisms/ml as shown in Fig. 1.

Harvesting and preparation of extracts with a Hughes press

All centrifugation procedures were carried out at 4°C. A Hughes Press (Hughes, 1951) was used to prepare extracts for measurements of enzyme activities (Fig. 1). Cells (1 litre) were collected under an atmosphere of nitrogen in centrifuge pots containing chloramphenicol (1 g) and cycloheximide (100 mg). The suspension was centrifuged for 10 min at 1500g (r_{av} , 24 cm) in the 6 × 1-litre rotor of an MSE Mistral centrifuge. The pellet was suspended in 0.25M-sucrose – 2 mM- MgCl_2 – 10 mM-tris – HCl (pH 7.4) and the cells were broken in a Hughes press. The suspension was centrifuged for 5 min at 2000g (r_{av} , 7.6 cm) in an 8 × 50 ml rotor of an MSE 17 centrifuge and the supernatant used for determination of enzyme activities.

Harvesting and preparation and disruption of sphaeroplasts

Sphaeroplasts were formed as previously described (Cartledge & Lloyd, 1972a), but precautions to exclude oxygen and thus prevent respiratory adaptation were taken. Chloramphenicol (1 mg/ml) and cycloheximide (0.1 mg/ml) were added to the growth vessel 10 min before harvesting. Processing of the cells was carried out under an atmosphere of nitrogen in an anaerobic cabinet. Suspensions were removed from the cabinet for centrifugation only after transfer to sealed centrifuge pots. These precautions were relaxed only after breakage of sphaeroplasts, which was effected by using six cycles of a Teflon-glass hand homogenizer (clearance 0.01 cm). Partially digested cells, intact sphaeroplasts and nuclei were removed by centrifugation for 5 min at 2000g (r_{av} , 7.6 cm) in an 8 × 50 ml rotor of an MSE 17 centrifuge, and the supernatant (termed the whole homogenate) was decanted.

Fractionation by high-speed zonal centrifugation

Zonal centrifugations were performed in a B XIV zonal rotor as described previously (Cartledge & Lloyd, 1972a).

Enzyme assays

NAD(P)H-cytochrome *c* oxidoreductases (EC 1.6.2.1, EC 1.6.2.3), cytochrome *c* oxidase (EC 1.9.3.1), catalase (EC 1.11.1.6), acid *p*-nitrophenyl phosphatase (EC 3.1.3.2), AMPase (EC 3.1.3.5), ADPase (EC 3.1.3.7), ATPase (EC 3.6.1.4) and pyrophosphatase (EC 3.6.1.1) activities were assayed as described previously (Cartledge & Lloyd, 1972a). α -Galactosidase (EC 3.2.1.22), α -glucosidase (EC 3.2.1.20) and β -galactosidase (EC 3.2.1.23) were assayed as described by Cartledge & Lloyd (1972b). Succinate-, D(-)-lactate- (EC 1.1.2.4) and L(+)-lactate- (EC 1.1.2.3) cytochrome *c* oxidoreductases were assayed by the method used for NADH-cytochrome *c* oxidoreductase (Cartledge & Lloyd, 1972a) except that 186 μM -NADH was replaced by 10 mM-succinate, 5 mM-D(-)- or L(+)-lactate.

Other determinations

Cytochromes. Total cytochrome contents ($\text{Na}_2\text{S}_2\text{O}_4$ -reduced-oxidized) of homogenates were calculated from measurements of peak heights on spectra recorded at room temperature with a Cary model 14 recording spectrophotometer fitted with a fine slide wire. The absence of cytochromes *a*+*a*₃ was confirmed by spectra recorded at the temperature of liquid nitrogen.

Protein. Protein was measured by the method of

Lowry *et al.* (1951), with bovine serum albumin as standard.

Glucose and ethanol. These were determined with Sigma reagent kits numbers 510 and 330-UV respectively.

Electron microscopy. Electron micrographs were obtained as described by Cartledge & Lloyd (1972a).

