Subcellular Fractionation of *Candida stellatoidea* after Growth with Glucose or *n*-Hexadecane

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Spheroplasts of glucose grown and *n*-hexadecane-grown *Candida stellatoidea* were prepared using snail-enzyme or Zymolyase-5000 and the resultant cell extracts fractionated on sucrose or metrizamide gradients. Organelles from *n*-hexadecane-grown cells were more fragile than those from glucose-grown cells and organelle integrity was maintained only after spheroplast formation using Zymolyase-5000. Isopycnic density gradient centrifugation through metrizamide gradients yielded more complex distributions and markedly higher percentage sedimentabilities of marker enzymes than with sucrose gradients. The zone containing cytochrome *c* oxidase and all tricarboxylic acid cycle enzymes assayed was readily identified. The density of microbodies appears to be similar to that of mitochondria on either gradient material; on metrizamide a second catalase peak at $\rho = 1.07 \text{ g ml}^{-1}$ was also observed. This zone was shown by electron microscopy to contain organelles up to $1 \mu\text{m}$ diameter, and activities of carnitine acetyltransferase and long chain alcohol and aldehyde dehydrogenases. The first enzyme was located mainly in zones containing mitochondria and microbodies; the last two enzymes were multilocational and of differing distributions, but were found mainly in mitochondrial and microsomal fractions. The possibility that cells grown on *n*-hexadecane contain two populations of microbodies is discussed. Most lysosomes were disrupted on sucrose gradients but sedimented to a density of 1.12 g ml^{-1} on metrizamide gradients.

INTRODUCTION

Growth of many yeasts with *n*-alkanes leads to increased development of microbodies and elevated activities of several enzymes (Osumi & Fukui, 1972; Osumi *et al.*, 1974; Teranishi *et al.*, 1974). In *n*-alkane-grown *Candida tropicalis*, catalase, D-amino acid oxidase, isocitrate lyase, malate synthase and NADP-linked isocitrate dehydrogenase (Kawamoto *et al.*, 1977) occur in microbody-enriched fractions. Malate dehydrogenase, citrate synthase, aconitase and NAD⁺-linked isocitrate dehydrogenase (Kawamoto *et al.*, 1977), however, were found in mitochondria-enriched fractions. Several other enzymes, namely those of β -oxidation (Kawamoto *et al.*, 1978*a*), carnitine acetyltransferase (Kawamoto *et al.*, 1978*b*) and long chain alcohol and aldehyde dehydrogenases (Yamada *et al.*, 1980) were considered to be distributed between mitochondria and microbodies, and possibly to have yet other locations. Delaissé *et al.* (1981) studied the distribution of various enzymes from extracts of *Candida lipolytica* grown on *n*-hexadecane and concluded that a ω -hydroxylase is bound to a membrane that also contains cytochrome P450 and NADPH-cytochrome *c* oxidoreductase.

All of these studies used either differential or sucrose density gradients in swing-out rotors. Zonal rotors have been used with some success to investigate the subcellular distribution of enzymes from *Saccharomyces carlsbergensis* (Cartledge & Lloyd, 1972*a, b*; 1973). In addition, several workers have utilized different gradient materials to fractionate cell extracts from various yeasts, for example Urografin (Matile & Bahr, 1968), sorbitol (Szabo & Avers, 1969;

Neal *et al.*, 1970) and Ficoll (Roggenkamp *et al.*, 1975). In addition, metrizamide has been used successfully to separate lysosomes from mitochondria (Aas, 1973) and peroxisomes from mitochondria (Collot *et al.*, 1976) from rat liver homogenates.

In this paper, we compare the sedimentation of enzymes from spheroplasts of *Candida stellatoidea* prepared using either snail-enzyme or Zymolyase-5000 and investigate the effect of Triton X-100 on the activities of these enzymes. In addition, the subcellular distributions of several marker enzymes and selected enzymes involved in *n*-hexadecane assimilation are investigated using zonal centrifugation in both sucrose and metrizamide gradients. Enzyme specific activities, recovery data and percentage sedimentabilities are shown after fractionation of extracts of *C. stellatoidea* grown on glucose or *n*-hexadecane.

METHODS

Maintenance and growth. *Candida stellatoidea* was maintained on potato dextrose agar slopes. The medium of Veenhuis *et al.* (1978) was employed for growth, except that yeast extract was added at 0.025%. Glucose or *n*-hexadecane was added at 0.8% (w/v) and 0.6% (v/v), respectively. Cultures (200 ml) were grown in 1 l conical flasks at 30 °C and 200 r.p.m. on a rotary orbital incubator (Gallenkamp). Starter cultures grown to mid-exponential phase were used as inocula.

Organisms were counted in an Improved Neubauer haemocytometer (Hawksley, Lancing, Surrey, U.K.) after suitable dilution and mixing to separate clumps. Mean generation times in the exponential phase of growth on glucose or *n*-hexadecane were 0.93 h and 2.63 h, respectively. Cell concentration in stationary phase was 1.0×10^8 cells ml⁻¹ for growth on glucose and 1.8×10^7 cells ml⁻¹ for growth on *n*-hexadecane. Cultures were harvested between 1.5 and 2.0×10^7 organisms ml⁻¹ for glucose-grown cells and between 9.0 and 9.8×10^6 organisms ml⁻¹ for *n*-hexadecane-grown cells.

