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Formation of Yeast Mitochondria III: Biochemical Properties of Mitochondria Isolated from a Cytoplasmic Petite Mutant*

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Abstract

A number of biochemical properties of mitochondria from a cytoplasmic petite mutant of *Saccharomyces cerevisiae* with an extremely high adenine plus thymine content have been studied.

When such particles are isolated by means of standard procedures developed for use with wild-type yeasts they are grossly contaminated by non-mitochondrial membrane fragments. Further enrichment of mitochondria is achieved by non-equilibrium centrifugation in sucrose gradients.

Throughout this purification procedure the particles can be shown to retain an outer limiting, as well as a non-cristate inner membrane. In many of their morphological and physical features (size, shape, buoyant density) they resemble mitochondria isolated from the wild type.

Although enzymes of the respiratory chain are absent from the mutant particles, their content of L-malate dehydrogenase, NADP-dependent isocitrate dehydrogenase, and ATPase is comparable to that found in the wild type. The mitochondrial ATPase in this mutant strain is cold stable, oligomycin insensitive, Dio-9 sensitive, and susceptible to inhibition by the F_1 inhibitor of beef heart. The enzyme can be rendered cold labile by its detachment from the membrane, followed by fractionation with protamine sulfate and ammonium sulfate.

The existence of mutant particles that are incapable of function in oxidation and phosphorylation but resemble their functional homologues in many other ways raises the possibility that mitochondria are required in the cellular economy for purposes not directly linked to oxidative phosphorylation and electron transport. This hypothesis has led us to suggest that, contrary to models currently under discussion, mitochondria did not evolve as a consequence of endosymbiosis. We propose as an alternative that the mitochondrial organelle evolved as a means of improvement of existing subcellular structures in the primordial (perhaps eukaryotic) cell. Partial autonomy may thus constitute a relatively recent modification; the present-day mitochondrial genome had its origin in nuclear DNA and may have been amplified in a manner not unlike the amplification of ribosomal RNA cistrons in developing oocytes of *Xenopus*.

Introduction

Although the cytoplasmic "petite" mutation of *Saccharomyces cerevisiae* has now been known for a number of years[1–3] and its potential usefulness in studying nuclear-cytoplasmic relationships and their influence on electron transport and phosphorylation

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recognized by many investigators, this application has been vitiated by the pleiotropic phenotypic effect of the mutation. The absence of the normal array of respiratory pigments in whole cells[4,5] and, in particular, in isolated membrane fractions[6,7] supported the notion that much of the respiratory apparatus was encoded in mitochondrial genes.[8]

This conclusion has been weakened somewhat in recent years by the findings that F_1 ATPase[9,10] is present in crude mitochondrial preparations from petite cells and that cross reacting material to cytochrome oxidase[11] as well as cytochrome oxidase apoprotein[12] is present in these fractions.

Although the biochemistry of such mitochondrial fractions obtained from homogenates of mutant cells[5,13–18] and the morphology of the particles in cell sections *in situ*[19–21] have been investigated to a certain extent, no detailed characterization of the intact mitochondrial particle themselves has yet been reported.

This communication describes the purification and characterization of the mitochondria of one haploid strain of petite cells. Our results suggest that these particles strongly resemble the corresponding particles from wild-type cells even though they exhibit the expected respiratory lesions. These particles represent an important extreme case in the study of the nature of the mitochondrial gene products; we also find that studies on mutant mitochondria may lead to a new awareness of the functions of their normal homologues other than oxidation and phosphorylation.

Methods

Culture Conditions

A haploid wild-type strain, 4D, and a petite derivative of it, 4D-21, (supplied by C. Avers, Rutgers University) were used in these studies. Cells were grown on a semi-synthetic medium (see ref. 22 for details) using glucose (4D and 4D-21) or galactose (4D-21) as energy source, to stationary phase, unless stated otherwise in the text.

Cell Fractionation

See ref. 23. All manipulations were done at 0–4° unless stated otherwise in the text.

Analytical Methods

Protein was assayed by a modification of the method of Lowry *et al.*,[24] or by A_{280} for gradient fractions.

Assay procedures for L-malate dehydrogenase, NADP-linked* isocitrate dehydrogenase, and citrate synthase were as described in ref. 23.

Succinate dehydrogenase was measured using the PMS-assisted INT reductase assay of Nachlas *et al.*[25]

ATPase Assay

ATPase was assayed at 30° in buffer containing 0·5 mM EDTA, 80 mM KCl, 4 mM $MgCl_2$, and 50 mM TES-Cl adjusted to pH 7·4 with an ATP-regenerating system

* Abbreviations: A, adenine; ATP, adenosine triphosphate; C, cytosine; CAP, chloramphenicol; Cit Syn, citrate synthase; EB, ethidium bromide; EDTA, (ethylenedinitrilo) tetraacetic acid; G, guanine; GDH, L-glutamate dehydrogenase; INT, *p*-iodonitrotetrazolium violet; MDH, L-malate dehydrogenase; MTEA, 0·25 M mannitol, 20 mM Tris-sulfate (pH 7·4), 2 mM EDTA, 2 mM ATP; NAD(H), nicotinamide–adenine dinucleotide, (reduced); NADP(H), nicotinamide–adenine dinucleotide phosphate, (reduced); NADP-IDH, NADP-linked isocitrate dehydrogenase; PMS, phenazine methosulfate; T, thymine; TCA, trichloroacetic acid; TES, N-Tris (hydroxymethyl) methyl-2-amino-ethane sulfonic acid; Tris, Tris (hydroxymethyl) aminomethane.

consisting of pyruvate kinase (2 International units/ml) and phospho-enol-pyruvate (1·6 mg/ml). The enzyme sample is preincubated with buffer and/or inhibitors for 5 min at 30° before starting the reaction by the addition of neutralized ATP to a final concentration of 4 mM. The reaction was stopped by the addition of ammonium molybdate and H_2SO_4 and phosphate was assayed according to ref. 26.

The phosphate determination is markedly inhibited by the presence of small quantities of mannitol and less so by sorbitol (0·027 ml of 70% sorbitol inhibits 50%). For this reason sucrose was used in all buffers except when conditions used in other laboratories were being examined.

Whenever an inhibitor was added in ethanol, the small effect of ethanol alone was accounted for. No more than 10 λ of ethanol was ever used.

Inhibition of ATPase activity by F_1 inhibitor was done as follows: a sample of enzyme (0·25–0·5 μmoles/min) of enzyme was incubated in 20 mM Tris-TES buffer (pH 6·85) containing 0·2 mM ATP and 0·2 mM Mg^{2+} plus 5–25 μg F_1 inhibitor (total volume of 0·5 ml) at 23° for 20 min. Then an aliquot was removed for assay. A control sample treated exactly the same but without F_1 inhibitor, was always assayed as well.

Electron Microscopy

Intact cells were fixed with 2% $KMnO_4$ for 30 min at 0° and then with 1% $K_2Cr_2O_7$ plus 1% uranyl nitrate for 30 min at 0°. The cells were then dehydrated with a series of ethanol solutions, embedded in Epon, sectioned with a Porter-Blum microtome and examined with a Siemens Elmiskop at 80 KV.

Spheroplasts and other fractions were fixed in suspension with 3% glutaraldehyde (0·1 M PO_4, pH 7·0), containing sufficient sucrose or sorbitol to stabilize conformation (1 M for spheroplasts, 0·4 M for other fractions), for 2 h at 0°. They were then sedimented at 20,000 $\times g$ for 20 min. Excess glutaraldehyde was removed by repeated washes with phosphate-sucrose. Samples were then fixed as a pellet with 1% OsO_4 (in 0·1 M PO_4, pH 7·4 + 0·32 M sucrose) for 1 h at 0°. Samples were then embedded, stained with lead citrate and examined as described above.