Chemicals

All enzyme substrates and inhibitors (antimycin A, ouabain and oligomycin) and also ergosterol were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Bovine serum albumin was from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. Difco yeast extract was from Baird and Tatlock, Landore, Swansea, Glam., U.K., and mycological peptone from Oxoid Ltd., London S.E.1, U.K. *Helix pomatia* were obtained from Haig and Co., Beambrook, Surrey, U.K.

Presentation of results

Enzyme units are expressed as nmol of substrate transformed/min or as nmol of measured product formed/min. In the graphical presentation (Figs. 3 and 4) vertical lines divide the diagrams at density intervals of 0.05 g/ml. Percentage sedimentability is arbitrarily expressed as that proportion of the total enzyme units that had sedimented beyond $\rho = 1.10$ g/ml after centrifugation for a minimum of 6×10^6 g-min.

Results

Changes in enzyme activities during growth

The glucose concentration decreased during exponential growth (mean generation time 1.25 h) from 560 mM to 500 mM, and there was a gradual accumulation of ethanol (Fig. 1a). A more rapid utilization of glucose in the stationary phase of growth was accompanied by a sudden increase in the concentration of ethanol. The following enzymes were not detectable in homogenates prepared from cells sampled at any point during anaerobic growth [limits of detectability, expressed as specific activities (units/mg of protein), given in parentheses]: cytochrome *c* oxidase (<0.05), succinate-ferricyanide oxidoreductase (<0.9), succinate-cytochrome *c* oxidoreductase (<0.05), L(+)-lactate-cytochrome *c* oxidoreductase (<0.09), catalase (<0.3). Other enzymes present but not assayed as a routine included NADH- and NADPH-ferricyanide oxidoreductases, D(-)-lactate-cytochrome *c* oxidoreductase, alkaline phosphatase (EC 3.1.3.1) and IDPase (EC 3.6.1.6). The activities of NADH- and NADPH-cytochrome *c* oxidoreductases (which were both virtually in-

sensitive to the addition of $3.6 \mu\text{M}$ -antimycin A) remained constant during exponential growth, but the NADH-linked activity increased in the stationary phase whereas that of NADPH-cytochrome *c* oxidoreductase fell (Fig. 1b). Activities of α -glucosidase and α -galactosidase (Fig. 1c) and ADPase, AMPase and pyrophosphatase (Fig. 1d) all increased gradually during the stationary phase of growth. Acid *p*-nitrophenyl phosphatase and β -glucosidase (Fig. 1e) both increased in activity during exponential growth and declined when growth ceased. These results were representative of 12 different batch cultures.

Cytochromes of anaerobically grown cells

The absence of mitochondrial cytochromes was confirmed by the examination of difference spectra of whole cell suspensions both at room temperature (Fig. 2) and at the temperature of liquid N₂. No detectable cytochromes *a*+*a*₃, *c* or *c*₁ were present, nor was it possible to detect cytochrome *a*₁ (α -band at 590 nm) or cytochrome *c* peroxidase (absorption maxima at 439, 558 and 590 nm; Ishidate *et al.*, 1969). An unidentified absorption maximum at 445 nm (also observed by Ishidate *et al.*, 1969, in *S. cerevisiae*) cannot be due to the Soret band of cytochrome oxidase, as no corresponding α -band was seen. Absorption maxima at 422, 527 and 555 nm indicated the presence of cytochrome *b*₁ (Slonimski, 1953; Chaix & Heyman-Blanchet, 1957; Lindenmayer & Estabrook, 1958; Criddle & Schatz, 1969; Ishidate *et al.*, 1969). No contribution from cytochrome *b*₂ (Lindenmayer & Estabrook, 1958) was possible, as L(+)-lactate-cytochrome *c* reductase was not detectable. Of the total cytochrome *b* reducible by dithionite (as measured from difference spectra, 424–410 nm) only 19% was NADH-reducible, whereas further addition of NADPH gave 46% reduction. CO-difference spectra (dithionite-reduced+CO minus dithionite-reduced, Figs. 2c and 2d) indicated the presence of cytochrome *P*-450 and at least one other haemoprotein (at 414–420 nm), which has not been further identified, but which corresponds to cytochrome *P*-420 of Ishidate *et al.* (1969).