Harvesting, preparation and disruption of spheroplasts. All centrifugation procedures were carried out at 4 °C except during harvesting of glucose-grown cells. A temperature of 4 °C during harvesting of *n*-hexadecane-grown cells was necessary to remove the residual *n*-hexadecane in solid form.

Organisms were harvested by centrifugation for 2 min in a 6 × 250 ml rotor of an MSE 18 centrifuge at 6000 *g* for glucose-grown cells or 10000 *g* for *n*-hexadecane-grown cells.

Spheroplasts were prepared by one of two methods. (a) The method of Duell *et al.* (1964) was used except that the snail-enzyme (an extract from the digestive tract of *Helix pomatia*) was prepared in our laboratory. Incubation was at 30 °C for 1 h (pH 4.5). (b) Cells were incubated at 30 °C for 15 min in 0.05 M-potassium phosphate buffer (20 ml, pH 7.2) containing 0.9 M-sorbitol, 0.1 M-2-mercaptoethanol and 100 units Zymolyase-5000 (Kogyo Co., Tokyo, Japan). Unless otherwise stated, method (b) was employed.

With both methods, greater than 80% spheroplast formation was accomplished. After washing twice spheroplasts were disrupted in buffer containing 0.25 M-sucrose, 2 mM-MgCl₂ and 10 mM-Tris/HCl (pH 7.4). Breakage of spheroplasts was effected by using 15 cycles of a Teflon hand homogenizer, care being taken to avoid cavitation. Whole cells and intact spheroplasts were removed by centrifugation at 600 *g* for 5 min. The supernatant, termed the cell extract, was carefully decanted.

Fractionation of cell extract by differential and zonal centrifugation. Differential centrifugation was carried out in an 8 × 50 ml rotor of an MSE 18 centrifuge at 4 °C. Cell extract was centrifuged at 20000 *g* for 20 min and the supernatant was retained. The pellet was washed once in disruption buffer, supernatants were combined and the volume recorded. The pellet was resuspended in disruption buffer to a known volume.

Zonal centrifugations were carried out in an MSE 50 Superspeed centrifuge. Fractionations through sucrose gradients were performed in a B XIV aluminium rotor by the method described by Cartledge & Lloyd (1972*a*). Volume of cell extract was 10 ml. Sucrose concentrations were measured using a refractometer. Centrifugation was at 25000 *g* for 236 min. Fractionations through metrizamide gradients containing 0.25 M-sucrose, 10 mM-Tris/HCl (pH 7.4) and 2 mM-MgCl₂ were carried out in a B XXIX aluminium rotor. Metrizamide gradients were constructed in 2.5% (w/v) steps, each of 20 ml from 5 to 50% (w/v) with 35 ml 60% (w/v) metrizamide as cushion. The overlay contained 30 ml of each of 6% and 4% (w/v) sucrose solutions containing Tris/HCl and MgCl₂. The volume of cell extract was 10 ml. The B XXIX rotor was filled with Tris/HCl (10 mM, pH 7.4) containing 2 mM-MgCl₂. Overlay, cell extract, gradient and cushion were pumped to the outside of the rotor in the order given, at a loading speed of 3000 r.p.m. Centrifugation was carried out at 33000 *g* for 180 min. Fractions (10 ml) were collected from the outside of the rotor after displacement by Tris/HCl containing MgCl₂ solution. Metrizamide concentrations were measured using a sugar refractometer and converted to density using a calibration curve.

Enzyme assays. The following assays were carried out by previously published methods: aconitase (EC 4.2.1.3) (Fansler & Lowenstein, 1969); citrate synthase (EC 4.1.3.7) (Srere *et al.*, 1963); malate synthase (EC 4.1.3.2) and isocitrate lyase (EC 4.1.3.1) (Dixon & Kornberg, 1959); catalase (EC 1.11.1.6) (Lück, 1963). NAD⁺-linked isocitrate dehydrogenase (EC 1.1.1.4) was measured according to Kawamoto *et al.* (1978*a*), except