Materials

Adenine sulfate, ATP (Sigma grade), *p*-iodonitrotetrazolium violet (Grade I), DL-isocitric acid, trisodium salt (Type 1), NADH (Grade III), NADP (Sigma grade), NADPH, *cis*-oxalacetic acid (Grade I), and phenagine methosulfate were purchased from Sigma Chemical Company; acetyl coenzyme A, trilithium trihydrate (A grade), 2-phosphoenol pyruvic acid (A grade), pyruvate kinase (rabbit muscle) (A grade), and TES were from CalBiochem; glusulase from Endo Laboratories; RNAase-free sucrose from Mann Chemical Co.; yeast extract and Bacto-peptone from Difco Laboratories.

All other chemicals were of reagent grade.

F_1 inhibitor was provided by Dr. E. Racker and Dio-9 was provided by Dr. R. Guillory.

Results

Does Glucose Repression Occur in Petites?

Mounolou *et al.* have reported that one may obtain minimal glucose repression while still allowing net growth of petites by growing the cells in a chemostat in which the

glucose concentration is maintained at 0·1%.[27] In the past we have approximated this regime by growing the cells in a maximum concentration of 0·1% glucose by adjusting the glucose concentration to that level whenever it had dropped to zero.[22]

Because of the inconvenience of maintaining these conditions we examined alternative ones. We found that growth of wild-type strain (4D) on 1% galactose or 1% mellibiose was characterized by a moderately high oxygen uptake which was maintained throughout the exponential phase (see also ref. 5); when the sugar was exhausted, cellular respiration was derepressed further to such an extent that oxygen uptake using glucose (OU) increased approximately two-fold. Since strain 4D showed only a low level of repression under these conditions, we reasoned that 4D-21 (isogenic in all respects other than the $\rho^+ \rightarrow \rho^-$ mutation) would respond similarly.

Table I shows an analysis of enzyme levels in homogenates (S_{2000}) of 4D and mutant 4D-21 grown under a variety of conditions. From it we may conclude that enzyme levels in exponential phase petite cells are less repressed than are those of wild-type cells grown to the same stage under identical conditions. Although certain enzyme levels in 4D-21 are slightly higher on galactose than on glucose, it is clear that these petites grown on 1 or 5% glucose are *not* so severely repressed as wild-type strain. Recent studies of Woodward *et al.*[28] comparing enzyme levels in wild-type *Neurospora* with those in two respiratory deficient mutants, one cytoplasmic (*mi-1*) and one nuclear (*cyt-1*) show a similar phenomenon, namely that certain, but not all, derepressible enzymes are present at derepressed levels in the mutant, so that, upon respiratory adaptation (change from 1% sucrose to acetate), little or no further increase occurs.

TABLE I. Specific activity of enzymes in yeast homogenates (S_{2000})

	Wild-type 4D			Petite 4D-21		
Carbon source	1 or 5% glucose	3% lactose	1% galactose	1% glucose	5% glucose	1% galactose
Stage of growth	log	log	log	log or stat	log	log
	Repressed	Derepressed	—	—	—	—
Catalase*	<30	5750	3135	1052	540	520
GDH(NAD)	7·35	50·9	43·5	20	25	36
GDH(NADP)	77	97·4	116·8	51	88	73
MDH	318	3460	1161	500	500	1110
NADP-IDH	44	86	66·4	36	36	36
Citrate synthase	67	295	166·2	89	89	135
Isocitrate lyase	2·1	57·8	3·34	1·0	1·0	2·1

* $\Delta O.D._{250} . min^{-1} mg^{-1}$.
Units are $nmoles . min^{-1} . mg^{-1}$.
Levels of enzyme activity, assayed in extracts of wild-type yeast, under various physiological conditions of growth were compared to levels in extracts of a petite strain grown on the same energy sources. Two to five grammes of cells, wet weight, were used for each analysis. Cells were broken by shaking with glass beads, and debris were removed by a 2000 × *g* spin for 10 min at 4°.

Careful analysis of Table I reveals that glucose repression is a composite of several phenomena. In agreement with Polakis *et al.*[30] we find that respiratory and certain other enzymes in the wild type are repressible by glucose but not by galactose; certain others are repressed by either sugar.[29] Enzymes in the latter class are found in the mutant

in levels corresponding to repressed wild type; those in the former class are either absent entirely or, if present, they occur at levels that exceed those found in repressed wild type. In studies to be described in detail elsewhere,[31,32] we find that the release from glucose repression of many enzymes begins well before glucose has become depleted.* This component of derepression is independent of the demand for respiration and the resultant ATP generation since it occurs normally (in wild type) even in the presence of 1 μg of antimycin A/ml, a drug which abolishes growth on non-fermentable carbon sources by inhibiting respiration. This component of the derepression phenomena (as well as the aerobic component of derepression) is absent in petites, whose enzyme level is comparable to the level in the wild type when glucose has been completely exhausted. Therefore we conclude that in this instance the petite mutation has led to a release from this particular kind of catabolite repression found in the wild type.

We, therefore, routinely grow cells on 1 or 3% galactose although glucose would be equally acceptable.

Isolation and Purification of Petite Mitochondria

Although investigators in several laboratories have studied certain constituents of mitochondrial fractions of petites (F_1 ATPase,[9,10] membrane[11–13,16] and "structural" proteins,[33,34] DNA,[22,27,35] and RNA[36]) not one, ourselves included, has yet submitted the mitochondria contained in these crude fractions to a systematic characterization.

We isolate particulate fractions from spheroplast lysates according to the protocol developed for the wild type.[23] An electron micrograph of such a fraction of petite mitochondria is shown in Fig. 1; it consists of approximately 10–20% particles containing an intact two-membrane structure. An analogous fraction isolated from the wild-type (4D) cells after derepression is composed of intact mitochondria to the extent of 70–90%.[23]

Clearly a further enrichment of this fraction is necessary if we wish to study the composition of petite mitochondria.

Gradient Fractionation

Density gradient centrifugation is the usual method employed for the analysis and further resolution of crude particulate fractions.[37–39] In Fig. 2 we compare the patterns obtained by non-equilibrium centrifugation (10 min of centrifugation) to that obtained by isopycnic centrifugation (60 min of centrifugation).

The particular marker enzymes shown are used because we have previously found them to be authentic mitochondrial markers in 4D[23] and because they are also present in homogenates of mutant 4D-21 (Table I).

From these data we may conclude:

1. The fraction placed on the gradient shown in Fig. 1 contains non-mitochondrial membranes with a buoyant density very close to that of mitochondria. There is a slight difference in size between mitochondria and contaminating membranes which may be exploited to enrich the content of mitochondria in the fraction.

2. Isopycnic density gradient centrifugation is not useful for further enriching the mitochondrial content of the crude mitochondrial pellet.

* Glucose becomes depleted when the whole cell protein concentration in the medium is about 0·45 mg/ml while derepression for many enzymes begins when there is 0·25 mg of protein/ml of medium. During that 2 hr period the specific activity of respiratory enzymes increases three- to ten-fold (while total activity in the culture increases six- to twenty-fold); cytochrome oxidase increases in total activity but not in specific activity.

3. The four enzymes used conform to the criteria required for markers representing a particular fraction. They are present in, and can be used as markers for, petite mitochondria. This experiment was performed at 23° and the ATPase assayed immediately

