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Changes in Enzyme Activities and Distributions during Glucose De-repression and Respiratory Adaptation of Anaerobically Grown *Saccharomyces carlsbergensis*

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1. During anaerobic glucose de-repression the respiration rate of whole cells of *Saccharomyces carlsbergensis* remained constant and was insensitive to antimycin A but was inhibited by 30% by KCN. Aeration of cells for 1 h led to increased respiration rate which was inhibited by 80% by antimycin A or KCN. 2. Homogenates were prepared from sphaeroplasts of anaerobically grown, glucose de-repressed cells and the distribution of marker enzymes was investigated after zonal centrifugation on sucrose gradients containing $MgCl_2$. These homogenates contained no detectable cytochrome *c* oxidase or catalase activity. The complex density distributions of NADH– and NADPH–cytochrome *c* oxidoreductases and adenosine triphosphatase(s) [ATPase(s)] were very different from those of anaerobically grown, glucose-repressed cells. 3. The specific activity of total ATPase was lowered and sensitivity to oligomycin decreased from 58 to 7% during de-repression. 4. Cytochrome *c* oxidase and catalase activities were detectable in homogenates of cells after 10 min aeration. Zonal centrifugation indicated complex, broad sedimentable distributions of all enzyme activities assayed; the peaks of activity were at 1.27 g/ml. 5. Centrifugation of homogenates of cells adapted for 30 min and 3 h indicated a shift of density of the major sedimentable peak from 1.25 g/ml (30 min) to 1.235 g/ml (3 h). After 30 min adaptation a minor zone of oligomycin-sensitive ATPase and 15% of the total cytochrome *c* oxidase activities were detected at $\rho = 1.12$ g/l; these particles together with those of higher density containing cytochrome *c* oxidase, ATPase and NADH–cytochrome *c* oxidoreductase activities were all sedimented at $10^5 g$-min. 6. Electron microscopy indicated that the mitochondria-like structures of anaerobically grown, glucose-de-repressed cells were similar to those of repressed cells. After 10 min of respiratory adaptation highly organized mitochondria were evident which resembled the condensed forms of mitochondria of aerobically grown, glucose-de-repressed cells. High-density zonal fractions of homogenates of cells after adaptation also contained numerous electron-dense vesicles 0.05–0.2 μm in diameter. 7. The possibility that the 'promitochondria' of anaerobically grown cells may not be the direct structural precursors of fully functional mitochondria is discussed.

The process of respiratory adaptation, which occurs when suspensions of anaerobically grown yeast cells are aerated (Ephrussi & Slonimski, 1950), has been used extensively as a system for the study of mitochondrial development. Increasing respiratory activity of cells during adaptation is paralleled by increased activities of mitochondrial enzymes (Slonimski, 1953; Tustanoff & Bartley, 1964; Rouslin & Schatz, 1969), and increasing complexity of mitochondrial-membrane organization (Plattner *et al.*, 1970; Watson *et al.*, 1970). Studies of the time-course of formation of mitochondrial enzymes, e.g. oligomycin-sensitive ATPase* (Somlo, 1968) and cytochrome *c* oxidase (Chen & Charalampous, 1969; Sels & Verhulst, 1971), have revealed that this is a rapid process occurring within 1 h of the onset of aeration. The production of catalase, an enzyme which is partly located in peroxisomes in yeast (Avers & Federman, 1968; Cartledge *et al.*, 1969), also occurs rapidly during respiratory adaptation (Chantrenne & Courtois, 1954).

The progress of the integration of newly synthesized cytochrome components into functional electron-transport chains has been investigated by measurement of the kinetics of cytochrome oxidations in mitochondria isolated at various stages in the adaptation process (Cartledge *et al.*, 1972). It was shown that the *a*- and *c*-type cytochromes of homogenates, prepared from cells at various time-intervals after the commencement of adaptation, were quantitatively recovered in a fraction which was

* Abbreviation: ATPase, adenosine triphosphatase.

completely sedimentable in 0.25M-sucrose buffer at $10^5 g$-min (Cartledge *et al.*, 1972).

In the present paper we investigate the extensive changes in activities and subcellular distributions of some membrane-bound marker enzymes which occur during respiratory adaptation. The complexity of these changes suggests a massive reorganization of membrane distribution within the yeast cell during the transition from the anaerobic to the aerobic phenotype.

Materials and Methods

Maintenance and growth

Saccharomyces carlsbergensis (N.C.Y.C. 74S) was maintained and grown under strictly anaerobic conditions in a complex growth medium in the presence of 10% (w/v) glucose and lipid supplements (Tween 80 and ergosterol) as previously described (Cartledge & Lloyd, 1972*c*).

Respiratory adaptation of cells

Anaerobic glucose de-repression and respiratory adaptation were carried out at a cell density of 5×10^7 cells/ml in a medium containing (%, w/v): glucose (0.4), yeast extract (0.2), $CaCl_2$ (0.033), KH_2PO_4 (0.9), $MgSO_4$ (0.05), and $(NH_4)_2SO_4$ (0.6) at pH 5.0 as previously described (Cartledge *et al.*, 1972).

Harvesting of cells and preparation of extracts

Cells were harvested and, for the determination of total enzyme activities, extracts were prepared by using a Hughes Press (Hughes, 1951) as described previously (Cartledge & Lloyd, 1972*c*). Preparation of sphaeroplasts and subsequent disruption and centrifugation procedures for the production of whole homogenates used in all cell-fractionation studies were identical with those described by Cartledge & Lloyd (1972*a*).

Fractionation by high-speed zonal centrifugation

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

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*-nitrophenylphosphatase (EC 3.1.3.2) and ATPase (EC 3.6.1.4) activities were assayed as described previously (Cartledge & Lloyd, 1972*a*).

Other determinations

O_2 uptake. O_2 uptake was measured polarographically by using the equipment described by Lloyd & Brookman (1967).

Protein. Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

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

Chemicals

All enzyme substrates, oligomycin, ouabain, antimycin A and ergosterol were obtained from Sigma (London) Chemical Co., London, S.W.6, U.K. Dio-9 was supplied by Dr. D. E. Griffiths, University of Warwick, 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 was from Oxoid Ltd., London S.E.1, U.K. *Helix pomatia* were obtained from Haig and Co., Beambrook, Surrey, U.K.

