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Subcellular Fractionation by Differential and Zonal Centrifugation of the Trypanosomatid *Crithidia fasciculata*

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SUMMARY

Homogenates of *Crithidia fasciculata* were fractionated by differential centrifugation. Mitochondria were sedimented quantitatively at 10^4 *g*-min and accounted for approximately 10% of the total recovered protein. Catalase was found exclusively in the supernatant fraction whilst NADH: cytochrome *c* oxidoreductase and *p*-nitrophenylphosphatase were found in all the fractions. Zonal centrifugation confirmed that catalase was non-sedimentable. Clean separation of mitochondria was obtained in both high-speed and rate zonal experiments, but no NADH: cytochrome *c* oxidoreductase activity could be detected in these organelles. Separation of large lysosomal vacuoles which contained *p*-nitrophenylphosphatase activity was obtained and these were clearly resolved from mitochondria by both high-speed and rate zonal centrifugation.

INTRODUCTION

Satisfactory analytical subcellular fractionation studies of trypanosomes have not been reported, mainly due to the resistance of these parasites to conventional cell disruption methods (Simpson, 1972) which may be due to the presence of pellicular microtubules (Angelopoulos, 1970). Previous studies with *Crithidia fasciculata* used relatively harsh cell breakage methods, such as a French pressure cell or sonication (Toner & Weber, 1967), grinding with alumina (Hill & White, 1968) or using a Polytron blender after treatment with digitonin (Kusel & Storey, 1972). Such methods have mainly been used in an attempt to isolate intact mitochondria from trypanosomes, a task made difficult as the organism contains a single large mitochondrion which convolutes and branches throughout the cytoplasm.

In the present investigation we have achieved good separation of subcellular organelles by density-gradient zonal centrifugation of cell-free homogenates prepared by rupturing digitonin-treated suspensions of *C. fasciculata* in a Chaikoff press.

METHODS

Growth, harvesting and preparation of cell-free extracts. Crithidia fasciculata was maintained, grown, counted, harvested and disrupted as previously described (Edwards & Lloyd, 1973).

Fractionation of whole homogenates by differential centrifugation. The whole homogenate was centrifuged for 10 min at 10000 *g* ($r_{av.}$ = 7·6 cm) in the 8 × 50 ml rotor of an MSE 18 centrifuge. The supernatant fluid (S1) was carefully decanted, leaving the first pellet (P1), and then centrifuged for 20 min at 20000 *g* in the same centrifuge. This supernatant fluid (S2) was decanted from the first 'microsomal' pellet (P2) and centrifuged for 60 min at

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106000 g ($r_{av.}$ = 6 cm) in the 8 × 10 ml rotor of an MSE 40 centrifuge, giving a pellet (P3) and a final supernatant fluid (S3). The pellets were uniformly resuspended in known volumes of the homogenization buffer immediately on collection. All procedures were carried out at 4 °C.

Fractionation by zonal centrifugation. High-speed zonal centrifugation was done in a BXIV rotor (Anderson *et al.*, 1967) in an MSE 40 centrifuge. The rotor was loaded at 2500 rev. min^{-1} with a linear sucrose gradient [15 to 60% (w/w), total vol. 400 ml], the remainder of the rotor being filled with 60% (w/w) sucrose. The whole homogenate was pumped into the centre of the rotor followed by 30 ml of 6% (w/w) sucrose, and then 30 ml of 4% (w/w) sucrose overlay. The rotor was accelerated to 35000 rev. min^{-1} for 165 min (6×10^6 g-min at the sample zone). The rotor was unloaded at 2500 rev. min^{-1} and fractions (10 ml) were collected at 4 °C. Rate zonal centrifugation was done in an MSE HS zonal rotor in an MSE 18 centrifuge. The procedure was identical to that for high-speed zonal centrifugation except that the rotor was loaded and unloaded at 600 rev. min^{-1} and was accelerated to 10000 rev. min^{-1} for 30 min. All sucrose solutions used for zonal centrifugation in rotors contained 2 mM-$MgCl_2$ and were buffered to pH 8·0 with 10 mM-Tris/HCl. The gradients were generated and loaded into the rotor using an Isco 'Dialagrad' Pump (Shandon Southern Instruments, Camberley, Surrey) at a flow rate of 15 to 30 ml min^{-1}. Sucrose concentrations were measured using a refractometer and densities were calculated from the data of De Duve, Berthet & Beaufay (1959).