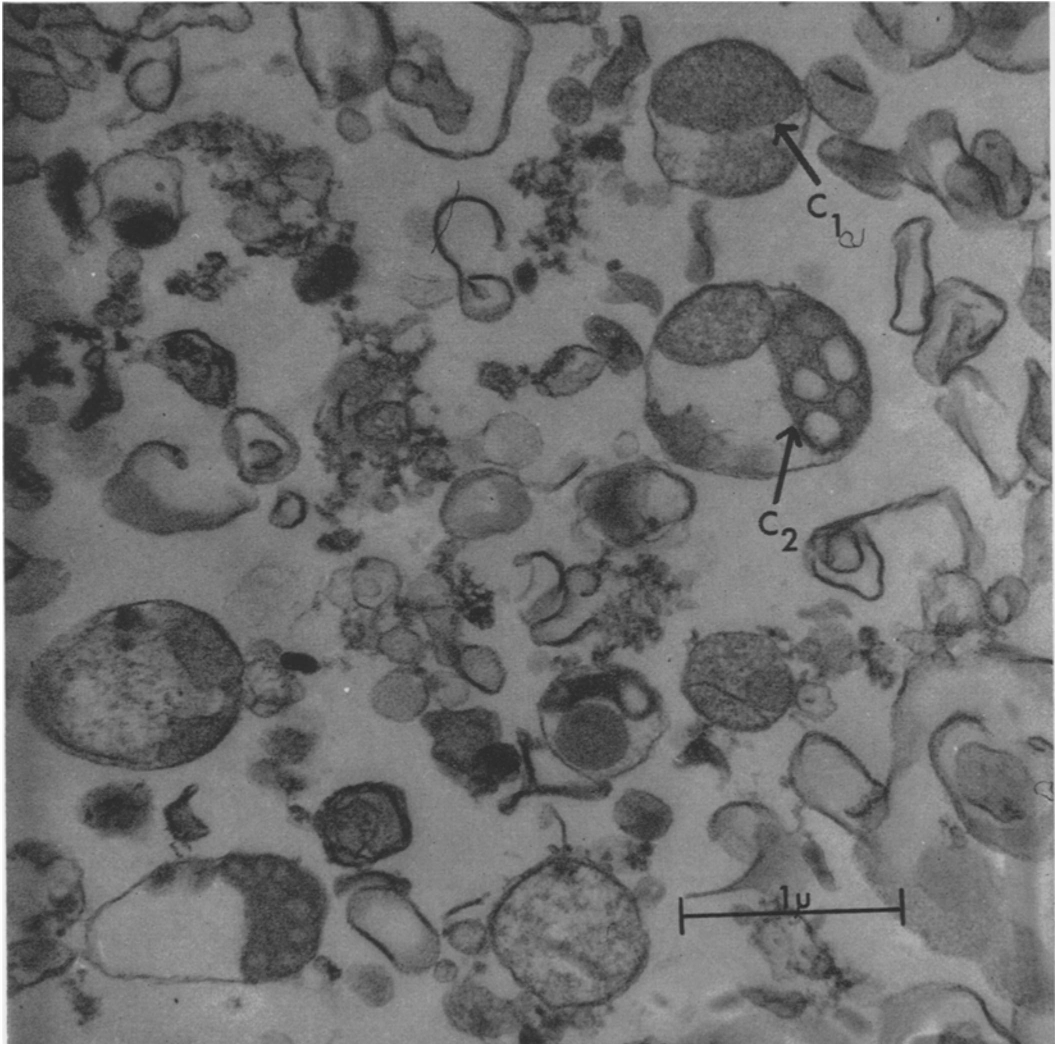

Figure 1. Electron micrograph of a crude mitochondrial fraction isolated from petite spheroplasts. Arrows denote typical two-membrane structures found in fractions of this type.

after fractionation, so that this activity would correspond to the "cold-labile" ATPase, shown by Schatz[9] to be very similar—and probably identical with—F_1 (see also section on ATPase).

4. Since MDH (and probably also citrate synthase and NADP-IDH) is easily extracted from intact mitochondria (e.g. by osmotic shock) and since it remained with the membranes during sedimentation in the gradient we may conclude that the mito-

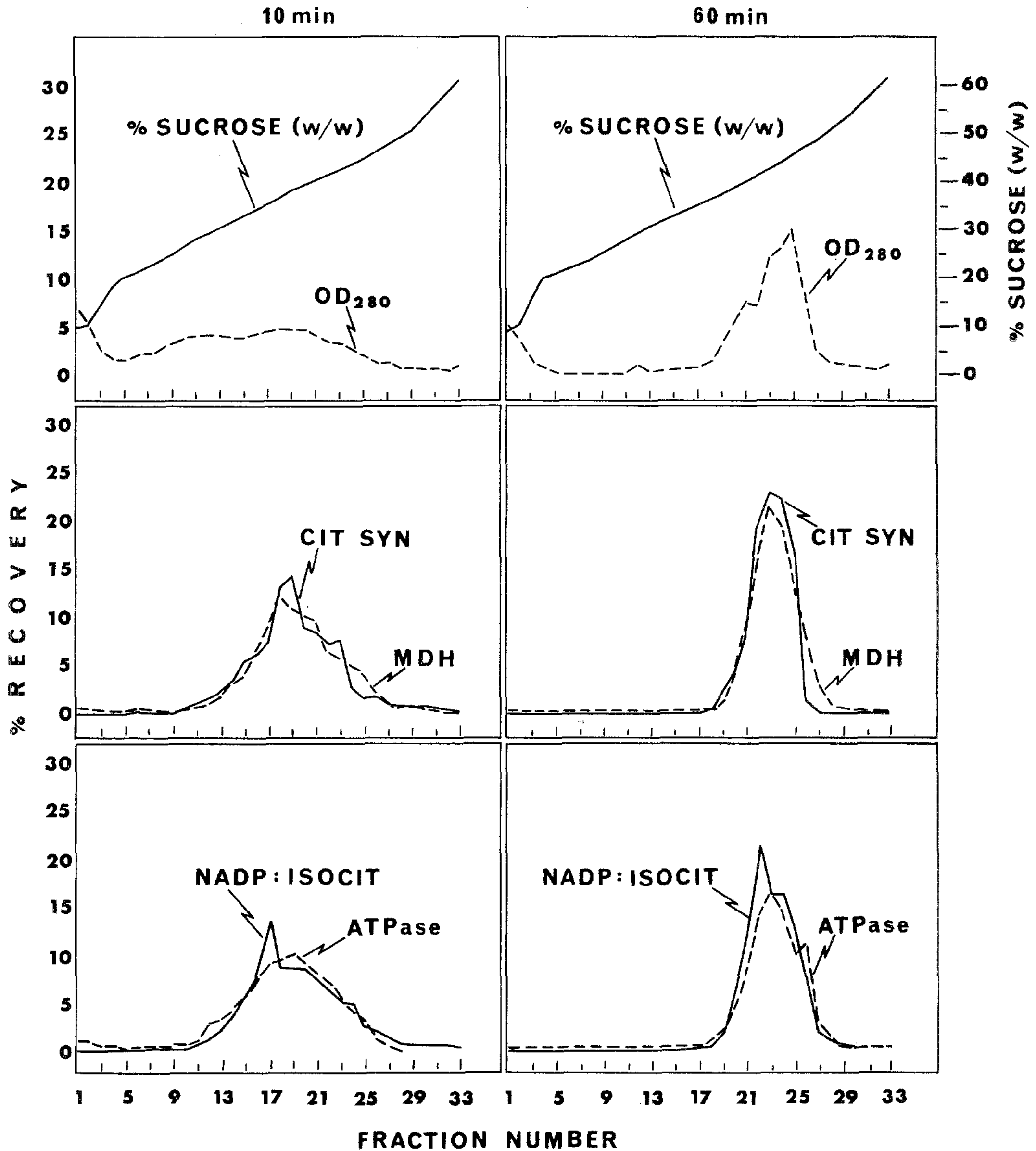

Figure 2. Distribution of various characteristic activities from a crude mitochondrial fraction isolated from the petite mutant, 4D-21, is placed on a linear (1·059–1·258 g/ml) sucrose gradient and centrifuged at 23° at 63,000 × *g* for either 10 or 60 min. Totals loaded were for protein, IDH, ATPase, MDH and Cit Syn: 40 (A_{280}), 0·77, 6·0, 20·1 and 11·16 units (μmoles/min), respectively. Recoveries were, after 10 min of centrifugation, 85, 117, 79, 87 and 75%, respectively; after 60 min of centrifugation 97, 114, 87, 109 and 72%, respectively.

chondria present in these fractions isolated by differential centrifugation remained intact.

5. The coincidence of all four enzymes even under non-equilibrium conditions strongly suggests that petite cells contain a single fairly homogeneous population of mitochondria—in good agreement with Avers *et al.*[40] and with our findings with 4D (wild type) under both repressing and non-repressing conditions of growth.[41]

Morphology of Petite Mitochondria

Figure 3 shows the morphological characteristics of the membranes found in the mitochondrial region of the non-equilibrium gradient. The fraction is more homogeneous than that represented by Fig. 1, and the particles denoted by the arrows in Fig. 1 appear to be the only recognizable structure, present in both fractions, that has been enriched by the additional step.