Presentation of results

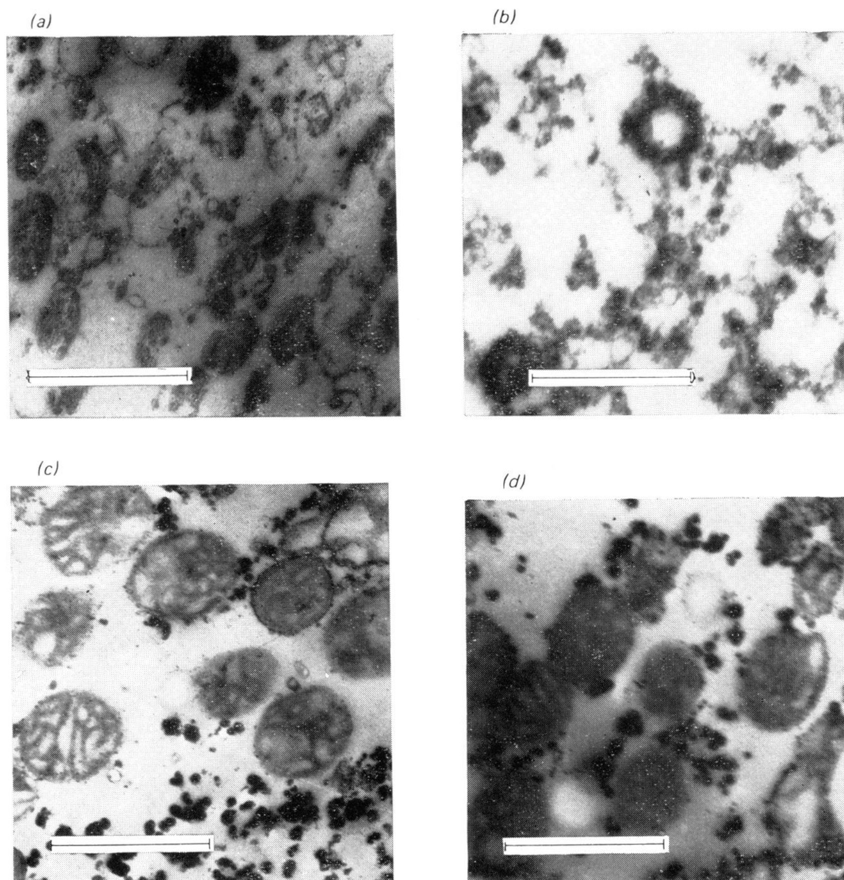
Enzyme units are expressed as nmol of substrate transformed/min or as nmol of measured product formed/min. In Figs. 2–5 vertical links divide the diagrams at density intervals of 0.05 g/ml. Percentage sedimentability is arbitrarily expressed as that proportion of the total enzyme units that had sedimented beyond $\rho = 1.10$ g/ml after centrifugation for a minimum of $6\times10^6 g$-min.

Results

Changes in respiration of whole-cell suspensions and enzyme activities of homogenates

On anaerobic de-repression, no increase was observed in respiration rates (Fig. 1*a*), but an immediate and rapid increase was produced when the cell suspension was aerated. Throughout the phase of anaerobiosis, antimycin A (2 μg/ml) gave no inhibition of O_2-uptake rates; 1 mM-KCN produced about 30% inhibition of the respiration of the anaerobically grown cells. Respiratory adaptation for 1 h led to the development of respiratory activity which was more than 80% inhibited by these inhibitors of mitochondrial respiratory chain electron transport.

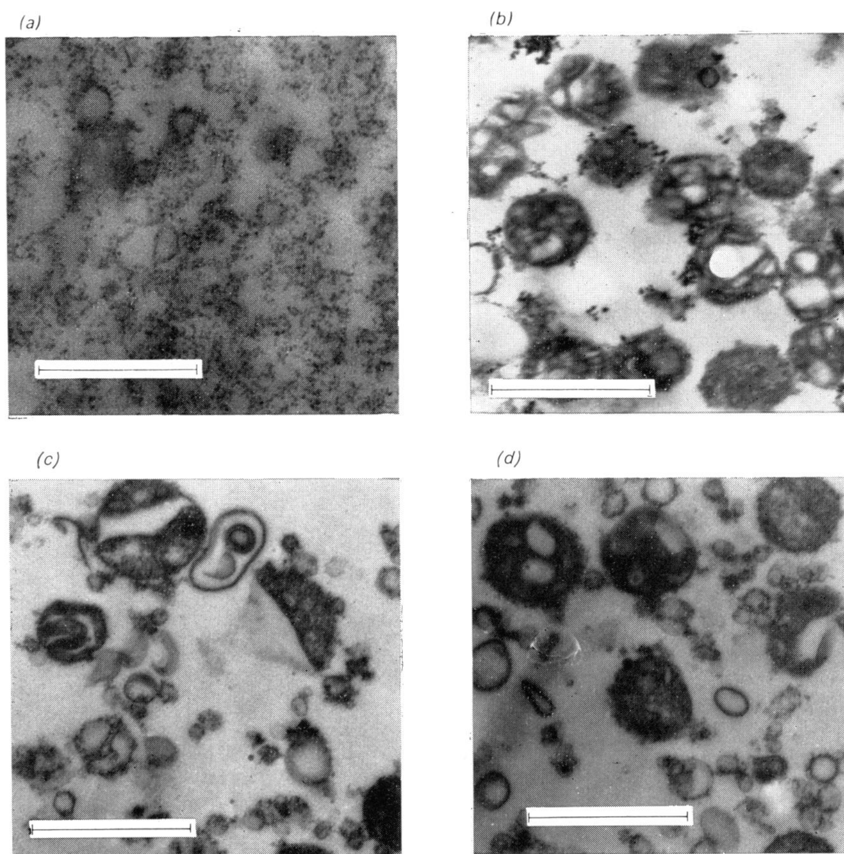
Cytochrome *c* oxidase activity was not detectable (specific activity <0.05 nmol/min per mg of protein) in homogenates of anaerobically grown cells, even after glucose de-repression, but showed a dramatic increase during the first 5 h of respiratory adaptation



EXPLANATION OF PLATE I

Electron micrographs of sections of material from the experiments shown in Figs. 2 *and* 3

(*a*) Fraction 41 (Fig. 2); (*b–d*) fractions 39, 43 and 46 respectively (Fig. 3). In all cases the bar represents 1.0μm. Material was fixed in glutaraldehyde at the equilibrium density of sucrose in the fractions. For further details see the text. Magnification $\times$20000.



EXPLANATION OF PLATE 2

Electron micrographs of sections of material from the experiments shown in Figs. 4 *and* 5

(*a* and *b*) Fractions 22 and 37 respectively (Fig. 4); (*c* and *d*) fractions 32 and 34 respectively (Fig. 5). In all cases the bar represents 1.0μm. Material was fixed in glutaraldehyde at the equilibrium density of sucrose in the fractions. For further details see the text. Magnification ×20000.

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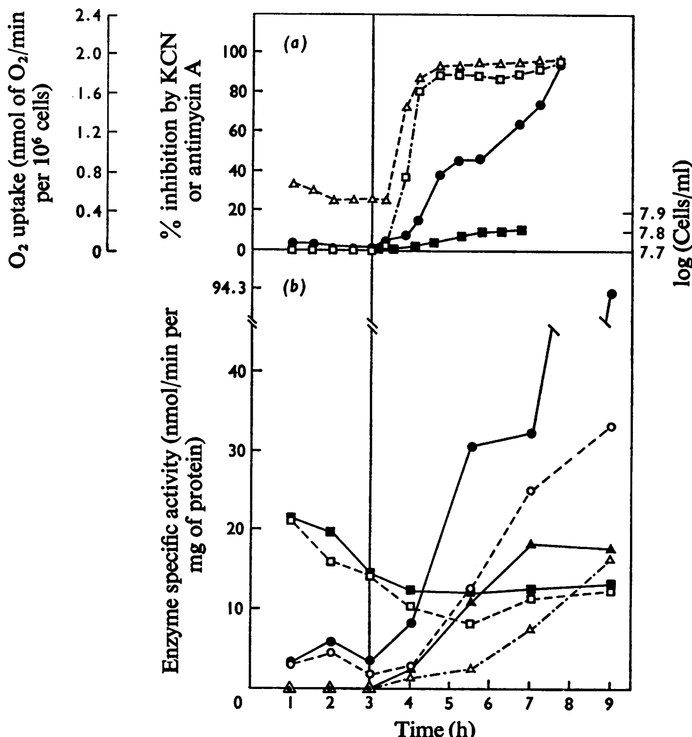