that the reaction volume was 1 ml. Carnitine acetyltransferase was measured according to Kawamoto *et al.* (1978*b*) except that the final reaction volume was 1 ml. Malate dehydrogenase (EC 1.1.1.37) was assayed as described by Kitto (1969) except that 0.1 M-potassium phosphate buffer, pH 7.8, was used. Cytochrome *c* oxidase (EC 1.9.3.1) was assayed as described by Smith (1955) except that cytochrome *c* was previously reduced with dithionite and passed through a G25 Sephadex column. NADPH-cytochrome *c* oxidoreductase (EC 1.6.2.4) was assayed by the method of Duppel *et al.* (1973) in the presence of 10 µg Antimycin-A. Palmitoyl-CoA dependent reduction of NAD⁺ was measured as described by Mishina *et al.* (1978). Long chain alcohol dehydrogenase and long chain aldehyde dehydrogenase were measured by the method of Lebeault *et al.* (1970) with slight modification: the reaction mixture (1 ml) consisted of 44.6 mM-Tris/HCl buffer (pH 8.5), 3.3 mM-NaN₃, 3.3 mM-NAD⁺, extract and 67 µl 11.94 mM-decanol in acetone (or decanal for aldehyde dehydrogenase). Ethanol dehydrogenase (EC 1.1.1.1) was assayed using the same system but with 67 µl 6.6 M-ethanol added instead of decanol. Due to the high UV absorbance of metrizamide, catalase was assayed in metrizamide gradient fractions by the titanium colour reaction for hydrogen peroxide, as described by Fukui *et al.* (1975), except that 0.02 M-potassium phosphate buffer, pH 7.4, was used. Acid *p*-nitrophenyl phosphatase (EC 3.1.3.2) and alkaline *p*-nitrophenyl phosphatase (EC 3.1.3.1) were assayed by the method of Torriani (1960).

Triton X-100 (final concentration 0.01%) was routinely added to enzyme incubation mixtures with the exception of those for catalase, long chain alcohol and aldehyde dehydrogenases and palmitoyl-CoA dependent reduction of NAD⁺. Acid and alkaline phosphatase assays were carried out in the presence of 0.2% Triton X-100. All spectrophotometric assays were carried out at 30 °C using a Pye-Unicam SP1800 spectrophotometer.

Preliminary experiments were carried out with cell extracts to ensure that the enzyme activities were proportional to the amount of extract protein used and were not limited by substrate concentration. Where necessary appropriate dilutions of cell extracts and fractions were used to obtain a suitable enzyme rate.

Protein was measured by the Lowry method with bovine serum albumin as standard. Metrizamide interference in protein determinations was excluded by precipitating the protein in 10% (w/v) cold TCA. The protein pellet produced by centrifugation at 10000 *g* for 10 min in the MSE 18 was washed in 10% cold TCA.

Electron microscopy. Material in fractions was fixed by the addition of 1.5 ml 24.8% (w/v) glutaraldehyde (Agar Aids, Bishop's Stortford, Herts, U.K.) to 3 ml of fraction and 2.5 ml disruption buffer. Fixation was for 2 h at 4 °C during centrifugation at 17000 *g*. Post-fixation was with 2% osmium tetroxide in 0.05 M-potassium phosphate buffer (pH 7.3).

Fixed pellets were stained with 1.5% uranyl acetate for 90 min, dehydrated through an ethanol series and embedded in Araldite. Sections were cut at a nominal 60 nm thickness using a Reichert OM U2 ultramicrotome. The sections were stained with saturated uranyl acetate for 15 min and Reynolds lead citrate for 1.5 min. All specimens were viewed on a AEI 802 electron microscope at 60 kV.

Chemicals. All enzyme substrates were obtained from Sigma. Hexadecane was obtained from Aldrich and metrizamide from Nyegaard and Co., Oslo, Norway. All other chemicals were obtained from BDH in AnalaR form.

RESULTS

Differential centrifugation of cell extracts produced from spheroplasts of cells treated with either snail-enzyme or Zymolyase-5000

Following growth on *n*-hexadecane, approximately 10 times more catalase and malate dehydrogenase and three times more cytochrome *c* oxidase were sedimented from cell extracts prepared from Zymolyase-5000 than from those prepared using snail-enzyme (Table 1). Extracts of glucose-grown cells showed little difference in the amounts of both catalase and cytochrome *c* oxidase sedimented irrespective of the methods of extract preparation. Malate dehydrogenase, however, was six times more sedimentable in these extracts following Zymolyase-5000 treatment.

Low sedimentability of enzyme activities for snail-enzyme treated cells suggests loss of enzyme from damaged organelles; consequently Zymolyase-5000 was employed for further preparations of cell extracts.

Effect of Triton X-100 or metrizamide on enzyme activity

Triton X-100 (final concentration 0.01%) increased the activity of a number of enzymes in cell extracts prepared from both glucose and *n*-hexadecane-grown cells (Table 2). This effect was probably caused by release of hitherto latent activity from cell organelles. An increase in Triton X-100 final concentration from 0.01 to 0.03% produced some inhibition of enzyme activity. The

Fig. 1. Zonal centrifugation of a cell extract of glucose-grown *C. stellatoidea* on a sucrose gradient. Cell extract (10 ml) containing 75 mg protein, was loaded on the gradient. Centrifugation was at 28000 r.p.m. for 236 min (6×10^6 g-min at the sample zone; $\int_0^t \omega^2 dt = 1.45 \times 10^{11}$ rad² s⁻¹). Enzyme activities shown on the ordinates are milli units ml⁻¹, where one unit represents μ mol substrate used or product formed min⁻¹. Where indicated in the text percentage sedimentability refers to the amount of enzyme activity detected beyond $\rho = 1.10$ g ml⁻¹. (a) Sucrose density gradient (○), light scattering at 520 nm (—), 260 nm (---) and protein (●). (b) Alkaline phosphatase (○) and acid phosphatase (●). (c) Cytochrome c oxidase (○), malate dehydrogenase (●) and aconitase (□). (d) Catalase (○), carnitine acetyltransferase (●) and citrate synthase (□). (e) Long chain alcohol dehydrogenase (○), ethanol dehydrogenase (●).