We conclude that this structure is typical of mitochondria isolated from petite cells. The gradient procedure has not altered their morphology. Two membranes are seen, neither of which shows the structure of a typical unit membrane. The ratio of outer to inner membrane may be smaller in petites compared to wild-type isolated and fixed in this manner but it is clear that a well-defined inner membrane is present.

Although these results appear to be in conflict with the findings of Yotsuyanagi[19] we wish to point out that he studied sectioned *whole* cells which had been fixed with potassium permanganate, a far harsher and qualitatively different fixative from the one employed by us, and that we examine *isolated particles* (see Damsky *et al.*[42] for a concise discussion of various fixatives and stains used for biological samples). Whole petite cells fixed with permanganate (Fig. 4) do show a few profiles per cell which correspond to Yotsuyanagi's description of petite mitochondria.

We have observed that the inner membrane of these particles may assume at least two conformations both of which may be seen within a single mitochondrion (Fig. 1, arrow marked C_1 and C_2). We have attempted to effect their interconversion by treating fractions with ATP, with calcium ion plus ATP, and with 10 mM EDTA and, as yet, have only negative results. We are pursuing this matter further.

We have tried to examine whole cell sections of spheroplasts fixed and stained in a manner similar to the cell-free fractions but are plagued by a lack of contrast. While mitochondrial and certain other membranes stand out nicely in permanganate fixed cells we find that our poor contrast is due, not to a failure of mitochondrial membranes to stain, but to the increased staining of reticular membranes and ribosomes.

Preliminary findings with spheroplasts from wild-type cells (where the increased number of mitochondria makes them easier to distinguish after fixation by glutaraldehyde) show that, *in situ*, the mitochondria look the same regardless of the fixative used (see Fig. 5). However, in partially lysed spheroplasts, the mitochondria resemble those in isolated fractions. With this in mind, we would expect petite mitochondria as seen in sectioned spheroplasts to show a single limiting membrane with an unstructured (but not empty) interior (Fig. 6).

Enzyme Localization in Petites

The distribution of a number of enzymes found in both the mitochondria and the cytosol of petites was studied in a way analogous to that previously reported for de-

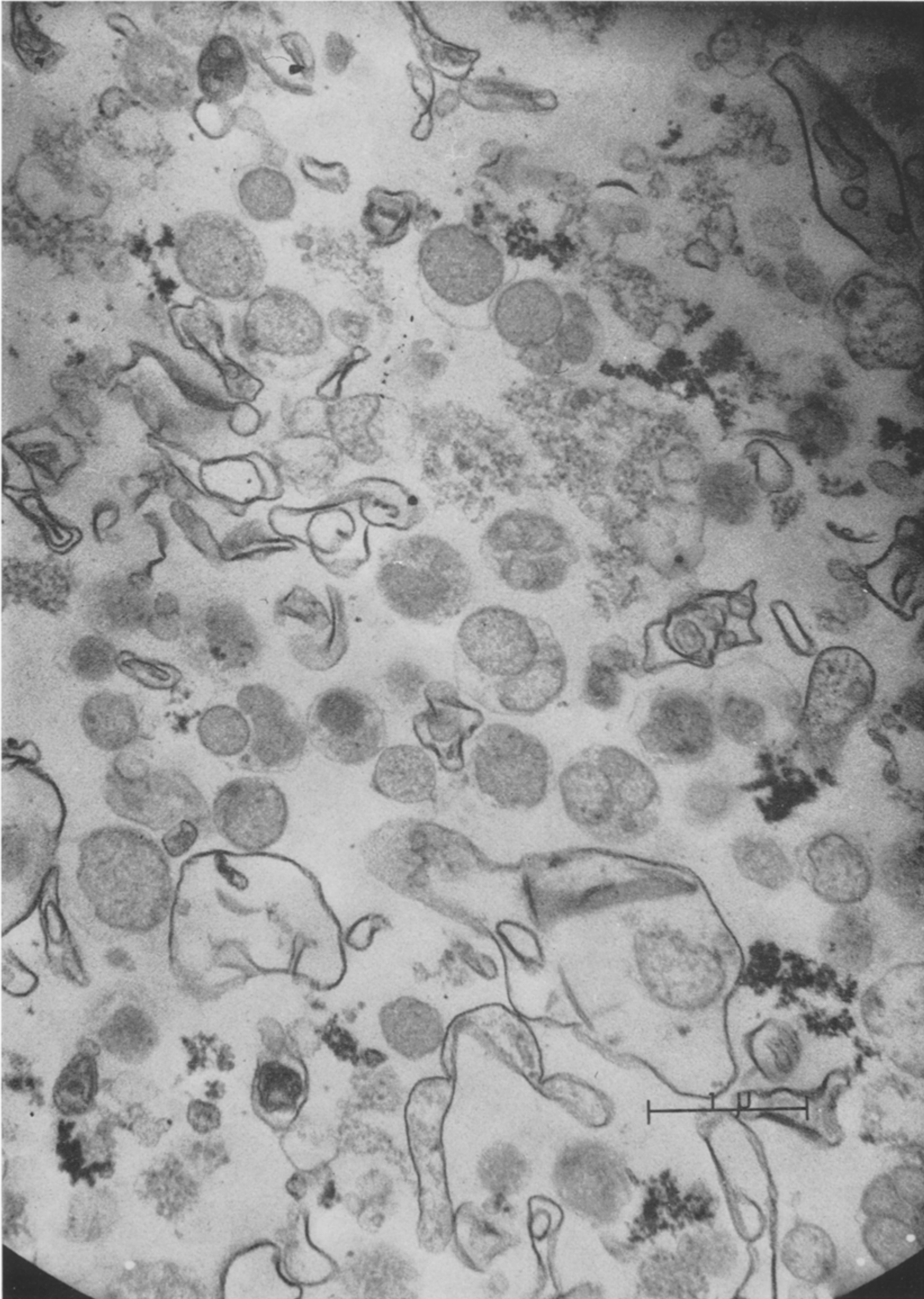

Figure 3. Electron micrograph of a mitochondrial fraction from petite cells which had been enriched by a non-equilibrium density gradient step.

repressed cells of 4D.[23] It is important to stress that the estimate derived is a qualitative one: because of a variable and uncontrolled entrapment in the low speed pellet, the fraction placed on the gradient (the $600 \times g$ supernatant does not necessarily and *accurately* represent the intracellular contents. In keeping with a rationale presented elsewhere,[23] these data represent a minimum estimate of the percentage of the total of a given enzyme which is localized in the mitochondria.

Figure 7 shows the distributions of a number of enzymes in a linear sucrose gradient