Fig. 1. *Changes in respiration rates of whole cells and enzyme activities of homogenates during glucose de-repression and respiratory adaptation of anaerobically grown S. carlsbergensis*

The period (0–1 h) indicates the time taken to harvest (at 6×10^7 cells/ml) and resuspend cells in adaptation medium; 1–3 h is the period of anaerobic de-repression and 3–9 h is the 6 h of adaptation. (*a*) Concn. of cells [log(cells/ml), ■], O_2 uptake (nmol of O_2/min per 10^6 cells, ●), and % inhibition of respiration in the presence of 2 µg of antimycin A/ml (□), or 1 mM-KCN (△); (*b*) specific activities of cytochrome *c* oxidase (▲), catalase (△), NADH–cytochrome *c* oxidoreductase (●), antimycin A-insensitive NADH–cytochrome *c* oxidoreductase (○), NADPH–cytochrome *c* oxidoreductase (■), and antimycin A-insensitive NADPH–cytochrome *c* oxidoreductase (□).

(Fig. 1*b*); at this time the activity was similar to that of aerobically grown, glucose-de-repressed cells. The activity of NADH–cytochrome *c* oxidoreductase showed little change during anaerobic glucose de-repression, but increased 27-fold over the first 6 h of adaptation; this increase was due mainly to an increase in the antimycin A-sensitive activity which finally accounted for more than 60% of the total activity. The specific activity of NADPH–cytochrome *c* oxidoreductase decreased during anaerobic de-repression, but remained virtually unchanged during respiratory adaptation. Catalase activity was not detectable (<0.9 nmol/min per mg of protein) during anaerobic de-repression, but showed a biphasic increase during adaptation; its activity increased slowly over the first 2.5 h, and was still rising more rapidly after 6 h. Little net cell growth was observed during respiratory adaptation; cell counts indicated an increase from 5×10^7 to 6.5×10^7 cells/ml over the 6 h period.

Aeration of anaerobically grown, glucose-repressed cells in growth medium also led to respiratory adaptation, whereas no adaptation was observed when these cells were oxygenated under conditions of limited cell growth.

Fractionation of homogenate of anaerobically grown glucose-de-repressed cells

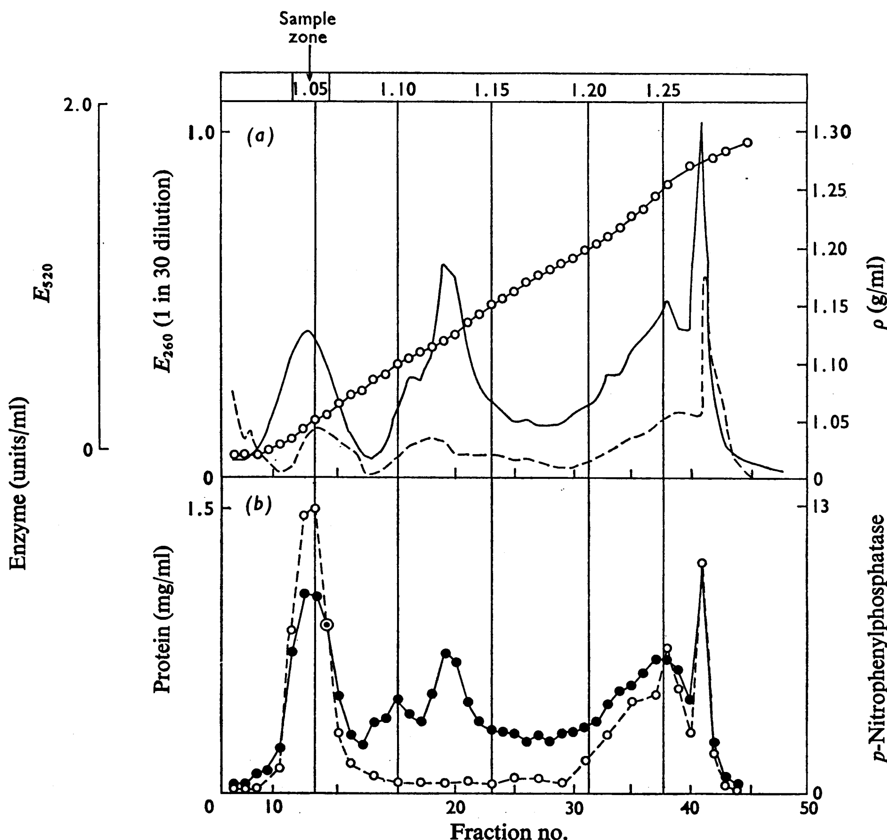
Centrifugation in the B XIV rotor was for 35000 rev./min for 165 min (a minimum of 6×10^6 *g*-min at the starting zone). The profiles of absorbance at 520 and 260 nm were complex (Fig. 2*a*) particularly in the region $\rho > 1.10$ g/ml. The starting zone still

contained 35% of the protein together with 46% of the acid *p*-nitrophenylphosphatase (Fig. 2*b*), less than 10% of the total NADH–cytochrome *c* oxidoreductase and 18% of the NADPH–cytochrome *c* oxidoreductase activities (Fig. 2*c*). Populations of particles containing NADH–cytochrome *c* oxidoreductase activity occurred at $\rho = 1.10$, 1.18, 1.23, 1.25 and 1.27 g/ml; this distribution was different from that of the NADPH-linked enzyme which was found mainly at $\rho = 1.25$ and 1.27 g/ml but also showed a broad zone between $\rho = 1.07$ and 1.2 g/ml. Oligomycin-sensitive ATPase activity was found in a single fraction at $\rho = 1.27$ (Fig. 2*d*) which corresponds with the position of particles containing *p*-nitrophenylphosphatase and the two oxidoreductase activities. The remaining ATPase activity (which was oligomycin-insensitive) was found between $\rho = 1.17$ and 1.26 g/ml.

Electron-microscopic examination of fractions 11, 17, 23 and 34 indicated that all populations of particles in these fractions were less than 0.1 μm in diameter. Fraction 41 contained ellipsoid mitochondria-like structures 0.3–0.5 μm in length (Plate 1*a*), which showed poorly developed inner membranes and were similar in appearance to structures found in anaerobically grown, glucose-repressed cells (Cartledge & Lloyd, 1972*c*).

