HETEROGENEITY OF YEAST MITOCHONDRIAL FRACTIONS REVEALED BY AUTOMATED ANALYSIS OF SUCROSE GRADIENTS

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Mitochondrial fractions were prepared from protoplasts of *Saccharomyces carlsbergensis* by a modification of the method of Ohnishi, Kawaguchi and Hagihara [1] and analysed on sucrose gradients. The gradients were monitored by a Technicon Autoanalyzer, with instrument calibration, calculation, tabulation and plotting of results performed on an ATLAS computer, using a FORTRAN V program. Bacteria-free mitochondrial fractions labelled *in vitro* with ^{14}C-leucine [2] were also analysed. It was found that succinate dehydrogenase, total protein and radioactive protein sedimented closely together and showed a homogeneous distribution in the gradient, with reasonably symmetrical bands. Thus the bulk of the preparation consisted of yeast mitochondria that could incorporate amino acids *in vitro*. However, in identical gradients monitored at the same time, both NADH-ferricyanide reductase and L(+)lactate-ferricyanide reductase showed complex sedimentation patterns, with at least three major components, and several minor components. Although these may represent mitochondrial break-down products, it is more likely that they are non-mitochondrial components.

S. carlsbergensis was grown on a lactate medium [1] and early stationary phase cells were treated first with 0.14 M mercaptoethylamine/0.04 M EDTA [3] for 30 min at 30°, and then with snail juice in 1.3 M sorbitol/10 mM tris-maleate pH 5.7/0.1 mM EDTA [4] for 60 min at 30°. The protoplasts were washed and then disrupted in 0.6 M mannitol/0.1 mM EDTA pH 6.7 by 30 sec treatment with an overhead Waring-Blendor. Mitochondria were prepared as in ref. [1], with an additional layering over 1.5 M sucrose/0.1 mM EDTA pH 6.7 for 10 min at 31 500 rev/min in the Spinco SW 39L rotor. This removed residual intact cells and protoplasts. The mitochondria were then either analysed directly on a sucrose gradient or after incubation for 30 min at 30° in 0.1 M sucrose/0.1 M KCl/10 mM $MgCl_2$/10 mM KH_2PO_4/10 mM K succinate/2 mM ADP/0.1 μC per ml of U-^{14}C-leucine (165 mC per mmole). The medium was adjusted to pH 6.7 with tris base [2]. Mitochondrial fraction (1.0 ml) was layered over 28 ml of a linear sucrose gradient (20%-60% w/v) that contained 0.1 mM EDTA and was adjusted to pH 6.7 with tris base. After centrifuging at 25.000 rev/min in the Spinco SW 25.1 rotor (g_{av}: 63.581), the gradients were pumped directly into a Technicon Autoanalyzer, at the rate of 1.2 ml/min. A dual channel system was used, similar to that described previously [4], and the stream from the gradient tube was split in order to collect fractions for protein and radioactivity determinations [2,5]. The enzyme assays were at 30°, under O_2-free N_2 and the final reaction mixture contained 25 mM K phosphate/0.4 mM K_3 $Fe(CN)_6$/0.05% Triton-X-100 and was at pH 7.4. The final concentration of the three substrates were: 0.36 mM NADH, 1.8 mM L(+) Na lactate or 1.8 mM Na succinate. Assays were also run with the above reaction mixture with water instead if substrate. It was found that there was an increment in absorption at 420 mμ as the gradient was pumped through the analyzer in the absence of substrate (E_{420}).

The sedimentation patterns of the three enzymes and the E_{420} are shown in fig. 1. Succinate-ferricyanide reductase and E_{420} are in single, reasonably symmetrical bands with a peak buoyant density of about 1.18 g/cm^3, in agreement with other workers [6–8]. Since it is reasonable to take the succinate

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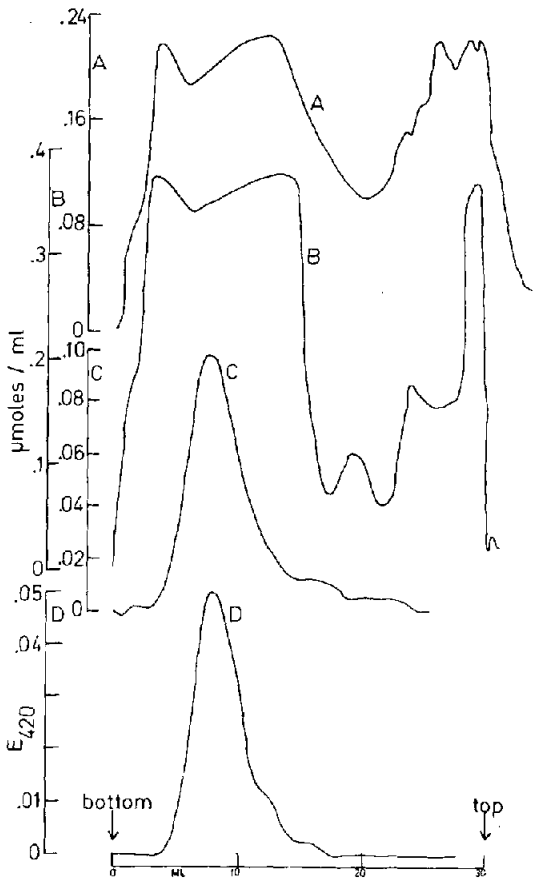

Fig. 1. Analysis of yeast mitochondrial fractions in sucrose Gradients. A: NADH-ferricyanide reductase; B: L(+) lactate-ferricyanide reductase; C: succinate-ferricyanide reductase; D: E_{420} (absorption in absence of substrate). See text for methods of fractionation and assay. After the gradient tube was emptied, 2 ml of 0.6 M mannitol-0.1 mM EDTA was added as a wash.