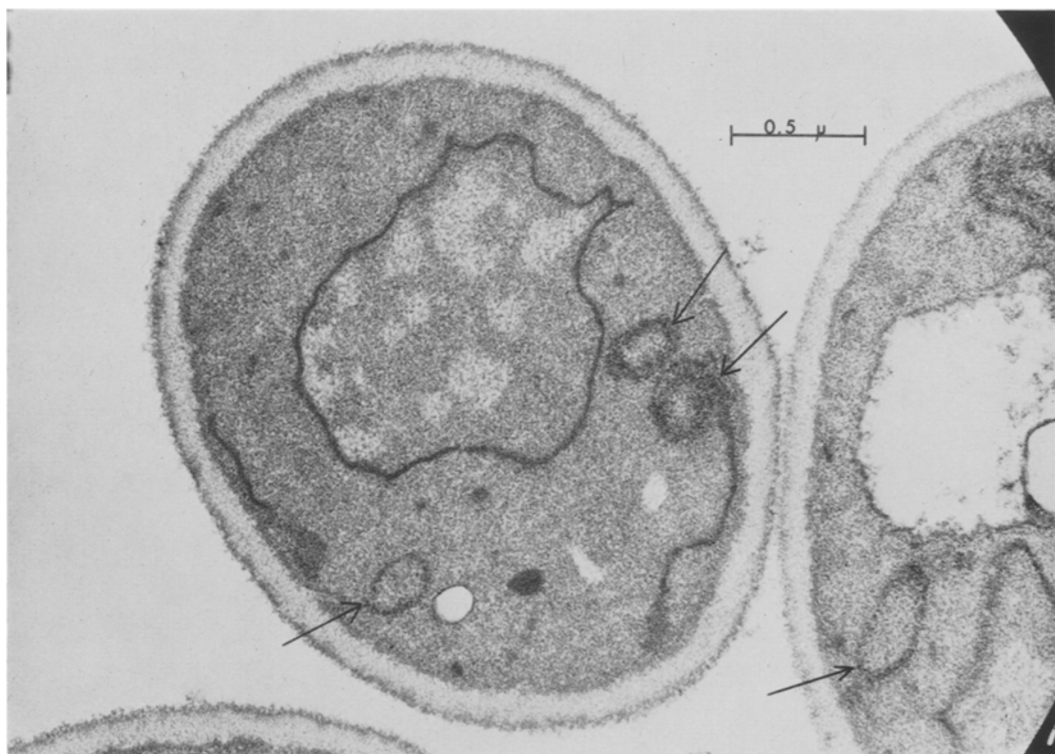

Figure 4. Cells of strain 4D-21 were grown to stationary phase on 1% galactose and fixed with $KMnO_4$ (see experimental). Typical mitochondria are denoted by arrows. No detail of the inner membrane is apparent. The mitochondria vary in size (0·2–0·6 μ, long axis) and in shape. There are about three such profiles per section.

which had been centrifuged for 1 h at $63{,}000 \times g$ at 4°. The mitochondria have reached their equilibrium buoyant density (see below). Under this set of conditions only mitochondrial and other large membranes have entered the gradient: the bulk of

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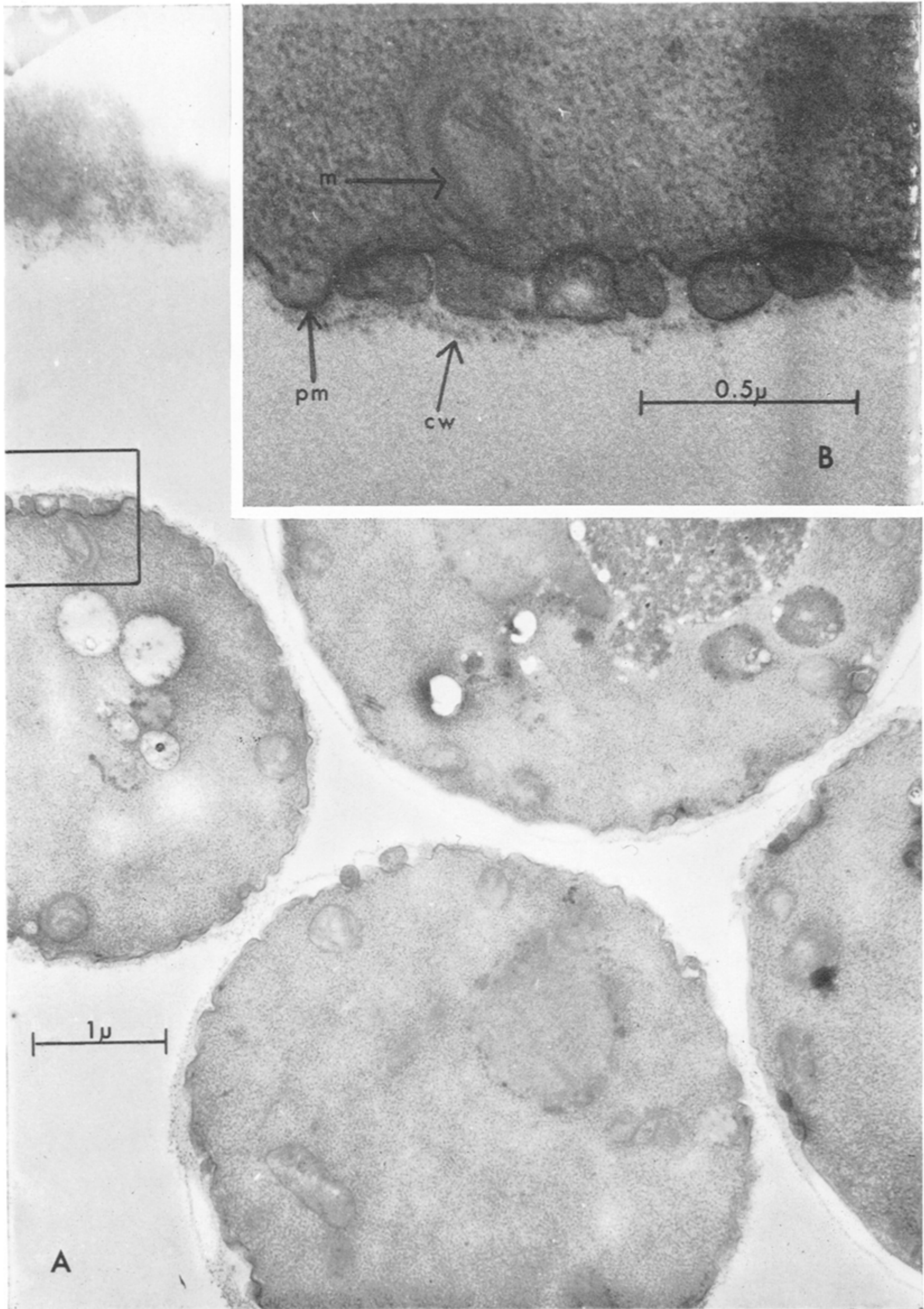
Figure 5. A. Spheroplasts of wild-type strain "Fleischmann" (50% derepressed) were fixed and stained in the same way that particulate subcellular fractions are treated. Mitochondria showing typical cristae are evident although the contrast is not as good as with $KMnO_4$-fixed cells. The area enclosed in the box is enlarged in B.
B. Note the particles on the periphery of the cell. These have a unit, limiting membrane (similar to the plasma membrane) and internal structure similar to the cytoplasm of the cell. They are located between the plasma membrane and the remnants of the cell wall. Since they are not seen in $KMnO_4$ fixed cells they may be artifactual pinching off of bits of cytoplasm caused by the spheroplasting or fixing procedures. They are seen in petite spheroplasts also. m, mitochondrion; pm, plasma membrane; cw, cell wall.