Fractionation of homogenates of cells after 10 *min respiratory adaptation*

After zonal centrifugation of a homogenate prepared from cells adapted for 10 min, the distributions of enzymes was markedly altered when compared with those found in the experiment with anaerobically grown, glucose-de-repressed cells. The profiles of absorbance at 520 nm and 260 nm (Fig. 3*a*) again showed a major broad zone at $\rho > 1.2$ g/ml. The 520 nm trace also revealed the presence of particles at $\rho = 1.18$ g/ml which corresponded with the fractions containing the highest activity of *p*-nitrophenylphosphatase (Fig. 3*b*). Both cytochrome *c* oxidase and catalase activities (Fig. 3*c*) were now detectable, and the sedimentable portions of these enzymes showed extremely heterogeneous distributions with the bulk of activities sedimenting to $\rho > 1.23$ g/ml. Non-sedimentable cytochrome *c* oxidase and catalase activities accounted for 12% and 22% respectively



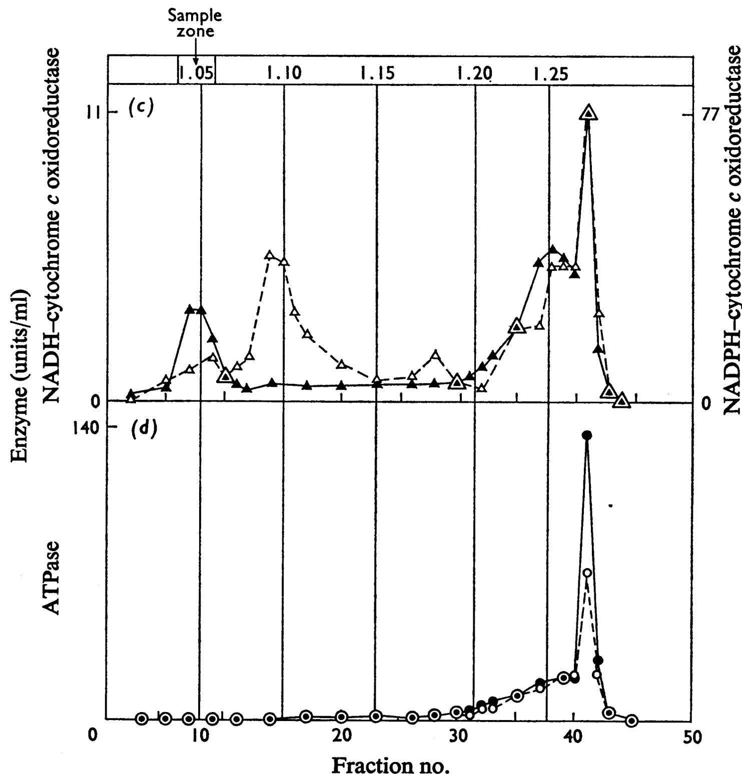


Fig. 2. *Fractionation by high-speed zonal centrifugation of a homogenate of anaerobically grown, glucose-de-repressed S. carlsbergensis*

The whole homogenate (30ml) containing 279mg of protein was loaded on to the gradient. The volumes of whole homogenate (diluted 1 in 20) and of fractions taken for assay were as follows: NADH– and NADPH–cytochrome *c* oxidoreductases, 0.1ml; ATPases and acid *p*-nitrophenylphosphatase, 0.2ml. Cytochrome *c* oxidase and catalase activities were not detectable. Centrifugation was at 35000rev./min for 165min ($6\times10^6 g$-min at the starting zone; $\int_0^t \omega^2 dt = 1.45\times10^{11}\,rad^2/s$). (*a*) Sucrose density gradient (○), light-scattering at 520nm (---) and 260nm (——); (*b*) protein (●) and acid *p*-nitrophenylphosphatase activity (○); (*c*) NADH–cytochrome *c* oxidoreductase (△) and NADPH–cytochrome *c* oxidoreductase (▲) activities; (*d*) ATPase (●) and oligomycin-insensitive ATPase (○) activities. Specific activities of enzymes in whole homogenate were as follows (recoveries in parentheses): protein (96%), NADH–cytochrome *c* oxidoreductase 8.7 (65%), NADPH–cytochrome *c* oxidoreductase 43 (69%), ATPase 24 (79%) and *p*-nitrophenylphosphatase 4.2 (97%).

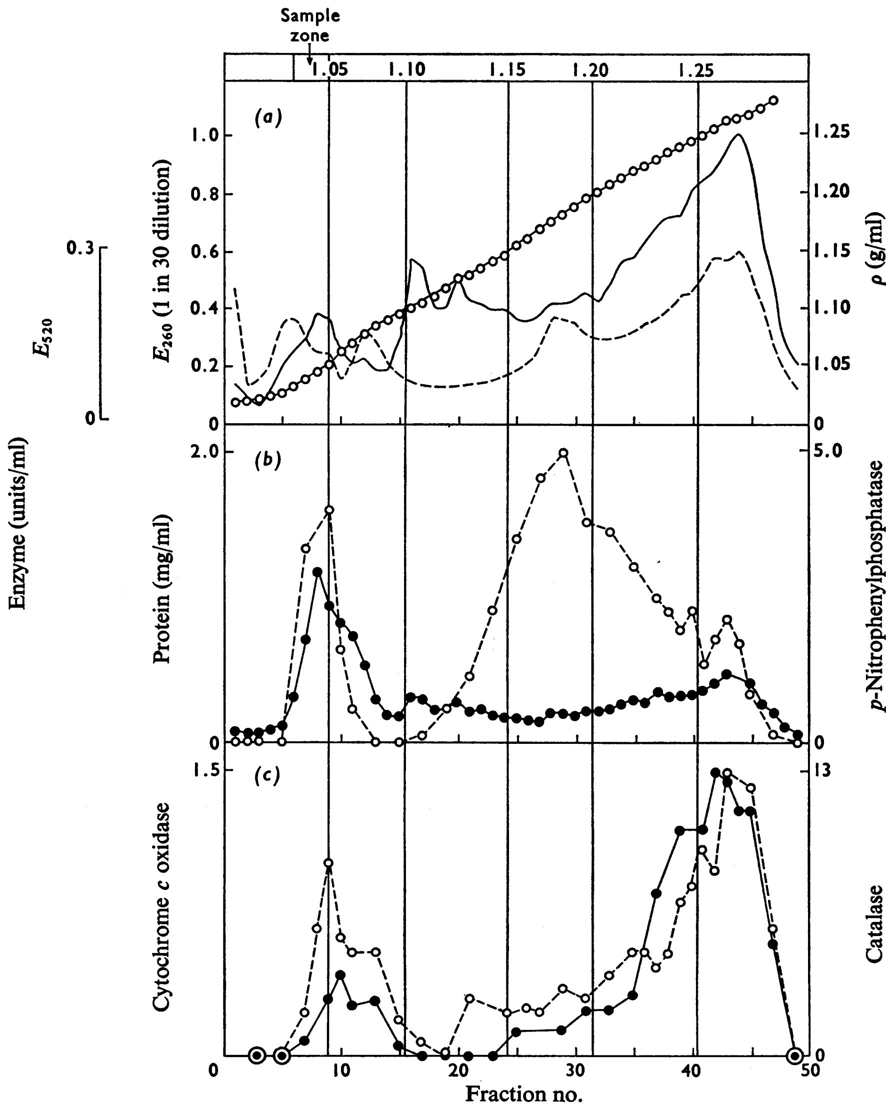
of the total enzyme units recovered. The overall distributions of the sedimentable nicotinamide nucleotide–cytochrome *c* oxidoreductases (Fig. 3*d*) were not markedly different from those found for homogenates of anaerobically grown, glucose-de-repressed cells, although the percentage sedimentability of the NADH-linked enzyme was decreased. Total ATPase activity (Fig. 3*c*) now showed a broad distribution above $\rho = 1.15$ g/ml and was almost entirely inhibited by oligomycin, dio-9 or dicyclohexylcarbodi-imide (Fig. 3*f*). That the inhibitor-insensitive portions of this enzyme showed different distributions may indicate that several different populations of particles contribute to the overall ATPase activity.