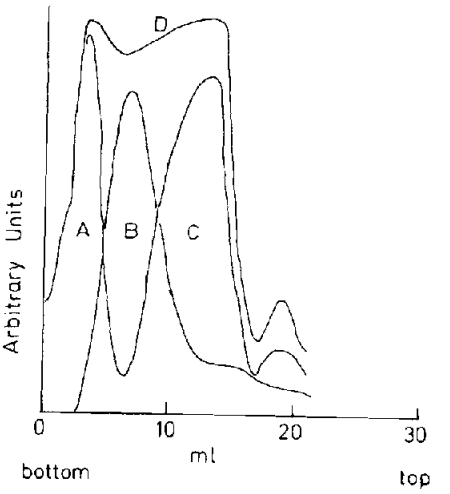

Fig. 2. Possible components in L(+) lactate-ferricyanide reductase containing particles. Curve B is an arbitrary curve of the same shape and position as previously observed with succinate-ferricyanide reductase. Curves A and C obtained by subtracting curve B from the observed curve D.

dehydrogenase as a mitochondrial marker, and the E_{420} follows it very closely, it is possible that the latter is due to Soret bands of mitochondrial cytochromes. In contrast to these results, however, complex sedimentation patterns are observed with NADH- and L(+)lactate-ferricyanide reductases, in the *same gradients*. If one assumes that some of the L(+)lactate-ferricyanide reductase is bound to the same particles that contain succinate dehydrogenase, the complex pattern in the lower part of the gradient tube may be resolved into three possible components (fig. 2); although until the actual L(+) lactate dehydrogenase of mitochondria can be determined, precise resolution of the pattern is not possible. When mitochondria labelled *in vitro* were analysed on the sucrose gradient, succinate dehydrogenase, total protein and radioactive protein all banded at precisely the same position in the gradient, again with reasonably symmetrical peaks (fig. 3). However, the same complex pattern was observed with L(+) lactate-ferricyanide reductase, except that some of the minor peaks along the upper part of the gradient were missing (probably as a result of the pre-incubation).

The results therefore confirm and extend previous findings that yeast mitochondria can incorporate radioactive amino acids *in vitro* [2,9]. The lactate dehydrogenase system of yeast is very complex [10], and the above results now add the complication that there may exist several lactate-dehydro-

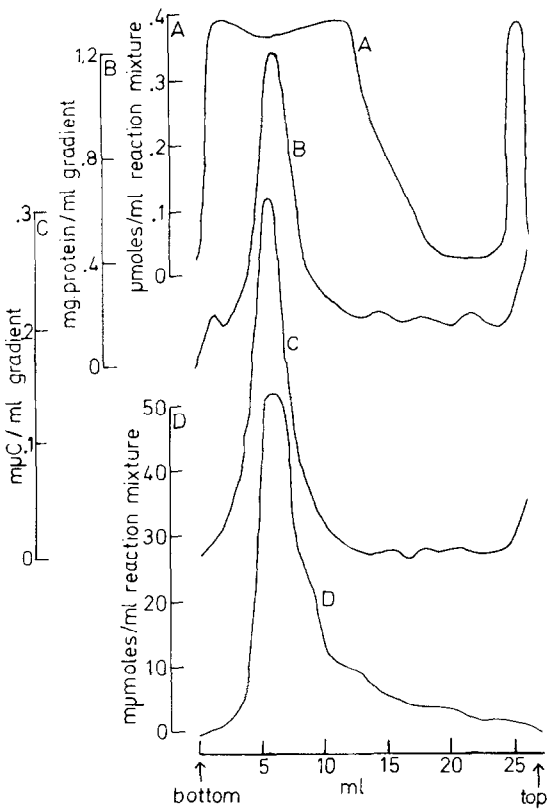

Fig. 3. Analysis of labelled yeast mitochondrial fractions in Sucrose gradients. A: L(+) lactate-ferricyanide reductase; B: total protein; C: radioactive protein; D: succinate-ferricyanide reductase. Mitochondrial fractions labelled *in vitro* with ^{14}C-leucine and then analysed on gradient. See text for details of method of fractionation and assay.

genase containing particles in the yeast cell. The demonstration of heterogeneity of the particles containing NADH-ferricyanide reductase may also call for some re-assessment of current views on the system for oxidation of NADH in yeast [11,12] because of the possibility that not all the oxidation of NADH observed by isolated mitochondrial fractions may have been due to mitochondria.

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