m
pm
cw
0.5μ
B
1μ
A

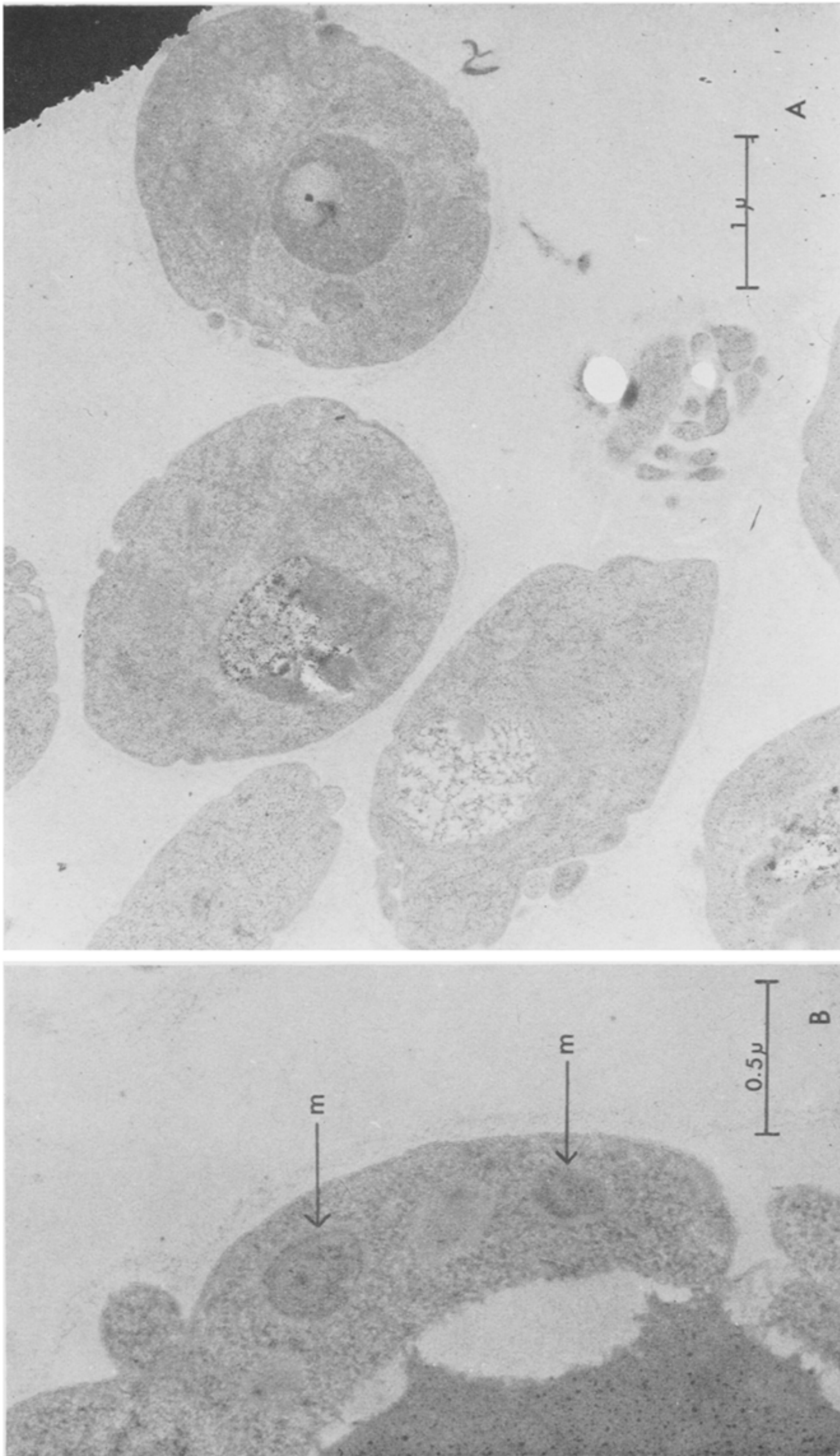


Figure 6. A. Spheroplasts of 4D-21 grown on 1% galactose to stationary phases prepared as in Fig. 5. The absence of mitochondrial inner membrane structure (cristae) makes visualization of mitochondria more difficult. The inset (B) shows an enlarged mitochondrial profile.

NADPH: cytochrome *c* reductase-containing membranes (called "microsomes" by Schatz and Klima[43]) remains in the first five fractions.

The A_{280} profile reveals no clearcut peak in the region of the gradient containing the mitochondrial enzyme, F_1 ATPase. Although 7·2% of the total material absorbing at 280 nm is present between fraction 15 and 28, it is obvious that only a small proportion of this is actually mitochondrial; therefore there are membranes present in petite cells

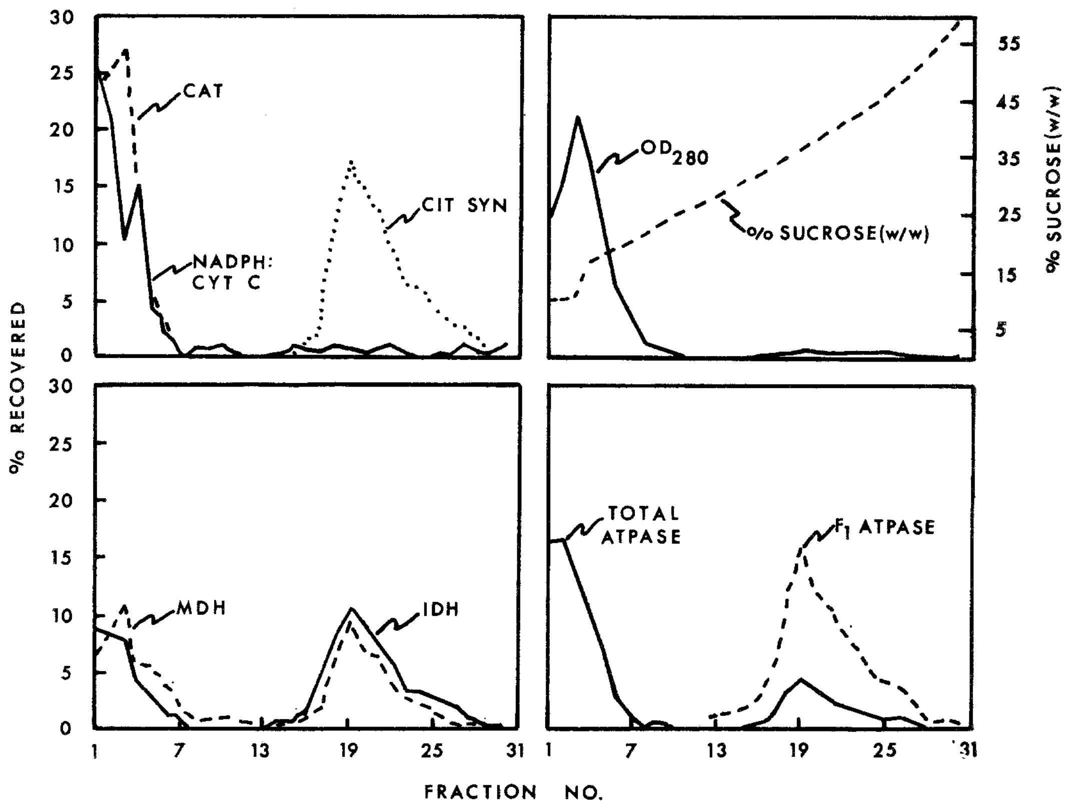


Figure 7. Enzyme distribution in petite "homogenate" (600 g supernatant): Three ml of homogenate containing about 30 mg of protein was loaded onto a linear (1·059–1·258 gm/ml) sucrose gradient and centrifuged at 4° at 63,000 × *g* for 60 min. Units recovered for protein (A_{280}), catalase, cit synth, NADPH: cytochrome *c* reductase, MDH, IDH, total ATPase and F_1 ATPase were 103, 18·25, 0·169, 0·216, 6·5, 0·256, 2·86 and 0·79, respectively.

(and in wild type as well) with a size and buoyant density similar to that of mitochondria. We estimate that in the petite no more than 2% of this homogenate fraction (S_{600}) is comprised by mitochondrial material.

Most of the MDH, NADP-linked IDH and citrate synthase activity is localized in the mitochondria. We were unable to detect citrate synthase activity at the top of the gradient due to interfering UV-absorbing material—we did not, however, recover in the mitochondrial region more than 50% of the activity placed on the gradient, which suggests the presence of some activity at the top of the gradient.

Of the total ATPase, only 28% was inhibited to an extent greater than 90% by 50 μg F_1 inhibitor/ml (generously provided by Professor E. Racker). However, none of the activity at the top of the gradient was inhibited by that agent, and all of it in the mitochondrial peak was.

Catalase activity, which is present at a level 25% that in a fully derepressed isogenic wild type, is localized strictly in the cytosol. In contrast to the latter[23,44] there do not appear to be any catalase-containing particles in this strain of petite.

NADPH: cytochrome *c* reductase is not a mitochondrial activity in this strain.

Enzyme Activities in Isolated Mitochondria

Table II compares the specific activities of several enzymes in mitochondrial fractions isolated from derepressed wild type and petite cells. Both sets of data are minimum values since neither fraction is homogeneous. However, it is clear that the mitochondrial content of these four enzymes is quite similar in the two strains. Of course, petite mitochondria lack the entire respiratory chain in a functional form; with this strain we have been able to detect succinate dehydrogenase at very low levels in isolated mitochondrial fractions but it is indetectable in the homogenate. It has been reported that some petites contain measurable activity of this enzyme while in other strains it seems to be absent.[14]

TABLE II. Specific activity* of enzymes in isolated mitochondrial fractions

	MDH	NADP-IDH	ATPase	Purity (%)†
4D	10·5	0·16	3·8	90
4D-21 (galactose) (glusulase preparation)	5·4	0·152	1·82	40–50
4D-21 (galactose) (mechanical preparation)	1·65	0·098	1·07	Not done

* Units are μmoles.min^{-1}.mg protein^{-1}.
† Based on electron microscope studies.