Electron-microscopic examination of fractions 8, 15 and 22 indicated that most particles were less than 0.1 μm in diameter, although in all these fractions some larger vesicles occurred. Fraction 39 contained ribosomal aggregates often in close association with

vesicles 0.05–0.2 μm in diameter (Plate 1*b*). Fraction 43 contained highly developed mitochondria-like structures of 0.5–1.0 μm diameter (Plate 1*c*). These resembled the condensed forms of mitochondria of aerobically grown cells and were of similar size (Cartledge & Lloyd, 1972*a*). Fraction 46 also contained mitochondria-like organelles (Plate 1*d*). Both fractions contained numerous densely osmiophilic vesicles about 0.1 μm in diameter.

Fractionation of homogenates of cells after 30 *min respiratory adaptation*

After 30 min respiratory adaptation the profiles of absorbance at 520 nm and 260 nm (Fig. 4*a*) and the distribution of protein (Fig. 4*b*) indicated that the densities of particles predominated at $\rho = 1.25$ rather than at $\rho = 1.27$ g/ml as found with homogenates adapted for 10 min (Fig. 3). Sedimentable acid *p*-nitrophenylphosphatase activity (Fig. 4*b*) again showed an extremely broad density distribution whereas the major sedimentable zones of cytochrome *c* oxidase and catalase (Fig. 2*c*), NADH- and NADPH–cytochrome *c* oxidoreductase (Fig. 2*d*) and ATPase (Fig. 2*e*) activities were found at $\rho = 1.25$. Virtually all the cytochrome *c* oxidase activity was sedimentable, but its distribution was bimodal, with the minor zone (accounting for 15% of the total enzyme units) having a median buoyant density of 1.12 g/ml; some oligomycin-sensitive ATPase activity



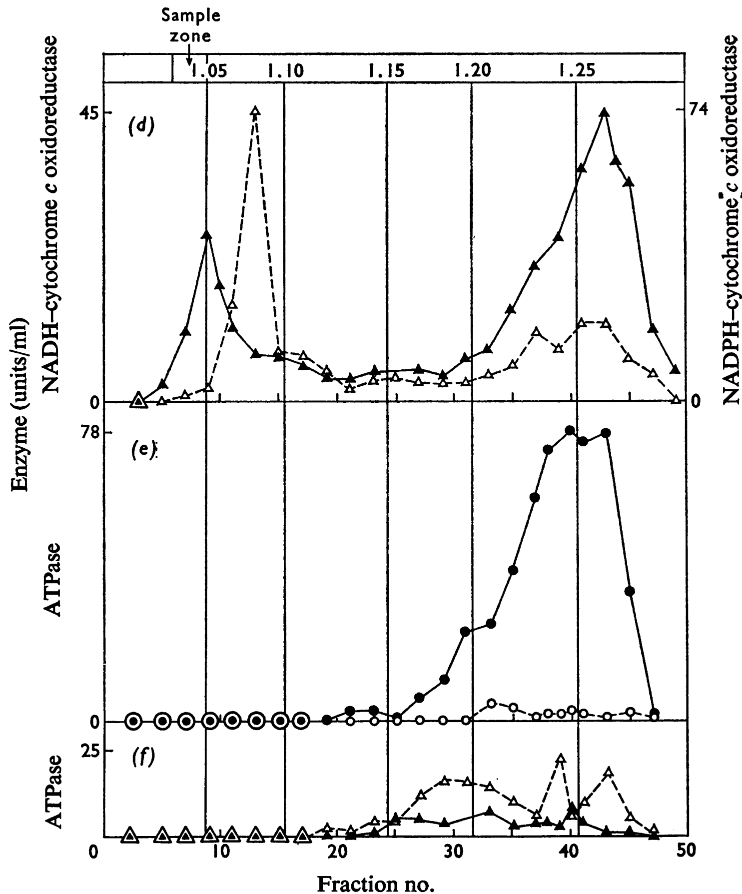


Fig. 3. *Fractionation by high-speed zonal centrifugation of a homogenate of cells after respiratory adaptation for* 10 *min*

The whole homogenate (30 ml) containing 168 mg of protein was loaded on to the gradient. The volumes of whole homogenate (diluted 1 in 20) and of fractions taken for assay were as follows: NADH– and NADPH–cytochrome *c* oxidoreductases, cytochrome *c* oxidase and catalase, 0.1 ml; ATPases and acid *p*-nitrophenylphosphatase, 0.2 ml. Centrifugation was at 35000 rev./min for 16 min (6×10^6g-min at the sample zone; $\int_0^t \omega^2\cdot dt = 1.45\times10^{11}$ rad²/s). (*a*) Sucrose density gradient (○), light-scattering at 520 nm (– – –) and 260 nm (——); (*b*) protein (●) and acid *p*-nitrophenyl phosphatase activity (○); (*c*) cytochrome *c* oxidase (●) and catalase (○) activities; (*d*) NADH–cytochrome *c* oxidoreductase (△) and NADPH–cytochrome *c* oxidoreductase (▲) activities; (*e*) ATPase (●) and oligomycin-insensitive ATPase (○) activities; (*f*) dio-9-insensitive ATPase (▲) and dicyclohexylcarbodi-imide-insensitive ATPase (△) activities. Specific activities of enzymes in whole homogenate were as follows (recoveries in parentheses): protein (92%), NADH–cytochrome *c* oxidoreductase 21.4 (93%), NADPH–cytochrome *c* oxidoreductase 72.4 (83%), cytochrome *c* oxidase 8.9 (110%), catalase 12.9 (90%), ATPase 57.3 (90%) and acid *p*-nitrophenylphosphatase 5.9 (94%).

was also detected in this region of the gradient. Sedimentable catalase activity also showed a bimodal density distribution.