Table 1. *Sedimentability of selected enzymes in cell extracts prepared by snail-enzyme and Zymolyase-5000 treatment*

Sedimentability was calculated as a percentage of the total recovered activity present in the pellet formed following centrifugation at 20000 *g* for 20 min. Recoveries of enzyme activity were between 75 and 100%.

Enzyme	Sedimentability (%)			
	<i>n</i> -Hexadecane-grown		Glucose-grown	
	Snail-enzyme	Zymolyase-5000	Snail-enzyme	Zymolyase-5000
Catalase	5	59	73	68
Cytochrome <i>c</i> oxidase	31	90	91	96
Malate dehydrogenase	7	62	13	84

Table 2. *Effect of Triton X-100 (final concentration 0.01%) on enzyme activity*

Enzyme	Increase in activity (%) in extract	
	Glucose-grown	<i>n</i> -Hexadecane-grown
Catalase	19	0
Cytochrome <i>c</i> oxidase	376	156
Malate dehydrogenase	400	212
Aconitase	669	163
Carnitine acetyltransferase	400	104
NAD ⁺ -linked isocitrate dehydrogenase	285	78
Isocitrate lyase	ND	39
NADPH-cytochrome <i>c</i> reductase	0	0

ND, Not detectable.

presence of Triton X-100, even at a final concentration of 0.01% inhibited the activity of long chain alcohol and aldehyde dehydrogenase and palmitoyl-CoA dependent reduction of NAD⁺. Rapid freeze-thawing of cell extracts did not alter the activity of the first two enzymes but the activity of the last was lost. Assay mixtures of both acid and alkaline phosphatases employed 0.2% Triton X-100 to ensure that total (i.e. latent plus free) activities were measured.

Incubation of cell extract in either disruption buffer or disruption buffer containing 30% (w/v) metrizamide for 4 h at 4°C showed that metrizamide reduced the detectable activity of a number of enzymes: NAD⁺-linked isocitrate dehydrogenase activity was reduced by 20%; cytochrome *c* oxidase, malate dehydrogenase, citrate synthase and palmitoyl-CoA dependent reduction of NAD⁺ by 30 to 40% and carnitine acetyltransferase activity by more than 40% and all isocitrate lyase activity was lost. Activities of catalase and NADPH-cytochrome *c* oxidoreductase were not affected by metrizamide under these conditions. Metrizamide was shown not to affect reagents of the assay systems employed, therefore reductions in activity were caused by direct inhibition or inactivation.

Zonal centrifugation of an extract from glucose-grown cells on a sucrose gradient

The profiles of absorbance at 260 nm and 520 nm showed major peaks at $\rho = 1.09 \text{ g ml}^{-1}$ and $\rho = 1.21 \text{ g ml}^{-1}$, respectively (Fig. 1*a*). Approximately 50% of the protein remained in the sample zone, and relatively small accumulations of protein corresponded with major zones of absorbance at 260 nm and 520 nm. Acid and alkaline phosphatases were 24% and 49% sedimentable, respectively, forming zones of low activity over a broad density range (Fig. 1*b*).

long chain aldehyde dehydrogenase (●) and ethanol dehydrogenase (□). Specific activities [mU (mg protein)⁻¹] of enzymes in the cell extract were as follows (recoveries in parentheses): protein (122%), alkaline phosphatase 27 (102%), acid phosphatase 4 (124%), cytochrome *c* oxidase 262 (51%), malate dehydrogenase 2291 (95%), aconitase 27 (71%), catalase 19 (97%), carnitine acetyltransferase 30 (114%), citrate synthase 29 (64%), long chain alcohol dehydrogenase 11 (129%), long chain aldehyde dehydrogenase 41 (142%) and ethanol dehydrogenase 1 (76%).

Fig. 2. Zonal centrifugation of a cell extract of *n*-hexadecane-grown *C. stellatoidea* on a sucrose gradient. Cell extract (10 ml) containing 29 mg protein, was loaded on the gradient. Centrifugation was at 28000 r.p.m. for 236 min (6×10^6 g-min at the sample zone; $\int_0^t \omega^2 \cdot dt = 1.45 \times 10^{11}$ rad² s⁻¹). Enzyme activities shown on the ordinates are milli units ml⁻¹, where one unit represents μ mol substrate used or product formed min⁻¹. Where indicated in the text percentage sedimentability refers to the