Fractionation of whole homogenate by zonal centrifugation

Centrifugation in the B XIV rotor was for 35000 rev./min for 165 min (a minimum of 6×10^6 g-min at the starting zone). The profiles of absorbance at 520 and 260 nm were complex (Fig. 3a), particularly in the region $\rho < 1.10$ g/ml. The starting zone still contained over 60% of the protein, with more than 50% of the total activities of NADPH-cytochrome *c* oxidoreductase (Fig. 3b), AMPase (Fig. 3d), acid

Fig. 1. Changes in cell population, glucose and ethanol concentrations in the medium and specific activities of marker enzymes of homogenates from *S. carlsbergensis* during anaerobic growth

For experimental details see the text. (a) Concn. of cells [log(cells/ml), ●], concn. of glucose (Δ) and concn. of ethanol (▲); (b) activities of NADH-cytochrome *c* oxidoreductase (■, □) and NADPH-cytochrome *c* oxidoreductase (▲, Δ); broken lines show the antimycin A-insensitive portion of each enzyme; (c) activities of acid *p*-nitrophenyl phosphatase (●), α-galactosidase (▲), α-glucosidase (○) and β-glucosidase (Δ); (d) activities of AMPase (●), ADPase (■) and pyrophosphatase (□).

Fig. 2. Difference spectra of anaerobically grown *S. carlsbergensis*

Cells were grown to a density of 2×10^8 /ml, harvested in an atmosphere of nitrogen, and resuspended at a density of 6.7×10^8 cells/ml. Spectra were recorded at room temperature. (a) Oxidized-oxidized; (b) dithionite-reduced-oxidized; (c) dithionite-reduced-dithionite-reduced; (d) dithionite-reduced+CO-dithionite-reduced.

p-nitrophenyl phosphatase, α - and β -glucosidases (Fig. 3e). A major portion of the NADH-cytochrome *c* oxidoreductase (Fig. 3b) had moved with a zone of oligomycin-insensitive, ouabain-sensitive ATPase (Fig. 3c) to a density of 1.07 g/ml. This zone (D) corresponds to the major peak of absorbance at 520 nm seen around fraction 9. Electron microscopy of fraction 10 revealed the presence of a mixed population of particles (Plate 1a); the electron-dense elements that predominated were heterogeneous in size but generally less than $0.5 \mu\text{m}$ in diameter and possessed a distinct ultrastructure (Plate 1b). Two zones of material were present in the overlay, as indicated by the trace of absorbance at 520 nm. The lightest of these (A) was probably lipid granules; fraction 5 (zone B) also contained small pieces of membrane, all $<0.3 \mu\text{m}$ (Plate 1c). None of the enzymes assayed could be definitely assigned to either of these particles, as there was incomplete resolution from the non-sedimentable portions of enzymes remaining at the starting zone (C). The trace at 260 nm indicates that ribosome monomers have reached fraction 15; ATPase, ADPase and pyrophosphatase were also present in this fraction. Ribosome subunits were present in fraction 13 (zone E) and the ribosome