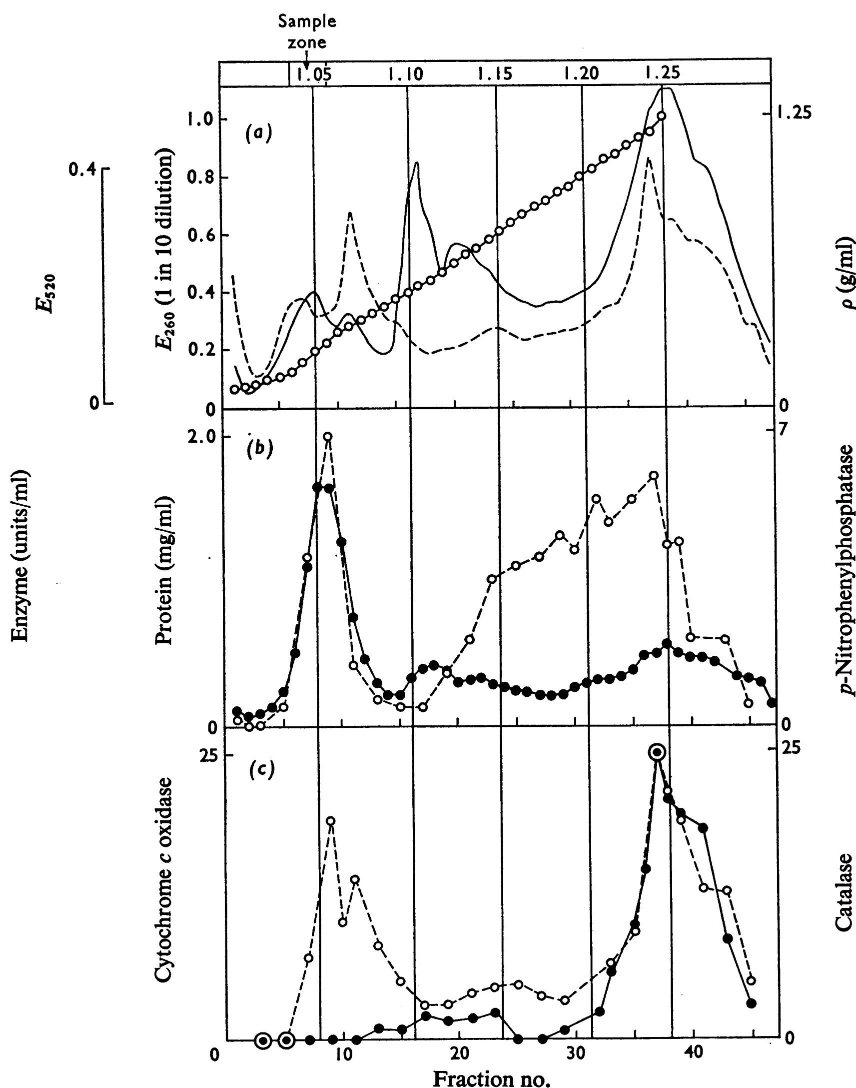
Electron micrographs of fraction 10 showed that most of the material was less than 0.1 μm in diameter, although a few vesicles of 0.3 μm diameter were present. Fraction 22 contained ribosomes and some vesicles (0.3 μm in diameter) closely associated with ribosomes (Plate 2*a*). Fractions 37 (Plate 2*b*) and 39 contained mitochondria-like structures of 0.3–0.8 μm

diameter which were of orthodox and condensed form similar to mitochondria of aerobically grown cells (Cartledge & Lloyd, 1972*a*). Fraction 39 also contained small electron-dense vesicles which appeared to be identical with those of fraction 43 of cells adapted for 10min.

Sedimentation of whole homogenates through gradients of Urografin gave very similar results to those obtained with the usual sucrose gradients, although the density of the mitochondria was 1.155g/ml in this case. The use of sorbitol-containing gradients was unsatisfactory as these led to extensive loss of mitochondrial integrity (as judged by increased non-sedimentability of cytochrome *c* oxidase activity and examination of fractions in the electron microscope).

Preparation of fraction S_1 and its subfractionation by zonal centrifugation

Whole homogenate of cells adapted for 30min was centrifuged for 10min at 10000g ($r_{av.}$ 7.6cm) in the 8×50ml rotor of an MSE 17 centrifuge. The supernatant (S_1) was decanted from the pellet (P_1). Fraction S_1 contained no detectable cytochrome *c* oxidase or ATPase activities and zonal centrifugation showed that no sedimentable activities of NADH–cytochrome *c* oxidoreductase or acid *p*-nitrophenyl-



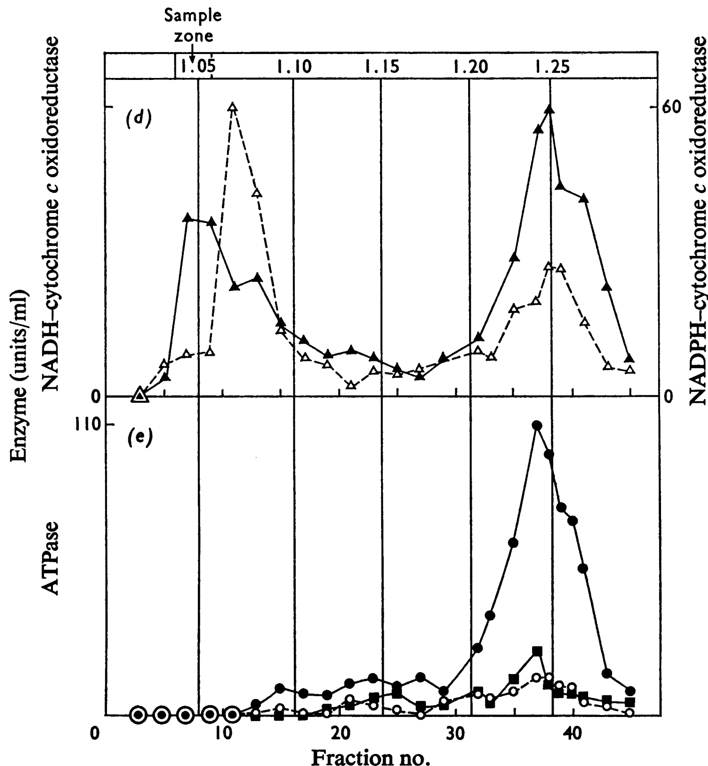


Fig. 4. *Fractionation by high-speed zonal centrifugation of a homogenate of cells after respiratory adaptation for 30 min*

The whole homogenate (30 ml) containing 195 mg of protein was loaded on to the gradient. The volumes of whole homogenate (diluted 1 in 20) and of fractions taken for assay were as follows: NADH– and NADPH–cytochrome *c* oxidoreductases, cytochrome *c* oxidase and catalase, 0.1 ml; ATPases and acid *p*-nitrophenylphosphatase, 0.2 ml. Centrifugation was at 35000 rev./min for 165 min ($6\times10^6 g$-min at the sample zone; $\int_0^t \omega^2\cdot dt = 1.45\times10^{11}\,rad^2/s$). (*a*) Sucrose density gradient (○), light-scattering at 520 nm (---) and 260 nm (——); (*b*) protein (●) and acid *p*-nitrophenylphosphatase activity (○); (*c*) cytochrome *c* oxidase (●) and catalase (○) activities; (*d*) NADH–cytochrome *c* oxidoreductase (△) and NADPH–cytochrome *c* oxidoreductase (▲) activities; (*e*) ATPase (●), oligomycin-insensitive ATPase (○) and dicyclohexylcarbodi-imide-insensitive ATPase (■) activities. Specific activities of enzymes in whole homogenate were as follows (recoveries in parentheses): protein (99.7%), NADH–cytochrome *c* oxidoreductase 36 (70%), NADPH–cytochrome *c* oxidoreductase 88 (51%), cytochrome *c* oxidase 12.7 (90%), catalase 12.7 (137%), ATPase 57 (95%) and acid *p*-nitrophenylphosphatase 6.1 (105%).

phosphatase were present (Table 1). Sedimentable NADPH–cytochrome *c* oxidoreductase activity was entirely located at $\rho > 1.2$ g/ml.