Enzyme assays. Assay conditions for acid *p*-nitrophenylphosphatase (EC 3.1.3.2), catalase (EC 1.11.1.6), NADH:cytochrome *c* oxidoreductase (EC 1.6.99.3) and NADPH: cytochrome *c* oxidoreductase (EC 1.6.2.4) have been described previously (Lloyd *et al.*, 1971). β-Glucosidase (EC 3.2.1.21) was assayed as described by Gatt (1969). Methods used for malate dehydrogenase (EC 1.1.1.37), succinate:cytochrome *c* oxidoreductase (EC 1.3.99.1) and succinate dehydrogenase (EC 1.3.99.1) were as described by Poole & Lloyd (1973). Assays for acid *p*-nitrophenylphosphatase were performed at 25 °C, and all spectrophotometric assays were carried out at room temperature (18 ± 2 °C) using a Cary model 14 recording spectrophotometer fitted with a fine slide wire which gave a full-scale deflexion corresponding to an extinction of 0·1.

Other determinations. Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Electron micrographs were obtained as described by Cartledge & Lloyd (1972).

Chemicals. All enzyme substrates were obtained from Sigma. Bovine serum albumin (fraction V from bovine plasma) was from Armour Pharmaceuticals, Eastbourne, Sussex.

Treatment and presentation of results. Enzyme units are expressed as nmol substrate transformed min^{-1} or nmol product formed min^{-1}. The vertical lines in Figs 2 and 3 divide the diagrams at density intervals of 0·05 g ml^{-1}. Percentage sedimentability in high-speed zonal centrifugation experiments is arbitrarily expressed as that proportion of total enzyme units recovered which had sedimented at densities greater than 1·10 g ml^{-1} after centrifuging for 6×10^6 g-min.

RESULTS

Changes in specific activities of enzymes during growth

Specific activities of all the enzymes assayed were at a maximum in homogenates of organisms harvested after 40 to 48 h, which corresponded to the late-exponential phase of growth and a density of approximately $1{\cdot}5 \times 10^8$ organisms ml^{-1} (Fig. 1). All experiments were performed with organisms from this phase of growth.

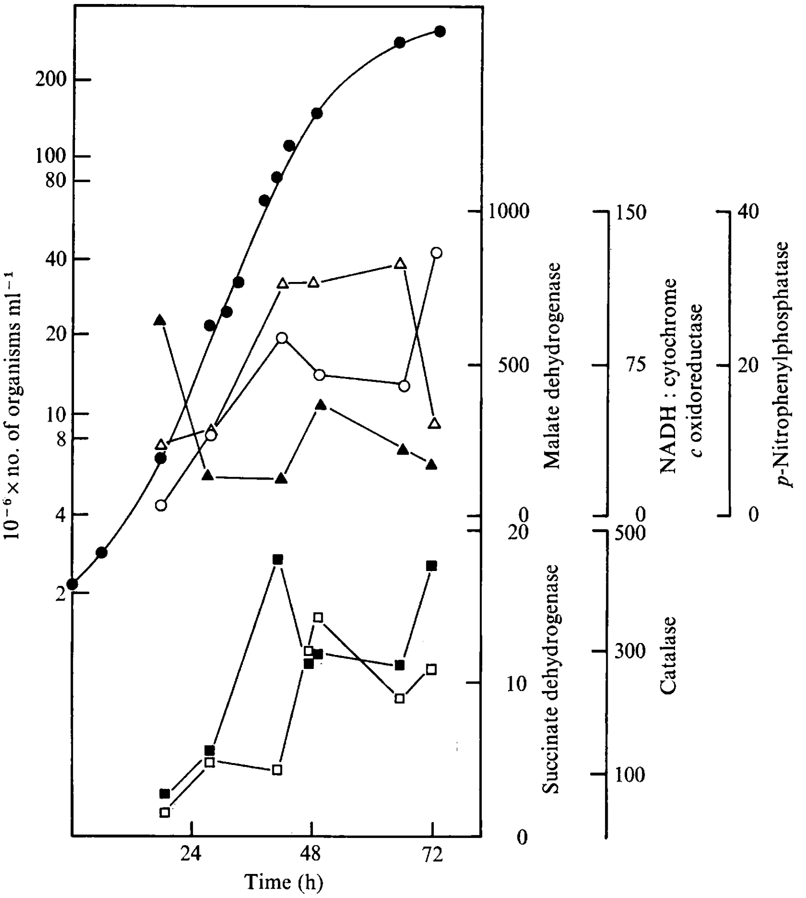

Fig. 1. Changes in the specific activities of malate dehydrogenase (△), NADH : cytochrome *c* oxidoreductase (▲), *p*-nitrophenylphosphatase (○), catalase (■) and succinate dehydrogenase (□) during growth of *Crithidia fasciculata.*