dimer was evident in fraction 19 (zone G). Ribosome subunit, monomer and dimer were identified on the basis of values of $s_{20,w}$ calculated by using a computer programme constructed by Mr. M. Statham. Sedimentable material was heterogeneously distributed through the gradient at densities >1.10 g/ml, and enzymes found associated with these particles included antimycin A-insensitive NADH- and NADPH-cytochrome *c* oxidoreductases (Fig. 3b), oligomycin-sensitive and ouabain-insensitive ATPase (Fig. 3c), ADPase, pyrophosphatase (Fig. 3d) and acid *p*-nitrophenyl phosphatase (Fig. 3e). α - and β -Glucosidases and AMPase were virtually entirely non-sedimentable. Major sedimentable peaks of activity of NADH- and NADPH-cytochrome *c* oxidoreductases occurred at $\rho = 1.22$ g/ml (zone H) and 1.23–1.24 g/ml (zone I) respectively. Both oligomycin-sensitive and -insensitive ATPases also gave major zones in this region, whereas acid *p*-nitrophenyl phosphatase was predominantly associated with particles distributed between $\rho = 1.15$ and 1.23 g/ml. Electron micrographs of fraction 36 reveal a mixed population of particles (Plate 2a). The largest organelles (generally 0.4 – $0.7 \mu\text{m}$ diam.) possessed an ultrastructure resembling that of mitochondria

and having a highly organized inner membrane (Plates 2*b* and 2*c*). These particles have a more highly organized structure than those isolated from *S. cerevisiae* after similar growth conditions as described by Watson *et al.* (1970).

Preparation of fraction S₁ and its subfractionation by zonal centrifugation

Whole homogenate was centrifuged for 10 min at 10000*g* (r_{av} , 7.6 cm) in the 8 × 50 ml rotor of an MSE 17 centrifuge. The supernatant (S₁) was de-

canted from the pellet (P₁). Fraction S₁ still contained high proportions of all the enzymes assayed (with the exception of ATPase) and cytochrome *b* (Table 1), and this fraction was then subjected to zonal centrifugation under conditions identical with those used for fractionation of whole homogenate. After centrifugation at 35000 rev./min for 165 min (6×10^6 g·min at the sample zone), the absorbance at 520 nm revealed a major peak at $\rho < 1.10$ g/ml (zone D) (Fig. 4*a*). On this occasion there was no separation from the ribosome subunit peak (zone E). NADH-cytochrome *c* oxidoreductase activity (Fig. 4*b*) was

EXPLANATION OF PLATE I

Electron micrographs of sections of material from fractions 5 and 10 as shown in Fig. 3. (a) Fraction 10 ($\times 20000$); (b) fraction 10 ($\times 50000$); (c) fraction 5 ($\times 20000$). In all cases the bar represents $1.0\mu\text{m}$. Material was fixed in glutaraldehyde at $\rho = 1.07\text{g/ml}$ (for *a* and *b*) and $\rho = 1.04\text{g/ml}$ (for *c*).

EXPLANATION OF PLATE 2

Electron micrographs of sections of material from fraction 36 as shown in Fig. 3. (a) $\times 20000$; (b) $\times 32000$; (c) $\times 60000$. In (a) and (b) the bar represents $1.0\mu\text{m}$ and in (c) $0.1\mu\text{m}$. All material was fixed in glutaraldehyde at the equilibrium density ($\rho = 1.23\text{g/ml}$).

EXPLANATION OF PLATE 3

Electron micrographs of sections of material from the experiment shown in Fig. 4. (a)–(e) are all $\times 20000$ and the bar represents $1\mu\text{m}$. All material was fixed in glutaraldehyde at the density of sucrose in the fractions. (a) Fraction 9; (b) fraction 12; (c) fraction 17; (d) fraction 21; (e) fraction 33.

Fig. 3. Fractionation of a whole homogenate of anaerobically grown *S. carlsbergensis*