Non-sedimentable activity was located mainly in the sample zone although acid phosphatase showed evidence of movement into the gradient. All cytochrome *c* oxidase and 94% malate dehydrogenase activity gave symmetrical peaks at $\rho = 1.20 \text{ g ml}^{-1}$ (Fig. 1c). Aconitase (Fig. 1c) and citrate synthase (Fig. 1d) sedimented to a higher density of 1.21 g ml^{-1} , with less than 10% of their recovered activity remaining in the sample zone. Carnitine acetyltransferase (Fig. 1d) was broadly distributed over a region occupied by catalase, cytochrome *c* oxidase and citrate synthase. Non-sedimentable activities of carnitine acetyltransferase (23%) and catalase (19%) remained in or around the sample zone. Particles containing sedimentable catalase (Fig. 1d) activity were found at a lower density ($\rho = 1.18 \text{ g ml}^{-1}$) than those containing cytochrome *c* oxidase. Over 90% of the long chain alcohol dehydrogenase and aldehyde dehydrogenase activities (Fig. 1e) were sedimentable forming broad, complex distributions. The most active fraction for long chain alcohol dehydrogenase was at $\rho = 1.21 \text{ g ml}^{-1}$ corresponding to the peak of citrate synthase activity, whereas the most active fraction for long chain aldehyde dehydrogenase was at $\rho = 1.18 \text{ g ml}^{-1}$ corresponding to the peak of catalase activity. Ethanol dehydrogenase activity (Fig. 1e) was manifest only in the sample zone.

Zonal centrifugation of an extract from n-hexadecane-grown cells on a sucrose gradient

The profile of absorbance at 260 nm was complex showing six distinct maxima (Fig. 2a). Protein distribution was similar to that for the extract from glucose-grown cells with approximately 50% remaining in the sample zone (Fig. 2a). The majority of the acid and alkaline phosphatase activities (Fig. 2b) were recovered from the sample zone although there was some movement of the former enzyme into the gradient. As shown for the extract from glucose-grown cells, ethanol dehydrogenase was manifest only in the sample zone (Fig. 2f). With the exception of citrate synthase, catalase and isocitrate lyase, the remaining 12 enzymes showed greater than 80% sedimentable activity with a maximum at $\rho = 1.21 \text{ g ml}^{-1}$. Maximum activity of citrate synthase (Fig. 2d; 94% sedimentable) was at $\rho = 1.23 \text{ g ml}^{-1}$ and that for catalase (Fig. 2e; 73% sedimentable) was $\rho = 1.23 \text{ g ml}^{-1}$. The most active fraction for isocitrate lyase was in the sample zone and sedimentable activity (75%) was distributed over a density range of 1.17 to 1.30 g ml^{-1} (Fig. 2e). Cytochrome *c* oxidase and malate dehydrogenase (Fig. 2c), palmitoyl-CoA dependent reduction of NAD^+ (Fig. 2d), malate synthase (Fig. 2e) and long chain aldehyde dehydrogenase (Fig. 2f) all showed either a shoulder or small peak at a higher density than their most active fraction. On the other hand, aconitase (Fig. 2c) and long chain alcohol dehydrogenase (Fig. 2f), together with citrate synthase and catalase, showed either a shoulder or peak of activity at a lower density than their most active fraction.

Zonal centrifugation of an extract from glucose-grown cells on a metrizamide gradient

Protein showed a complex distribution throughout the gradient (Fig. 3a). The profile of absorbance at 520 nm was also complex with three major zones at $\rho = 1.05$, 1.09 and 1.21 g ml^{-1} (Fig. 3a). Distributions of acid and alkaline phosphatases were dissimilar (Fig. 3b). Acid phosphatase activity was located in two major zones at $\rho = 1.05$ and 1.11 g ml^{-1} . Alkaline phosphatase activity, on the other hand, was virtually all non-sedimentable and manifest at a density lower than that of the theoretical sample zone. Cytochrome *c* oxidase, malate

amount of enzyme activity detected beyond $\rho = 1.10 \text{ g ml}^{-1}$. (a) Sucrose density gradient (○), light scattering at 260 nm (---) and protein (●). (b) Alkaline phosphatase (○), acid phosphatase (●) and NAD^+ -linked isocitrate dehydrogenase (□). (c) Cytochrome *c* oxidase (○), malate dehydrogenase (●) and aconitase (□). (d) Palmitoyl-CoA dependent reduction of NAD^+ (○), carnitine acetyltransferase (●) and citrate synthase (□). (e) Catalase (○), malate synthase (●) and isocitrate lyase (□). (f) Long chain alcohol dehydrogenase (○), long chain aldehyde dehydrogenase (●) and ethanol dehydrogenase (□). Specific activities [$\text{mU (mg protein)}^{-1}$] of enzymes in the cell extract were as follows (recoveries in parentheses): protein (93%), alkaline phosphatase 74 (118%), acid phosphatase 7 (102%), NAD^+ -linked isocitrate dehydrogenase 56 (77%), cytochrome *c* oxidase 257 (58%), malate dehydrogenase 4639 (87%), aconitase 61 (78%), palmitoyl-CoA dependent reduction of NAD^+ 36 (50%), carnitine acetyltransferase 412 (63%), citrate synthase 429 (64%), catalase 315 (69%), malate synthase 39 (61%), isocitrate lyase 25 (71%), long chain alcohol dehydrogenase 416 (86%), long chain aldehyde dehydrogenase 43 (155%) and ethanol dehydrogenase 9 (60%).