Fractionation by differential centrifugation

This procedure yielded a fraction P1 that contained 10·8 % of the total protein together with 101·8 % of the recovered units of succinate dehydrogenase (Table 1). NADH:cytochrome *c* oxidoreductase was distributed throughout the fractions, P1 containing more (40·9 %) than the other three fractions. NADPH:cytochrome *c* oxidoreductase was not detected in P3 and S3; P1 and P2 contained 24 and 14 % of the activity respectively. Recoveries of this enzyme were always low in differential centrifugation experiments and this could be due to the partitioning of an essential cofactor by the fractionation procedure. Almost all the catalase activity (98·4 %) and 50 % of the *p*-nitrophenylphosphatase activity were found in S3, the remainder of the activity of the latter being divided between the other three fractions. Protein and enzyme recoveries, except for NADPH:cytochrome *c* oxidoreductase, were well within an acceptable margin of error for this procedure and gave an indication of the subcellular distribution of protein and of the enzymes studied.

Table 1. *Distribution of marker enzymes in homogenates of Crithidia fasciculata after fractionation by differential centrifugation*

	Whole homogenate	P1	P2	P3	S3	Recovery (%)
Protein						
Concn (mg ml^{-1})	5·36	1·86	1·04	5·84	3·68	117·0
Weight (mg)	85·80	9·30	5·20	29·20	56·70	
% total	100	10·80	6·05	33·90	66·0	
NADH: cytochrome *c* oxidoreductase						
Specific activity	54	214	186·5	43·7	16·2	108·5
Activity (units)	4635	1890	970	1270	915	
Activity (% total)	100	40·9	21·0	27·6	19·8	
NADPH: cytochrome *c* oxidoreductase						
Specific activity	43·7	95·6	93·5	–	–	38·2
Activity (units)	3750	890	540	–	–	
Activity (% total)	100	23·7	14·4	–	–	
Succinate dehydrogenase						
Specific activity	1·06	10·0	0·68	0·25	–	113·0
Activity (units)	91·3	93·0	3·57	7·15	–	
Activity (% total)	100	101·8	3·90	7·82	–	
Catalase						
Specific activity	139	20·9	24·9	5·76	207	102·3
Activity (units)	11900	194·5	129·5	169·5	11700	
Activity (% total)	100	1·63	1·08	1·42	98·4	
p-Nitrophenylphosphatase						
Specific activity	34·4	52·5	41·3	18·80	26·1	92·5
Activity (units)	2950	490	215·5	550	1475	
Activity (% total)	100	16·6	7·60	18·65	50·0	

–, Not detected.

Fractionation of a whole homogenate by high-speed zonal centrifugation

After centrifuging whole homogenate for 165 min at 35000 rev. min^{-1} (6×10^6 *g*-min at the sample zone), analysis by light scattering at 260 nm revealed a complex distribution through the gradient, major peaks occurring at $\rho = 1·11$, probably due to ribosomal monomers, and at 1·20 (Fig. 2*a*). Protein analysis of the gradient showed that 56·8% was sedimentable and could be detected up to $\rho = 1·22$; a major peak at $\rho = 1·11$ corresponded to that of light scattering at 260 nm whilst the other major peak of protein (25% of the total recovered) remained at the sample zone (Fig. 2*a*). NADPH:cytochrome *c* oxidoreductase was distributed unevenly throughout the gradient and was 45% sedimentable; the bulk of the activity (39%) remained at the sample zone (Fig. 2*b*). Malate dehydrogenase was also unevenly distributed throughout the gradient and 43% of the recovered activity remained at the sample zone. However, peaks of activity were noted at $\rho = 1·10$, 1·15 and 1·21, the latter density probably marking the isopycnic density of mitochondria. β-Glucosidase was mainly soluble as 75% of the total activity remained at the sample zone. A large proportion (43%) of the NADH:cytochrome *c* oxidoreductase was recovered from the sample zone but 47·5% was sedimentable; a distinct peak of activity at $\rho = 1·20$ corresponded to the mitochondrial band (Fig. 2*c*). All the recovered catalase activity (approximately 100%) was found in the sample zone, suggesting that this enzyme is soluble in *C. fasciculata* and has no peroxisomal association. The major peak of activity of *p*-nitrophenylphosphatase occurred at $\rho = 1·22$ with a distinct shoulder at $\rho = 1·20$ corresponding to the proposed