Whole homogenate (22.5 ml) containing 131 mg of protein was loaded on to the gradient. The volumes of homogenate (diluted 1 in 20) and of fractions taken for assay were as follows: catalase, cytochrome *c* oxidase and succinate-, D(-)-lactate-, L(+)-lactate-, NADH- and NADPH-cytochrome *c* oxidoreductases, all 0.1 ml; acid *p*-nitrophenyl phosphatase, acid α - and β -glucosidases, ATPase, AMPase, ADPase and pyrophosphatase, all 0.2 ml. Centrifugation was at 35000 rev./min for 165 min ($6 \times 10^6 g$ -min at the sample zone; $\int_0^t \omega^2 \cdot dt = 1.45 \times 10^{11} \text{ rad}^2/\text{s}$). Fractions (10 ml) were collected at 5°C. (a) Sucrose density gradient ($\circ$), light-scattering at 520 nm (----), 260 nm (—) and protein ($\bullet$); (b) activities of NADH-cytochrome *c* oxidoreductase ($\blacksquare$), NADPH-cytochrome *c* oxidoreductase ($\blacktriangle$) and antimycin A-insensitive NADPH-cytochrome *c* oxidoreductase ($\triangle$); (c) activities of ATPase ($\blacksquare$), oligomycin-insensitive ATPase ($\square$) and ouabain-insensitive ATPase ($\bullet$); (d) activities of AMPase ($\bullet$), ADPase ($\blacksquare$) and pyrophosphatase ($\square$); (e) activities of acid *p*-nitrophenyl phosphatase ($\bullet$), α -glucosidase ($\circ$) and β -glucosidase ($\triangle$). Specific activities of enzymes in the whole homogenate were as follows (recoveries in parentheses): protein (97%), NADH-cytochrome *c* oxidoreductase 8.25 (137%), NADPH-cytochrome *c* oxidoreductase 59.5 (77%), ATPase 46.9 (75%), AMPase 14 (88%), ADPase 15.3 (81%), pyrophosphatase 8.25 (67%), acid *p*-nitrophenyl phosphatase 2.4 (81%), acid α -glucosidase 6.8 (98%) and acid β -glucosidase 1.16 (129%).

predominantly associated with zone D, and a striking feature of this experiment was that none of this enzyme sedimented beyond $\rho = 1.10 \text{ g/ml}$. Membranes containing NADPH-cytochrome *c* oxidoreductase (Fig. 4b), however, were very heterogeneous with respect to density, and gave a major zone at $\rho = 1.25 \text{ g/ml}$. Oligomycin-insensitive ATPase (Fig.

4c) appeared to be associated with the ribosome monomer and dimer peaks (zones F and G; Fig. 4a), and also with particles of $\rho 1.15$ – 1.20 g/ml ; some oligomycin-sensitivity was manifest at $\rho > 1.20 \text{ g/ml}$. AMPase, pyrophosphatase and the three acid hydrolases assayed all showed different complex distribution patterns (Fig. 4d and 4e). As above (Fig. 3d),

Table 1. *Fractionation of a whole homogenate of anaerobically grown S. carlsbergensis to give fractions P₁ and S₁*

A whole homogenate (WH) was centrifuged at 10^5 g-min to give fraction P₁ and a supernatant (S₁). Fraction S₁ was then centrifuged in the B XIV zonal rotor at 6×10^6 g-min (Fig. 4). Values are the percentages of total enzymes of whole homogenate in the fraction S₁, and the recovery (%) of activity in fractions P₁ + S₁ referred to the whole homogenate. Values for recoveries of enzymes from the zonal-centrifugation step (referred to the amount of S₁ loaded on to the rotor) are given in the caption to Fig. 4. Enzymes not detectable in the whole homogenate were cytochrome *c* oxidase, catalase and succinate-cytochrome *c* oxidoreductase.

	$\frac{\text{Units in S}_1}{\text{Units in WH}} \times 100$	$\frac{\text{Units in P}_1 + \text{S}_1}{\text{Units in WH}} \times 100$
Protein	82.8	99.6
Acid <i>p</i> -nitrophenyl phosphatase	87.8	97.4
α -Glucosidase	83.9	97.1
β -Glucosidase	89.5	98.1
NADH-cytochrome <i>c</i> oxidoreductase	65.0	100.6
NADPH-cytochrome <i>c</i> oxidoreductase	56.3	126.0
ATPase	25.0	128.0
ATPase (+80 μ M-oligomycin)	66.0	150.0
AMPase	70.8	91.1
Pyrophosphatase	85.0	99.6
Dithionite-reducible cytochrome <i>b</i>	90.0	118.0

pyrophosphatase (and oligomycin-insensitive ATPase) sedimented with the ribosome monomer (Fig. 4*d*). Acid *p*-nitrophenyl phosphatase gave the major sedimentable zone at around $\rho = 1.20$ g/ml, whereas α -glucosidase was found at densities as high as 1.30 g/ml. Electron micrographs of fractions 9, 12, 17, 21 and 33 (Plates 3*a-e*) failed to reveal any particles larger than 0.1 μ m diameter.