Fractionation of homogenate of cells after 3 h respiratory adaptation

After zonal centrifugation further changes in enzyme distributions were evident after adaptation for 3 h. The major zone of absorbance at 260 nm now occurred at $\rho = 1.09$ g/ml, whereas the trace at 520 nm showed a major zone at $\rho = 1.23$ g/ml (Fig. 5*a*). *p*-Nitrophenylphosphatase activity was now predominantly non-sedimentable (Fig. 5*b*), whereas cytochrome *c* oxidase activity was hardly detectable at any region of the gradient with $\rho < 1.2$ g/ml (Fig. 5*c*). The median density of the zone containing cytochrome *c* oxidase activity was 1.23 g/ml, whereas that of sedimentable catalase activity, which also now showed a unimodal distribution was 1.21 g/ml. The

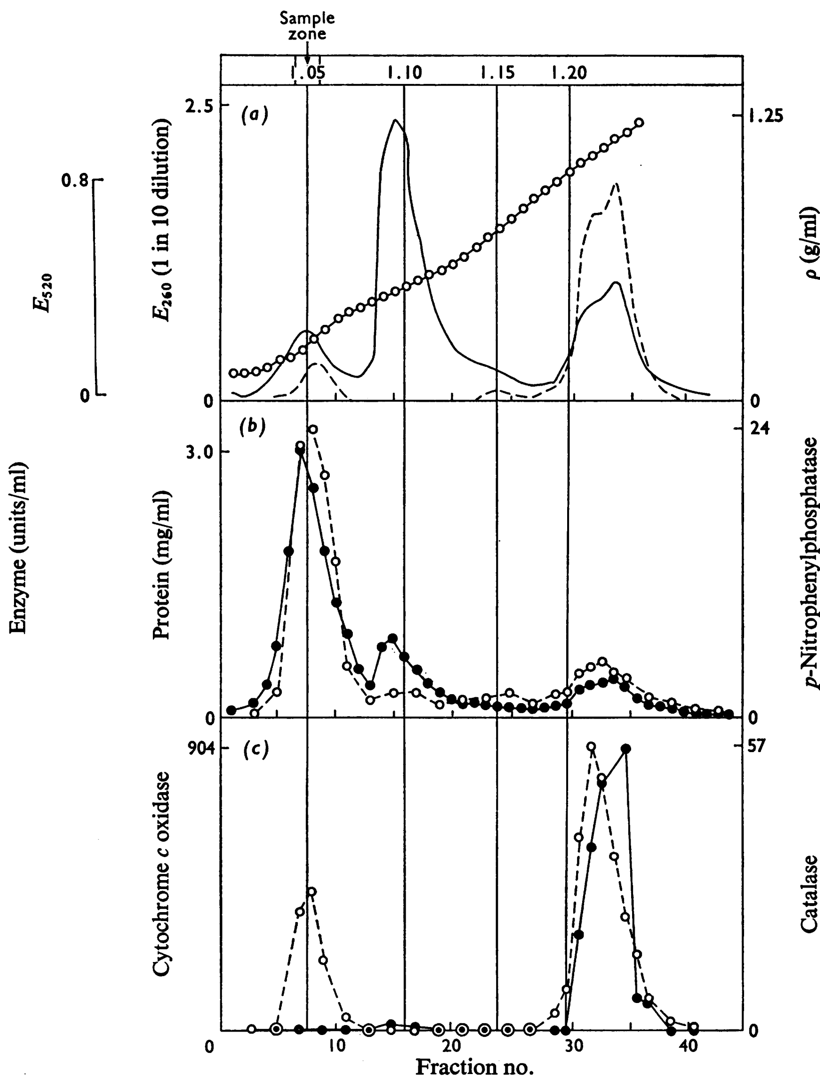
sedimentability of both NADH– and NADPH–cytochrome *c* oxidoreductases (Fig. 5*d*), had further increased over that of whole homogenates of 30min adapted cells. ATPase(s) showed complex distributions between $\rho = 1.20$ and 1.25g/ml (Fig. 5*e* and *f*).

Electron microscopy indicated that fraction 8 contained large vesicles up to 1.5μm in diameter. Fraction 32 contained mitochondria of 0.3–0.6μm diameter, together with large numbers of single-membraned electron-transparent vesicles of about 0.1μm diameter (Plate 2*c*). Fraction 34 was similar to fraction 32 and contained mitochondria together with vesicles of various sizes (0.05–0.2μm in diameter, Plate 2*d*).

A summary of percentage sedimentabilities of enzymes calculated from zonal centrifugation results on homogenates prepared from cells harvested at various stages of respiratory adaptation is presented in Table 2. Whereas the percentage sedimentable protein decreased throughout adaptation, the values for NADH–cytochrome *c* oxidoreductase activity show a gradual increase after the first 10min. Those for acid *p*-nitrophenylphosphatase activity show an increase in the first 10min followed by a decrease over the last 2.5h. Marked variations were not evident in the sedimentabilities of the other enzyme activities assayed.

Discussion

The respiration of anaerobically grown, glucose-repressed *S. carlsbergensis*, like that of *Saccharomyces*



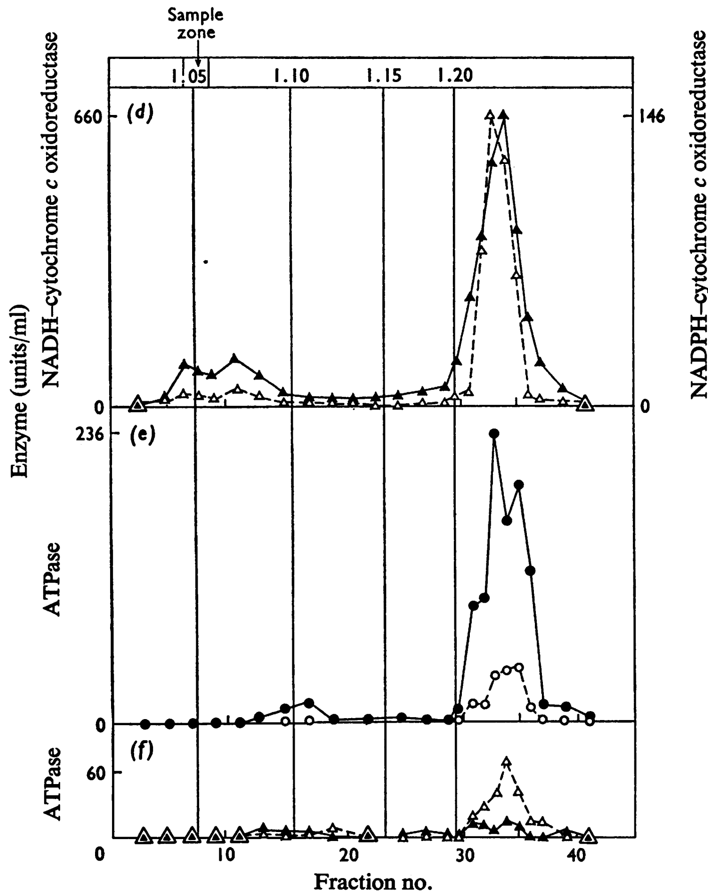


Fig. 5. *Fractionation by high-speed zonal centrifugation of a homogenate of cells after respiratory adaptation for 3h*

The whole homogenate (23ml) containing 235mg of protein was loaded on to the gradient. The volumes of whole homogenate (diluted 1 in 20) and of fractions taken for assay were as follows: NADH- and NADPH-cytochrome *c* oxidoreductases, cytochrome *c* oxidase and catalase, 0.1ml; ATPases and acid *p*-nitrophenylphosphatase, 0.2ml. Centrifugation was at 35000rev./min for 165min (6×10^6g-min at the sample zone; $\int_0^t \omega^2\cdot dt = 1.45\times10^{11}\,rad^2/s$). (*a*) Sucrose density gradient (○), light-scattering at 520nm (---) and 260nm (——); (*b*) protein (●) and acid *p*-nitrophenylphosphatase activity (○); (*c*) cytochrome *c* oxidase (●) and catalase (○) activities; (*d*) NADH–cytochrome *c* oxidoreductase (△) and NADPH–cytochrome *c* oxidoreductase (▲) activities; (*e*) ATPase (●) and oligomycin-insensitive ATPase (○) activities; (*f*) dio-9-insensitive ATPase (▲) and dicyclohexylcarbodi-imide-insensitive ATPase (△) activities. Specific activities of enzymes in whole homogenate were as follows (recoveries in parentheses): protein (99.5%), NADH–cytochrome *c* oxidoreductase 97.5 (94%), NADPH–cytochrome *c* oxidoreductase 45.7 (78%), cytochrome *c* oxidase 20.4 (71%), catalase 12.6 (114%), ATPase 45.7 (180%) and acid *p*-nitrophenylphosphatase 7.4 (95%).