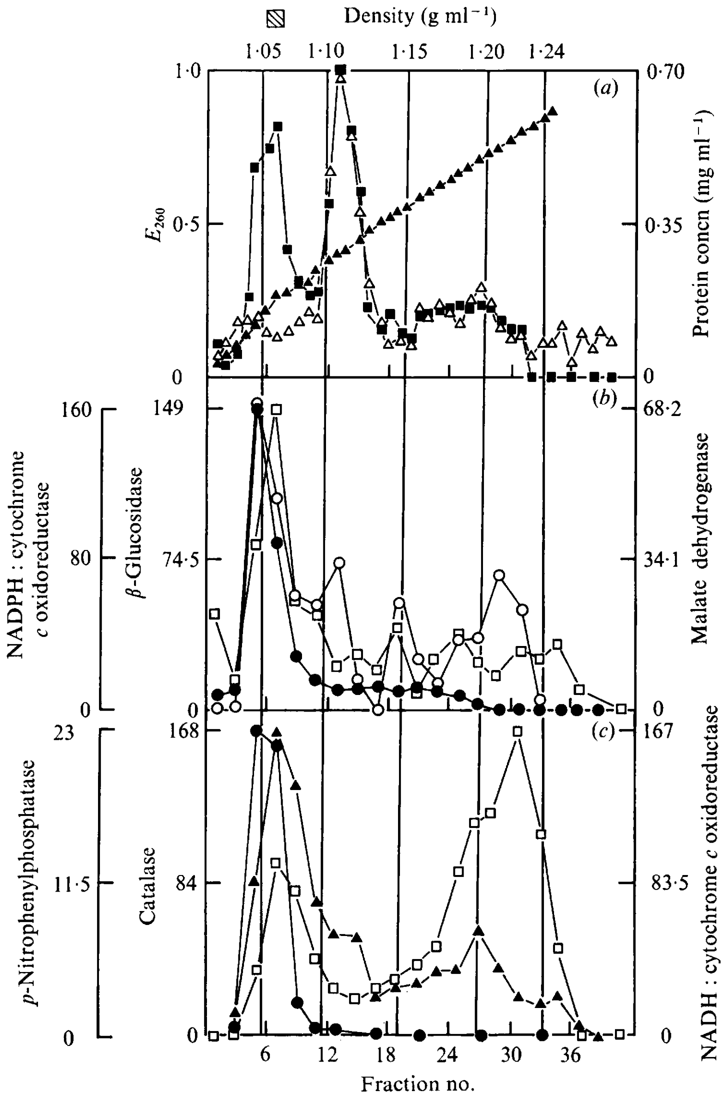

Fig. 2. High-speed subcellular fractionation of a whole homogenate of *Crithidia fasciculata* on a linear sucrose gradient in the MSE BXIV zonal rotor. Whole homogenate (14 ml), containing 116·2 mg protein, was loaded on to the gradient. The volumes of homogenate (diluted 1 in 20) and of fractions taken for assay were as follows: catalase, NADH: and NADPH:cytochrome *c* oxidoreductases, β-glucosidase, *p*-nitrophenylphosphatase and malate dehydrogenase, all 0·1 ml. Centrifugation was at 35000 rev. min^{-1} for 165 min (6 × 10^6 *g*-min at the sample zone; $\int_0^t \omega^2.dt = 1{\cdot}45 \times 10^{11}$ rad^2 s^{-1}). The shaded rectangle represents the sample zone before centrifugation.

(*a*) Sucrose density gradient (▲), light scattering at 260 nm after 1 in 30 dilution (△) and protein (■); (*b*) NADPH:cytochrome *c* oxidoreductase (□), malate dehydrogenase (○) and β-glucosidase (●); (*c*) NADH:cytochrome *c* oxidoreductase (▲), catalase (●) and *p*-nitrophenylphosphatase (□). Specific activities of enzymes in the whole homogenate were as follows (recoveries in parentheses): NADPH:cytochrome *c* oxidoreductase 57·7 (24·8 %); malate dehydrogenase, 79·5 (75 %); β-glucosidase, 5·1 (124 %); NADH:cytochrome *c* oxidoreductase, 63·2 (25·2 %); catalase, 51·5 (114 %); and *p*-nitrophenylphosphatase, 44·5 (58 %); protein recovery was 68 %. All enzyme activities are expressed as units ml^{-1}.