Fig. 3. Zonal centrifugation of a cell extract of glucose-grown *C. stellatoidea* on a metrizamide gradient. Cell extract (10 ml) containing 56 mg protein, was loaded on the gradient. Centrifugation was at 20000 r.p.m. for 180 min (6×10^6 g-min at the sample zone; $\int_0^t \omega^2 \cdot dt = 1.45 \times 10^{11}$ rad² s⁻¹). Enzyme activities shown on the ordinates are milli units ml⁻¹, where one unit represents μ mol substrate used or product formed min⁻¹. Where indicated in the text percentage sedimentability refers to the amount of

dehydrogenase (Fig. 3c) and citrate synthase (Fig. 3d) formed almost symmetrical peaks of activity at $\rho = 1.20$ to 1.22 g ml^{-1} . A large proportion of NAD^+ -linked isocitrate dehydrogenase activity (Fig. 3c) and carnitine acetyltransferase activity (Fig. 3d) was also located in this region of the gradient. In addition, however, NAD^+ -linked isocitrate dehydrogenase formed a zone of activity at $\rho = 1.07 \text{ g ml}^{-1}$ and approximately 50% carnitine acetyltransferase activity was distributed between $\rho = 1.03$ and 1.13 g ml^{-1} . The major portion of catalase activity (Fig. 3e) sedimented to a region denser than, but overlapping, that of cytochrome *c* oxidase activity. A second zone of catalase activity corresponded to the peak of carnitine acetyltransferase and NAD^+ -linked isocitrate dehydrogenase activity at $\rho = 1.07 \text{ g ml}^{-1}$. NADPH-cytochrome *c* oxidoreductase activity (Fig. 3e) was distributed throughout the gradient with a maximum at $\rho = 1.10 \text{ g ml}^{-1}$ and a large proportion of activity located in the same region as cytochrome *c* oxidase activity. For both NADPH-cytochrome *c* oxidoreductase and NAD^+ -linked isocitrate dehydrogenase, a pronounced shoulder of activity was evident at a density higher than cytochrome *c* oxidase activity. Long chain aldehyde dehydrogenase (Fig. 3f) was recovered from most regions of the gradient with the majority of activity forming a symmetrical peak at $\rho = 1.20 \text{ g ml}^{-1}$. Additional peaks of activity corresponded to the most active fraction of alkaline phosphatase, the most active fraction of NADPH-cytochrome *c* oxidoreductase and also the peak of carnitine acetyltransferase activity at $\rho = 1.05 \text{ g ml}^{-1}$.

Zonal centrifugation of an extract from n-hexadecane-grown cells on a metrizamide gradient

Protein (Fig. 4a) was recovered throughout the gradient with relatively large amounts located in fractions containing mitochondrial enzyme activity. Almost all alkaline phosphatase activity was located in a broad band at $\rho = 1.04 \text{ g ml}^{-1}$ (Fig. 4b). An electron micrograph of this fraction (Fig. 5a) indicates the presence of ribosomal material and the absence of membraneous organelles. Some acid phosphatase activity (Fig. 4b) was also recovered from this region, but the major zone of activity had sedimented to $\rho = 1.12 \text{ g ml}^{-1}$. Cytochrome *c* oxidase (Fig. 4c), citrate synthase (Fig. 4d) and NAD^+ -linked isocitrate dehydrogenase (Fig. 4c) formed almost symmetrical peaks of activity at $\rho = 1.17 \text{ g ml}^{-1}$. An electron micrograph of this region (Fig. 5b) of the gradient demonstrated the presence of mitochondria though these were contaminated with small membraneous vesicles, possibly rough endoplasmic reticulum. The last two enzymes also showed zones of lower activity at lower densities. Although the most active fraction for malate dehydrogenase (Fig. 4c) corresponded to that for cytochrome *c* oxidase, a substantial amount of activity was found throughout the rest of the gradient. Carnitine acetyltransferase (Fig. 4d) showed a bimodal distribution with activity manifest in fractions containing cytochrome *c* oxidase activity, and those containing all recovered activity of palmitoyl-CoA dependent reduction of NAD^+ at $\rho = 1.08 \text{ g ml}^{-1}$. Catalase activity (Fig. 4e) was also found in two regions of the gradient; a major zone at $\rho = 1.07 \text{ g ml}^{-1}$ and one at a slightly greater density than that of cytochrome *c* oxidase activity ($\rho = 1.19 \text{ g ml}^{-1}$). Electron micrographs of the region of the gradient at $\rho = 1.07 \text{ g ml}^{-1}$ (Fig. 6a) reveal the presence of organelles about $1 \mu\text{m}$ in length, whereas the region at $\rho = 1.19 \text{ g ml}^{-1}$ (Fig. 6b) contained what appear to be conventional microbodies with granular matrices and approximately $0.5 \mu\text{m}$ in length. NADPH-cytochrome *c* oxidoreductase activity (Fig. 4e) was recovered from most regions of the gradient with peaks of activity in fractions corresponding to those of long chain alcohol dehydrogenase and aldehyde dehydrogenase activity (Fig. 4f) between $\rho = 1.09$ and