A summary of sedimentabilities of enzymes in whole homogenate and in fraction S₁ is given in Table 2. A total of six density-gradient fractionation experiments with homogenates of anaerobically grown cells gave results essentially similar to those shown.

Discussion

Many of the enzymes present in aerobically grown glucose-de-repressed *S. carlsbergensis* (Cartledge & Lloyd, 1972*a*) cannot be detected when rigorous precautions are taken (*a*) to exclude oxygen during growth and harvesting, and (*b*) to inhibit protein synthesis before harvesting (Criddle & Schatz, 1969). After growth for 12–13 generations the inoculum accounts for less than 0.025% of the total cell numbers. Compared with homogenates of aerobically grown glucose-derepressed organisms, those prepared from the anaerobically grown glucose-repressed cells contain at least 800-fold less cytochrome oxidase and 30-fold less catalase (expressed as specific activities; units/mg of protein). None of the activities associated with the mitochondrial respiratory chain can be detected. This is in contrast

to the reports of Criddle & Schatz (1969) and Watson *et al.* (1970) that the 'promitochondria' of *S. cerevisiae* do contain considerable activities of succinate dehydrogenases (assayed with a number of different acceptors). We have been unable to demonstrate any such succinate-linked activities in homogenates of anaerobically grown *S. carlsbergensis*, and virtually no inhibition of NADH- and NADPH-cytochrome *c* oxidoreductases was produced on addition of antimycin A. Another characteristic of these homogenates is that, unlike those of aerobically grown cells, NADPH-cytochrome *c* oxidoreductase activity may be as much as eightfold that of the corresponding NADH-linked enzyme system.

The cytochrome complement appears similar to that reported for *S. cerevisiae* by Ishidate *et al.* (1969), except that we have been unable to demonstrate the presence of cytochrome *c* peroxidase. The major species include NADH- and NADPH-reducible cytochrome *b*₁, dithionite-reducible *b*-type cytochrome (which may also comprise part of the cytochrome *P*-420), cytochrome *P*-450 and an unidentified pigment at 445 nm that has no detectable absorption in the α -region.

Zonal centrifugation confirms the complexity of distribution of membrane components originally observed by Schatz (1965) and by Criddle & Schatz (1969) on density gradients of sucrose or Urografin; the present results enable further characterization of the various populations present.

Centrifugation of whole homogenates at 10^5 g-min sediments all the large particles, and the pellet (P₁)

produced contains the major portion of the ATPases with about half of the total units of NADPH-cytochrome *c* reductase, 35% of the NADH-linked enzymes and 30% of the AMPase. Less than 20% of the other enzymes assayed sedimented in this fraction. Comparison of the two zonal-centrifugation experiments presented indicates that these large particles are present in five zones (A, B, D, H and perhaps I, Figs. 3*a* and 4*a*). Particles in zone A must be lipid-rich ($\rho < 1.03$ g/ml). Zone B was not well resolved from the starting zone (C), and has not been further characterized. Zone D contains both

large and small particles, and the large particles contain oligomycin-insensitive, ouabain-sensitive ATPase. Large particles present in zones H and I contain oligomycin-sensitive ATPase, NADH- and perhaps NADPH-cytochrome *c* oxidoreductase.