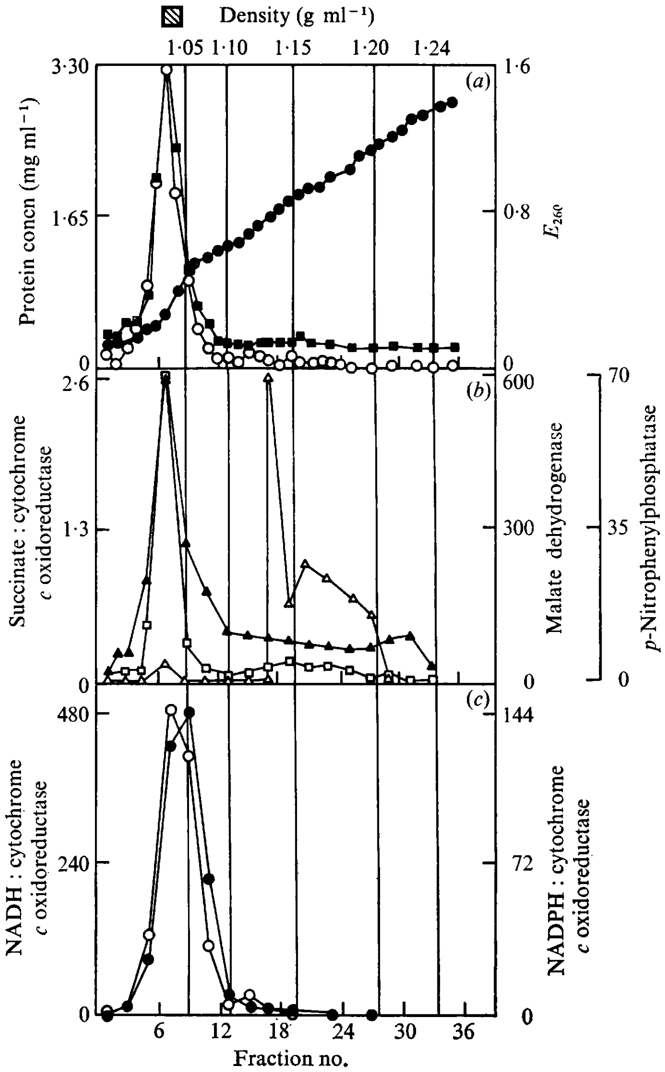

Fig. 3. Rate zonal separation of a whole homogenate of *Crithidia fasciculata* on a linear sucrose gradient in the MSE HS zonal rotor. The homogenate (20 ml) contained 166 mg protein. Whole homogenate was diluted 1 in 20 and volumes of diluted homogenate and of fractions taken for assay were as follows: succinate:cytochrome *c* oxidoreductase, malate dehydrogenase, *p*-nitrophenylphosphatase, NADH:cytochrome *c* oxidoreductase and NADPH:cytochrome *c* oxidoreductase, all 0·1 ml. After centrifuging at 10000 rev. min^{-1} for 30 min ($\int_0^t \omega^2 . dt = 2{\cdot}25 \times 10^9$ rad^2 s^{-1}), fractions (10 ml) were collected. The shaded rectangle represents the sample zone before centrifugation.

(*a*) Sucrose density gradient (●), light scattering at 260 nm after 1 in 30 dilution (■) and protein (○); (*b*) succinate:cytochrome *c* oxidoreductase (△), malate dehydrogenase (□) and *p*-nitrophenylphosphatase (▲); (*c*) NADH:cytochrome *c* oxidoreductase (●) and NADPH:cytochrome *c* oxidoreductase (○). Specific activities of enzymes in the whole homogenate were as follows (recoveries in parentheses): succinate:cytochrome *c* oxidoreductase, 0·8 (58·9 %); malate dehydrogenase, 120·8 (76·5 %); *p*-nitrophenylphosphatase, 30·1 (83 %); NADH:cytochrome *c* oxidoreductase, 153·1 (88·2 %); and NADPH:cytochrome *c* oxidoreductase, 59·9 (148 %); protein recovery was 74·8 %. All enzyme activities are expressed as units ml^{-1}.

isopycnic density of mitochondria. This enzyme was 76·4% sedimentable, only 20% of the total recovered activity remaining at the sample zone. Electron micrographs prepared from fractions 13, 18 and 28 indicated the increasing size of the various particles throughout the gradient. Fraction 28 contained structures up to 1 μm in diameter which showed characteristics of mitochondria, confirming the data obtained from enzymic analysis of this part of the gradient (ρ = 1·21).