enzyme activity detected beyond $\rho = 1.10 \text{ g ml}^{-1}$. (a) Metrizamide density gradient (○), light scattering at 520 nm (—) and protein (●). (b) Alkaline phosphatase (○) and acid phosphatase (●). (c) Cytochrome *c* oxidase (○) malate dehydrogenase (●) and NAD^+ -linked isocitrate dehydrogenase (□). (d) Carnitine acetyltransferase (●) and citrate synthase (□). (e) Catalase (○) and NADPH-cytochrome *c* oxidoreductase (●). (f) Long chain aldehyde dehydrogenase (●). Specific activities [mU (mg protein)⁻¹] of enzymes in the cell extract were as follows (recoveries in parentheses): protein (114%), alkaline phosphatase 51 (87%), acid phosphatase 4 (102%), cytochrome *c* oxidase 380 (36%), malate dehydrogenase 3230 (44%), NAD^+ -linked isocitrate dehydrogenase 122 (54%), carnitine acetyltransferase 64 (39%), citrate synthase 147 (49%), catalase 18 (60%), NADPH-cytochrome *c* oxidoreductase 43 (100%) and long chain aldehyde dehydrogenase 13 (155%).

Fig. 4. Zonal centrifugation of a cell extract of *n*-hexadecane-grown *C. stellatoidea* on a metrizamide gradient. Cell extract (10 ml) containing 32 mg protein, was loaded on the gradient. Centrifugation was at 20000 r.p.m. for 180 min (6×10^6 g·min at the sample zone; $\int_0^t \omega^2 \cdot dt = 1.45 \times 10^{11}$ rad² s⁻¹). Enzyme activities shown on the ordinates are milli units ml⁻¹, where one unit represents μ mol substrate used or product formed min⁻¹. Where indicated in the text percentage sedimentability refers to the

1.12 g ml⁻¹. The distribution of long chain alcohol dehydrogenase was extremely complex showing further major zones of activity in the sample zone and in fractions containing cytochrome *c* oxidase activity.

DISCUSSION

Using the criterion of sedimentability to indicate structural integrity of mitochondria and microbodies, there was no gross organelle comminution after snail-enzyme or Zymolyase-5000 treatment of glucose-grown cells, although malate dehydrogenase, an enzyme found largely in the mitochondrial matrix, was partially released after snail-enzyme treatment. For *n*-hexadecane-grown cells, the low sedimentability of the three enzymes shown in Table 1 following snail-enzyme treatment shows that damage is caused to both mitochondria and microbodies and thus Zymolyase-5000 was used in the preparation of spheroplasts.

The results show that care must be taken when incorporating Triton X-100 into enzyme assays. The majority of enzymes exhibit a greater degree of latency in glucose-grown cells than in *n*-hexadecane-grown cells, but are also inhibited by relatively low concentrations of this agent.

Complex distributions of organelles were found throughout the gradients. Mitochondria, as identified by cytochrome *c* oxidase activity formed symmetrical, sedimentable zones. Structural integrity of mitochondria on sucrose gradients was confirmed by the presence of the activities of established mitochondrial matrix enzymes (malate dehydrogenase, citrate synthase, aconitase and NAD⁺-linked isocitrate dehydrogenase) within the zone of cytochrome *c* oxidase activity. The location of some malate dehydrogenase and aconitase activity at regions of lower density, particularly in extracts of *n*-hexadecane-grown cells, may indicate that these mitochondria are more fragile than those from extracts of glucose-grown cells.

For all four fractionations, microbodies (as defined by the major regions of sedimentable catalase activity) were not well separated from the mitochondrial zone. These zones of catalase activity are generally more complex, and of differing densities but are less dense than mitochondria from extracts of glucose-grown cells on sucrose gradients and more dense than mitochondria for the other three conditions. On sucrose gradients, a second peak of activity was evident in the sample zone; more activity in this region from extracts of *n*-hexadecane-grown cells probably indicates increased fragility of microbodies. On metrizamide gradients, there was little activity in the sample zone, though major zones were evident at a density of 1.07 g ml⁻¹. Electron micrographs indicate the presence of organelles approximately 1 µm in length.

Isocitrate lyase and malate synthase were not detectable in extracts of glucose-grown cells. Fractionation in sucrose gradients of extracts of *n*-hexadecane-grown cells indicates that the enzymes are largely sedimentable beyond a density of 1.17 g ml⁻¹, though with different distributions.