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cerevisiae grown under similar conditions (Criddle & Schatz, 1969), is not inhibited by antimycin A and is only 30% inhibited by CN^-. During anaerobic glucose de-repression these characteristics were not altered and cytochrome *c* oxidase and catalase activities remained undetectable. The total ATPase activity of whole homogenates decreased during this period to a value one-half that found in homogenates

Table 1. *Fractionation of a whole homogenate of anaerobically grown S. carlsbergensis adapted to O_2 for 30min, to give fractions P_1 and a post-mitochondrial supernatant*

A whole homogenate (WH) was centrifuged at 10^5*g*-min to give fractions P_1 and a post-mitochondrial supernatant (PMS). The post-mitochondrial supernatant was then centrifuged in the B XIV zonal rotor at 6×10^6*g*-min. Values are the % of total enzyme of whole homogenate in the post-mitochondrial supernatant, and the recovery (%) of activity in fractions P_1+PMS referred to the whole homogenate.

	$\frac{\text{Units in PMS}}{\text{Units in WH}}\times100$	$\frac{\text{Units in } P_1+\text{PMS}}{\text{Units in WH}}\times100$
Protein	59	97
Acid *p*-nitrophenylphosphatase	56.6	99.6
Catalase	15	70
Cytochrome *c* oxidase	0	89.2
NADH–cytochrome *c* oxidoreductase	15.6	104
NADPH–cytochrome *c* oxidoreductase	26.1	106
ATPase	0	91

Table 2. *Percentage sedimentabilities of enzymes in whole homogenates of anaerobically grown, glucose-de-repressed cells of S. carlsbergensis after respiratory adaptation*

Percentage sedimentability is expressed as the % 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. Values presented are calculated from results shown in Figs. 2–5. Absence of a value indicates enzymes not detectable in whole homogenate.

	% sedimentability of enzymes in			
	Anaerobically grown, glucose-de-repressed cells	Cells adapted for 10min	Cells adapted for 30min	Cells adapted for 3h
Protein	64.7	57	45	28.5
Cytochrome *c* oxidase		88	97	99
Catalase		78.7	65	72
NADH–cytochrome *c* oxidoreductase	67.5	33	49	89.5
NADPH–cytochrome *c* oxidoreductase	72	78	66	78
ATPase	100	100	94	96
Oligomycin-insensitive ATPase	100	100	88	100
Acid *p*-nitrophenylphosphatase	53.3	83.1	75	36

of glucose-repressed cells. Of the ATPase in the repressed cells, 58% was inhibited by oligomycin, whereas only 7% inhibition was found after glucose-de-repression. The density of the major sedimentable zone containing oligomycin-sensitive ATPase activity, which has been shown to contain several different populations of particles (Cartledge & Lloyd, 1972*c*) had increased from $\rho = 1.235$ to $\rho = 1.27$g/ml. Comparison of electron micrographs of the mitochondria-like organelles found in the anaerobic glucose-repressed state (Cartledge & Lloyd, 1972*c*) with those presented here for the glucose-de-repressed organelles indicates that no marked morphological changes have accompanied this increased buoyant density.

After only 10min of respiratory adaptation cytochrome *c* oxidase was present in functional electron-transport chains and was found in particles large enough to be completely sedimented in 0.25M-sucrose after centrifugation for 10^5*g*-min (Cartledge *et al.*, 1972). These particles were located in two zones after density-gradient centrifugation, and the major sedimentable zone showed an extremely heterogeneous density distribution as did ATPase activity which was in this case 95% inhibited by oligomycin. Highly organized mitochondria were already present throughout this zone; these were completely different in ultrastructure from the organelles of the anaerobically grown cells that occurred in the fractions containing oligomycin-sensitive ATPase (Cartledge & Lloyd, 1972*c*) and have been referred to as promitochondria (Criddle & Schatz, 1969) or mitochondrial precursors (Watson *et al.*, 1970).

Enzyme distributions and electron micrographs indicate that several different populations of particles overlap in their distributions through the gradient, and cannot be separated by equilibrium density centrifugation. Highly osmiophilic vesicles of about 0.1 μm diameter, which have not been detected in anaerobically or aerobically grown cells, were of frequent occurrence throughout the fractions containing newly assembled mitochondria. The high E_{260}/protein ratio of these fractions suggests the presence of ribosomal aggregates involved in enzyme and membrane synthesis and that these are often intimately associated with membranes was confirmed by electron microscopy. The presence of sedimentable catalase activity indicates the presence of peroxisomes in these cells, whereas remarkable alteration of the subcellular distribution of *p*-nitrophenylphosphatase activity suggests an involvement of acid hydrolase-containing organelles (Cartledge & Lloyd, 1972*b*) in the turnover and reorganization of membrane distribution with the cell.

Further changes after 30min adaptation include the shift of cytochrome *c* oxidase and oligomycin-sensitive ATPase to $\rho = 1.25$ g/ml, although these enzymes are still present as broad zones of activity and a second zone containing both enzymes occurs at $\rho = 1.12$ g/ml. After 3h adaptation these mitochondrial marker enzymes were almost entirely confined to a zone at $\rho = 1.23$ g/ml, which corresponds to the density of mitochondria in aerobically grown, glucose-de-repressed cells (Cartledge & Lloyd, 1972*a*).

The conditions of protoplast disruption and subsequent gradient centrifugation employed in these fractionations do not lead to extensive release of mitochondrial-membrane marker enzymes into non-sedimentable or slowly sedimenting vesicles (Cartledge & Lloyd, 1972*a*), and the present work indicates that this is also the case with homogenates of cells adapted for 3h. We cannot discount the possibility that newly assembled organelles in the early stages of adaptation may be more susceptible to damage, but the finding that all the particles are large enough to be sedimented at 10^5*g*-min and have a wide range of densities suggests that they are not produced by membrane comminution. The particles of low buoyant density may represent a steady-state population of lipid-rich enzyme-containing vesicles, which fuse with particles of higher buoyant density, to give finally a population of intermediate density. Such a process could underlie the marked changes in density seen in these experiments. The currently favoured concept of the promitochondrion as a mitochondrial precursor would suggest that this organelle acts as a framework into which newly synthesized lipid and enzyme molecules are fitted (Plattner *et al.*, 1970). This hypothesis has not been rigorously tested and the present results suggest that assembly of fully functional mitochondria may involve fusion of quite large membrane units which do not pre-exist in anaerobically grown cells.

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