Small particles present in supernatant *S*₁ include those of zone D, which contains NADH-cytochrome *c* oxidoreductase and also some NADPH-cytochrome *c* oxidoreductase. This zone has no counterpart in fractionations of aerobically grown glucose-repressed *S. carlsbergensis* (Cartledge & Lloyd, 1972*a*). Small particles sedimenting from fraction *S*₁

Fig. 4. Subfractionation of fraction S_1 from anaerobically grown *S. carlsbergensis*

Fraction S_1 (30ml), containing 91 mg of protein, was loaded on to the gradient. The volumes of S_1 (diluted 1 in 20) and of fractions taken for assay were as follows: NADH- and NADPH-cytochrome *c* oxidoreductases, 0.1 ml; acid *p*-nitrophenyl phosphatase, acid α -glucosidase, acid β -glucosidase, ATPase, AMPase and pyrophosphatase, all 0.2 ml. Centrifugation was at 35000 rev./min for 165 min ($6 \times 10^6 g \cdot \text{min}$ at the sample zone; $\int_0^t \omega^2 \cdot dt = 1.45 \times 10^{11} \text{ rad}^2/\text{s}$). Fractions (10 ml) were collected. (a) Sucrose density gradient ($\circ$), light-scattering at 520 nm (----), at 260 nm (—) and protein ($\bullet$); (b) activities of NADH-cytochrome *c* oxidoreductase ($\blacksquare$) and NADPH-cytochrome *c* oxidoreductase ($\blacktriangle$); (c) activities of ATPase ($\blacksquare$) and oligomycin-insensitive ATPase ($\square$); (d) activities of AMPase ($\bullet$) and pyrophosphatase ($\square$); (e) activities of acid *p*-nitrophenyl phosphatase ($\bullet$), α -glucosidase ($\circ$) and β -glucosidase (Δ). Specific activities of enzymes in S_1 were as follows (recoveries in parentheses): protein (97.4%), NADH-cytochrome *c* oxidoreductase 5.2 (51%), NADPH-cytochrome *c* oxidoreductase 39 (82.3%), ATPase 18.2 (74%), AMPase 8.8 (106%), pyrophosphatase 1.2 (218%), acid *p*-nitrophenyl phosphatase 0.9 (152%), acid α -glucosidase 0.53 (60%) and acid β -glucosidase 3.9 (75%).

to beyond $\rho = 1.10 \text{ g/ml}$ contain no NADH-cytochrome *c* oxidoreductase, but do contain the NADPH-linked enzyme system and several ATPases.

It is difficult to compare these results with those obtained previously on gradients containing 2 mM-EDTA (Cartledge *et al.*, 1969; Criddle & Schatz, 1969; Cartledge *et al.*, 1971), as the replacement of this chelator by MgCl_2 ensures greater integrity of many membrane systems, and also alters density distributions. Although fraction P_1 contains particles

analogous to those termed 'promitochondria' by Criddle & Schatz (1969) and 'mitochondrial precursors' by Watson *et al.* (1970), it is also evident that zones H and I contain at least two populations that are not well separated on a density basis. The large number of different sedimentable subcellular structures from anaerobically grown yeasts can only be completely characterized when they have been individually isolated as 'pure' fractions. Their multiplicity suggests that caution is necessary at

Table 2. *Percentage sedimentabilities of enzymes in homogenates of anaerobically grown S. carlsbergensis*

Percentage sedimentability is expressed as the percentage of total enzyme units recovered that had sedimented beyond $\rho = 1.10$ g/ml after centrifugation giving a force-time equivalent to 6×10^6 g-min at the starting zone.

	Percentage sedimentability of enzyme	
	Whole homogenate	Fraction S ₁
Protein	31.5	52.2
Acid <i>p</i> -nitrophenyl phosphatase	42.9	26.9
α -Glucosidase	0.5	55.8
β -Glucosidase	0.5	20
NADH-cytochrome <i>c</i> oxidoreductase	39.0	0
NADPH-cytochrome <i>c</i> oxidoreductase	42.2	65.4
ATPase	87.6	85.4
ADPase	49.0	33.0
AMPase	5.2	82.9
Pyrophosphatase	55.6	47.0

present in attempting to correlate their structure and function.

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