Mitochondrial fractions obtained from a mechanical homogenate of mutant cells usually exhibit a lower specific activity for these enzymes. The lower MDH/ATPase ratio in such mechanically prepared petite mitochondria suggests that the particles are less intact (but not necessarily less homogeneous). This may be taken as indirect evidence that the ATPase of petite mitochondria is a membrane bound enzyme.

Buoyant Density and Size of Petite Mitochondria

When gradients of petite mitochondria are run at 4° (Fig. 2), the mitochondrial band is centered at about 38% sucrose (w/w) equivalent to 1·172 g/cm^3. This compares favorably with previous results using 4D, where the banding density equalled 1·172 g/cm^3.[23]

A direct comparison, using double-label density gradient analysis, rules out uncertainties present when comparing densities obtained in separate experiments. For example, petite and derepressed wild-type cells were labeled to isotopic equilibrium with ^{3}H and ^{14}C-phenyalanine, respectively. The cells were washed twice with distilled water, converted to spheroplasts in the usual fashion, and mitochondria were isolated

and washed once. The mixed mitochondria were loaded onto a gradient which was centrifuged for 1 h at 63,000 × g. The gradient was fractionated and each sample was counted for both labels. All samples were corrected for spillover and quenching using the method described by Henson *et al.*[45] Both corrections varied only slightly among the many fractions analyzed.

Figure 8 shows the results of this analysis: petite mitochondria were compared to mitochondria isolated from repressed and derepressed wild-type cells. We may conclude that all three types of mitochondria show essentially the same buoyant density, the peak fractions differing in position by at most one tube; any small differences observed may be ascribed to different amounts of heavy contaminants known to be present in mitochondrial fractions from repressed and petite, but not derepressed, cells. Since buoyant density is mainly a measure of the phospholipid to protein ratio in the membranes, this result suggests an invariance of this parameter.

We have shown (Fig. 2) that isolated petite mitochondria are nearly at their isopycnic position after only 10 min ($w^2t = 250 \times 10^7$) of centrifugation at 23,000 × g (including the acceleration time) using the SW 25 rotor. This is true for derepressed mitochondria as well. We therefore conclude that the size of petite mitochondria closely resembles that of wild-type mitochondria in agreement with the findings of Avers *et al.*[40] It is possible that petite and derepressed mitochondria vary in size (s value) and that their similar sedimentation behavior is a result of clumping during either the preparation or the gradient sedimentation. Any tendency to clump would tend to be minimized by the presence of 1 mM EDTA in the breakage medium and of 10 mM EDTA in the gradient.

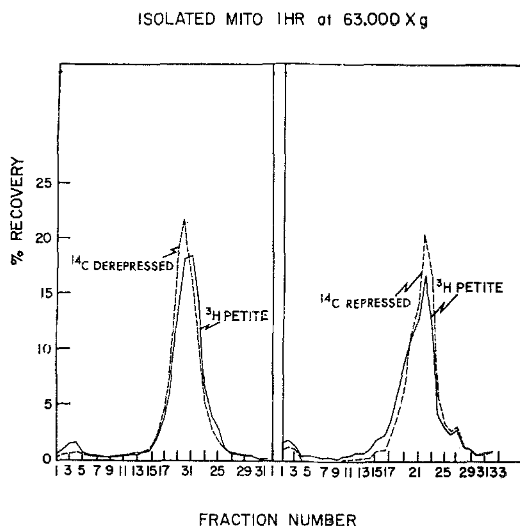


Figure 8. Direct comparison of buoyant density of isolated mitochondrial fractions. See text for details.

A. Petite (^{3}H) and derepressed (^{14}C): 142,000 counts/min of ^{14}C and 153,000 counts/min of ^{3}H were placed on the gradient and 92·5 and 77% were recovered respectively.

B. Petite (^{3}H) and repressed (^{14}C): 72,700 counts/min of ^{14}C and 117,200 counts/min of ^{3}H were placed on the gradient and 76 and 72% were recovered respectively.

We have, however, evaluated the sedimentation coefficient of these two kinds of mitochondria using the angle-rotor centrifugation method developed by Anderson.[46] We find that the value of $S_{5°, 0{\cdot}5\,M\,sorbitol}$ for mitochondria isolated from derepressed wild-type cells is around 5–7 × 10^3, while that of mitochondria isolated from petite cells is 8 × 10^3. This method for S-value estimation can be done using crude fractions; the sedimentation coefficient of mitochondria from derepressed cells is 5–7 × 10^3 in the homogenate as well as in the isolated fraction. It is clear that there is no meaningful difference between these values; we conclude, furthermore, that yeast mitochondria in these two strains are smaller than are mitochondria isolated from either *Tetrahymena pyriformis*[47] or rat liver.[48] This is in sharp contrast to the reports of Schatz and

co-workers[37,49] that yeast mitochondria may require upwards of 3 h of centrifugation at top speed ($w^2t = 2{\cdot}7 \times 10^{11}$) in the SW 37 rotor in order to reach their isopycnic density depending on the physiological state of the cells.

This is especially curious since mitochondria from cells grown anaerobically have been shown to be nearly as large as are mitochondria from derepressed cells—roughly 0·2–0·8 μ in diameter.[50] Any differences in the rate of sedimentation reported by them between different types of mitochondria (e.g. between repressed, anaerobic and derepressed) must be viewed with some reservation, since their particles were probably badly fragmented. We will show elsewhere that the mitochondrial population contained in a spheroplast lysate is much more homogeneous, with respect to buoyant density, than is that of an analogous fraction from cells broken by grinding with glass beads.[41] It has been well documented that inner membrane fragments and intact mitochondria have different sedimentation properties and buoyant densities.[51] Therefore a mixture of the two would yield a broader band in a sucrose gradient when compared to that of a homogeneous fraction of either one.

Petite Mitochondrial ATPase

Recently a number of reports have appeared stating that mitochondrial fractions from petite yeasts contain an activity which resembles F_1 ATPase found in the wild type.[9,10,52] The concensus of opinion is that the enzyme found in the petite cells is, in contrast to wild type, oligomycin insensitive and cold labile, but that, like wild type, it is inhibited by Dio-9 and by the F_1 inhibitor of Pullman and Monroy.[53]

We have undertaken a study of this enzyme found in the mitochondrial fractions of petite 4D-21.

Properties of the Enzyme in Intact Mitochondria

As mentioned earlier, part of the ATPase activity found in homogenates of mutant cells is localized in the mitochondrial region upon centrifugation in a density gradient.

We find that the particulate enzyme is unaffected by oligomycin at a concentration of 50 μg/ml, a level that inhibits by 90% or more the ATPase of wild-type particles. It is inhibited to the extent of 90% by 10 μg/ml of the F_1 inhibitor of Pullman and Monroy,[53] and it is sensitive to inhibition by Dio-9, an inhibitor specific for the mitochondrial ATPase of yeast[9]: 50 μg/ml inhibits the mutant enzyme 70%. From these data we conclude that the preponderant ATPase in this mutant strain, as well as of the wild type corresponds to F_1 ATPase.

The Na-K-stimulated ATPase mentioned by Schatz *et al.*[18] has not been encountered in our studies. Even in very crude mitochondrial fractions (20,000 × *g* pellet) the ATPase activity is inhibited 95% by F_1 inhibitor so that no corrections need be made. The activity is neither stimulated nor inhibited by NaCl or KCl or both and it is abolished by the absence of magnesium ion (4 mM).

With the enzyme of either mutant of wild-type mitochondria the extent of inhibition by oligomycin, Dio-9 or F_1 inhibitor is dependent on the amount of extraneous protein in the sample. The most highly purified fractions of ATPase were inhibited maximally by the lowest concentrations of inhibitor.

Cold Lability

We find that the ATPase activity in 4D-21 cells and isolated mitochondria is cold stable. In Table III we compare our preparation and growth conditions to those employed by Schatz,[9] who has documented cold lability of the same enzyme in another petite.