Fractionation of a whole homogenate by rate zonal centrifugation

Light scattering at 260 nm and protein show simple distributions, the main peaks for each occurring at the sample zone. Protein was only 16·9% sedimentable (Fig. 3*a*). Succinate:cytochrome *c* oxidoreductase activity was 98·5% sedimentable; this enzyme, a mitochondrial marker, had a peak of activity at ρ = 1·135 (Fig. 3*b*). Malate dehydrogenase occurred mainly at the sample zone, but there was a second peak of activity at ρ = 1·15 corresponding to that observed for succinate:cytochrome *c* oxidoreductase. The bulk of the *p*-nitrophenylphosphatase activity remained at the sample zone but 37% was sedimentable. A build-up of very large particles containing this enzyme is apparent at ρ = 1·22, corresponding to the major peak of this enzyme after high-speed zonal centrifugation (Fig. 2*c*). Both NADH: and NADPH:cytochrome *c* oxidoreductases showed similar distributions, the bulk of the activity remaining at the sample zone. Electron micrographs prepared from fractions 2, 6, 11, 15, 19 and 23 showed a distinct gradation of particle size. Mitochondrial structures were evident only in fractions 19 and 23 and coincided with the wide band of succinate:cytochrome *c* oxidoreductase activity (Fig. 3*b*). The mean diameter of the mitochondrial profiles in fraction 23 was 0·58 ± 0·13 μm (25 determinations).

DISCUSSION

Fractionation of extracts of the trypanosomatid *Crithidia fasciculata* produced by gentle disruption of digitonin-treated organisms suggests that there is good preservation of released organelles. Although microbodies have been observed by histochemical means in several *Crithidia* spp. (Müller, 1975), the present results confirm a previous observation (Cohen, 1972) that catalase in *C. fasciculata* is non-sedimentable and not associated with microbodies. Malate dehydrogenase is also primarily non-sedimentable, but a proportion of the activity is associated with mitochondria which sediment at ρ = 1·20 to 1·21 on equilibrium density centrifugation. The presence of two distinct isoenzymes of malate dehydrogenase, one cytosolic and one mitochondrial, has been reported in *C. fasciculata* by Bacchi *et al.* (1964). Neither NADH: nor NADPH:cytochrome *c* oxidoreductase could be assigned to a mitochondrial location; the low recoveries of these two enzymes from high-speed zonal centrifugation experiments suggest a progressive loss of activity which did not occur in rate zonal experiments due to the shorter centrifugation times and lower rotor speeds used. Good separation of *p*-nitrophenylphosphatase, a lysosomal marker, was achieved and this indicated the presence of lysosomal vacuoles sedimenting at approximately ρ = 1·22. A similar result has been obtained recently by McLaughlin, Meerovitch & Injeyan (1976), who observed a band of *p*-nitrophenylphosphatase activity at ρ = 1·22 after high-speed zonal centrifugation of a homogenate of *C. fasciculata* for 16 h.

The results from the rate zonal centrifugation experiments indicate good separation of mitochondria as judged by the distribution of succinate:cytochrome *c* oxidoreductase activity. The mitochondrial population moved well out of the sample zone; electron micrographs indicated a relatively homogeneous population with little contamination by membranes of non-mitochondrial origin. The lack of detectable NADH:cytochrome *c* oxidoreductase in mitochondria-containing fractions was unexpected, as in other protozoa this enzyme system has a multi-locational distribution which includes mitochondria (Cooper & Lloyd, 1972; Lloyd *et al.*, 1971). Rate zonal experiments confirm that *p*-nitrophenylphosphatase is associated with large, rapidly sedimenting vacuoles, which can easily be separated from mitochondria. The observation that these organelles survive the procedure used for disruption of the organism confirms that the techniques reported here are suitable for further characterization of trypanosome organelles.

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