After sucrose gradient fractionations, carnitine acetyltransferase activity was distributed largely between mitochondria and microbodies; a result in agreement with the findings of Kawamoto *et al.* (1978*b*). However, in our experiments, the remaining activity was in the sample zone and was not microsomal. After fractionation on metrizamide gradients, carnitine acetyltransferase activity was again located in the mitochondria and microbody region beyond a density of 1.15 g ml⁻¹ though half of the recovered activity was found associated with the microbodies at a density of 1.07 g ml⁻¹.

amount of enzyme activity detected beyond $\rho = 1.10$ g ml⁻¹. (a) Metrizamide density gradient (○) and protein (●). (b) Alkaline phosphatase (○) and acid phosphatase (●). (c) Cytochrome *c* oxidase (○), malate dehydrogenase (●) and NAD⁺-linked isocitrate dehydrogenase (□). (d) Palmitoyl-CoA dependent reduction of NAD⁺ (○), carnitine acetyltransferase (●) and citrate synthase (□). (e) Catalase (○) and NADPH-cytochrome *c* oxidoreductase (●). (f) Long chain alcohol dehydrogenase (○) and long chain aldehyde dehydrogenase (●). Specific activities [mU (mg protein)⁻¹] of enzymes in the cell extract were as follows (recoveries in parentheses): protein (116%), alkaline phosphatase 41 (84%), acid phosphatase 3 (61%), cytochrome *c* oxidase 280 (45%), malate dehydrogenase 4640 (45%), NAD⁺-linked isocitrate dehydrogenase 63 (51%), palmitoyl-CoA dependent reduction of NAD⁺ 37 (37%), carnitine acetyltransferase 239 (65%), citrate synthase 446 (57%), catalase 465 (75%), NADPH-cytochrome *c* oxidoreductase 43 (82%), long chain alcohol dehydrogenase 306 (100%) and long chain aldehyde dehydrogenase 89 (103%).

Fig. 5. Electron micrographs of section of material from experiment shown in Fig. 4. (a) Fraction 40, (b) fraction 20. In both cases the bar marker represents 1 μm .

Fig. 6. Electron micrographs of section of material from experiment shown in Fig. 4. (a) Fraction 36, (b) fraction 17. In both cases the bar marker represents 1 μ m.

After centrifugation of *n*-hexadecane-grown extracts in sucrose gradients, palmitoyl-CoA dependent reduction of NAD⁺ was found in the mitochondria and microbody region, though after centrifugation in metrizamide gradients all of the activity was found in the region containing microbodies at a density of 1.07 g ml⁻¹.

The term microbody used throughout this paper is a general term including peroxisomes and glyoxysomes. Peroxisomes are recognized as containing catalase and at least one flavin oxidase while the latter contain, in addition, isocitrate lyase and/or malate synthase (Cioni *et al.*, 1981; Tolbert & Essner, 1981). Our results indicate that whilst only one population of microbodies can be resolved in sucrose gradients, two populations differing in size, density and enzyme complement can be resolved in metrizamide gradients. The possibility that *C. stellatoidea* contains both peroxisomes and glyoxysomes within the same cell must be considered, though it is difficult to confirm this due to the problems in determining the distribution of isocitrate lyase and malate synthase on metrizamide gradients.

After fractionation of extracts on sucrose gradients, long chain alcohol and aldehyde dehydrogenases appeared to be largely associated with mitochondria and microbodies, agreeing with the findings of Yamada *et al.* (1980) using *C. tropicalis* and *C. lipolytica*. These authors concluded, however, that the enzymes are largely located in microbodies, whereas our results indicated that after growth on *n*-hexadecane long chain alcohol dehydrogenase and probably long chain aldehyde dehydrogenase are mitochondrial. After fractionation on metrizamide gradients of extracts of glucose-grown cells, long chain aldehyde dehydrogenase was again located in the mitochondria, though half of the activity was distributed between the sample zone, microbody zone at $\rho = 1.07 \text{ g ml}^{-1}$ and a zone at 1.10 g ml^{-1} . The latter zone corresponded to the peak of Antimycin-A insensitive NADPH-cytochrome *c* oxidoreductase activity which may indicate the presence of microsomal elements. After a similar fractionation on a metrizamide gradient of an extract from *n*-hexadecane-grown cells, the distribution of long chain aldehyde dehydrogenase was markedly different, the majority of activity corresponding to the zone of Antimycin-A insensitive NADPH-cytochrome *c* oxidoreductase activity. Long chain alcohol dehydrogenase activity was distributed between sample zone, microbody region at 1.07 g ml^{-1} , Antimycin-A insensitive NADPH-cytochrome *c* oxidoreductase zone and the mitochondrial region. Where assayed, ethanol dehydrogenase activity was located only in the sample zone.

Distributions of acid and alkaline phosphatase activity are similar to each other on sucrose gradients, but differ markedly on metrizamide gradients. In all four fractionations, alkaline phosphatase activity remained largely in, or near, the sample zone though the small amount of activity located in the mitochondrial zones may reflect microsomal contamination of these organelles. The considerable increase in sedimentable acid phosphatase activity on metrizamide gradients compared with that on sucrose gradients suggests that the former material should be used in experiments to isolate lysosomes.

The subcellular distribution of enzymes in *C. stellatoidea* is more easily investigated using extracts from Zymolyase-5000 produced spheroplasts by centrifugation on metrizamide gradients which preserve maximal sedimentability of many enzymes.

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