TABLE III. ATPase: test for cold lability in preparations of petite mitochondria

Carbon source	Galactose		Galactose		Glucose	
Method	Glusulase		Mechanical (Schatz[9])		Glusulase	
(Chris) t	23°	0°	23°	0°	23°	0°
S.A. at $t = 0$	1·213	—	0·13	—	0·75	—
2	100	100			100	100
$3\frac{1}{6}$	100	96	100	100	106	99
$4\frac{1}{2}$	96·9	95·6	107	99·5	109	93
18	100	91·5	108	93·2	129	96
$21\frac{1}{2}$	93	83	104	88	112*	89
$40\frac{1}{2}$	96·5	72·5	*	94		88
44	—	62·5	—	85·9		75
46	—	62·9	—	77		84
64	—	64·3	—	68·5		84

* Bacteria were growing.
Cells were grown at 30°, harvested at 23° and stored overnight at 23°. The entire preparation was done at 23° and samples were suspended in MTEA at 0° or at 23°. Activity was assayed with no ATP-regenerating system.

It is clear that neither Schatz's conditions for isolation (ATP added to all buffers; mannitol instead of sorbitol; cell breakage by grinding with glass beads) nor of growth (glucose instead of galactose) imparts cold lability to the ATPase. After $2\frac{1}{2}$ days at 0°, 65–84% of the activity remained; provided that 100 μg CAP/ml was present in the room temperature control to retard bacterial growth, 70–90% of the activity remained.

Although the activity associated with the mitochondria was cold stable it is possible that detachment of it from the membrane might render it immediately cold labile. A sample of petite mitochondria was sonicated in MTEA buffer for 2 min; it was centrifuged at 70,000 × g for 20 min at 23° to yield a clear sonic supernatant (S_{son}) and pellet (P_{son}). Table IV shows that the solubilized activity is still cold stable while the pellet now shows some slight cold lability after two days at 0°.

It was thought possible that the source of the cold stability was intrinsic to the F_1 protein(s) of this particular strain. Therefore, we examined the enzyme in the isogenic wild type for cold lability. We used the preparation procedure of Schatz *et al.*[54] but included 50 μg/ml of CAP to retard bacterial growth. This concentration of CAP does not inhibit the enzyme or otherwise interfere with the assay.

Approximately 50% of the activity was released from the wild-type (derepressed) mitochondria by shaking with glass beads. The supernatant was cold labile, losing

TABLE IV. Percentage of ATPase activity remaining

t	S_{son}		P_{son}	
	23°	0°	23°	0°
0	100	100	100	100
1	106	100	104	99
3	104	96	100	97
4	104	97	93	95
21⅓	102	86	100	72
24¾	—	89	—	71
26¾	—	85	—	72
45	—	84	—	60

80% of its activity within 13½ h in the cold. The second step, precipitation of contaminating protein by protamine sulfate, restored a degree of cold stability since the protamine sulfate supernatant lost only 30% of its activity during the same length of time. However, precipitation of the activity by ammonium sulfate rendered it spectacularly cold labile; it now was reduced by 68% after only 150 min at 0°. After 18½ h at 0° only 13% of the original activity remained compared to 89% in the control kept at room temperature. Activity in this ammonium sulfate pellet was inhibited 98% by 10 μg of F_1 inhibitor/ml.

We conclude that, in our hands, the wild-type F_1 ATPase behaves according to published observations.

We have repeated the above purification of ATPase from a crude particle fraction (20,000 × g for 20 min) of a mechanical homogenate of 4D-21. Eighty per cent of the activity was released from the membranes by shaking with glass beads. The ammonium sulfate pellet lost 64% of its activity after 21 h in the cold. The decrease was not so spectacular as with the wild-type enzyme but the conclusion is the same: petite ATPase is cold labile, in our hands, only after its separation from the mitochondrial membrane by rather drastic means followed by purification steps. The solubilized form of the enzyme is stabilized against cold inactivation by protamine sulfate and by 70% saturated ammonium sulfate.

The generation of cold-lability similar to that observed here has been well documented (although not explained) for the case of chicken liver pyruvate carboxylase.[55] Our data are in agreement with those of Kopaczyk *et al.*[56] who reported that the cold lability of rutamycin insensitive ATPase isolated from beef heart mitochondria was variable depending on the initial activity and the age of the preparation. We are thus unable to distinguish between the petite and wild-type ATPase on grounds other than their differing sensitivity to oligomycin. Our data suggest, however, that the petite ATPase may be bound to the mitochondrial membrane somewhat less strongly than is the wild-type enzyme.

We believe that the difference between mitochondrial ATPase in our strain and in the one used by Schatz[9] is evidence for heterogeneity in the extent of the lesion which results in the petite genotype. The presence or absence of succinate dehydrogenase activity in petite mitochondria may be another manifestation of this effect. It is important to note that one would expect the lesion in 4D-21 to be most severe since its mitochondrial

DNA has (probably) no information content. If it does possess more wild-type characteristics than does another strain with no less (and probably more) mitochondrially localized genetic information then one must consider this to be a regulatory phenomenon possibly unrelated to the mutation.

Discussion and Conclusions

We have reported here properties of purified mitochondrial fractions from petite mutants (containing at least 50% mitochondrial protein). Although these particles are non-functional with respect to respiration and its linkage to phosphorylation, they closely resemble their functional counterparts in the wild-type cell.

The strain studied has been shown to contain nonsense-type mitochondrial DNA.[22] If we assume that the mitochondrial genome is functionally deleted as a consequence of this extreme form of the petite mutation, it follows that the synthesis of these mitochondria is directed solely by nuclear genes. It is worth noting that the poly d(A-T) mitochondrial DNA found in this strain must itself be the product of a DNA polymerase activity again probably specified by a nuclear gene but found in the mitochondria; preliminary findings by Dr. D. South indicate that a DNA-dependent RNA polymerase activity may be present in these petite mitochondria as well. The mitochondrial amino-acid incorporation system appears to be absent from these petite mitochondria isolated under conditions which yield an active level of incorporation by wild-type mitochondria of radioactive leucine into hot TCA insoluble material.[57]

We have shown that, except for cytochrome oxidase, respiratory enzymes may be synthesized in the wild type in the presence of 4 mg of CAP/ml, a concentration of the drug that abolishes completely the ordinary increase in cellular oxygen uptake resulting in consequence of their release from glucose repression.[31,32] In addition we have also shown that these same enzymes, again except for cytochrome oxidase, may be synthesized in cells which have been rendered genotypically petite but are still phenotypically wild type as a result of treatment with the drug ethidium bromide (EB) for as long as four generations of growth using galactose as energy source.[13,59]* It is, of course, possible that the synthesis of relevant proteins continues in the petite cells but that their conversion to active enzymes ceases; although this has been reported to be the case for cytochrome oxidase[11,12] we have a considerable body of evidence that suggests that no part of the biosynthesis, transport, or activation of the other enzymes of the respiratory chain is CAP sensitive.

Why is it that as a consequence of the mutation $\rho^+ \rightarrow \rho^-$ the ability to produce respiratory enzyme is irreversibly lost while ATPase and certain other mitochondrial enzymes, in fact, mitochondrial particles themselves, continue to be made? It is likely that enzymes such as MDH, citrate synthase, and NADP-IDH play roles in metabolic pathways of petite cells other than those associated with oxidative metabolism. But why do they continue to be localized to at least the same extent in the mitochondria? A straight forward conclusion is that these mitochondrial particles (a) are subject to complete self-assembly, and (b) perform certain functions not related to oxidation and phosphorylation, functions which cannot be performed by the unorganized cytosol. It is

* Once these cells have assumed the petite phenotype, after about ten generations, their content of these enzymes is below the level of detection and is much lower than the level in repressed wild-type cells.

important to note that in these cells the transcription or translation of some *nuclear* messages, namely those for respiratory enzymes, appears to be shut off—probably because those enzymes cannot function in the absence of some mitochondrial gene product(s)—while the synthesis of other mitochondrial proteins, also specified by nuclear messages, whose integrated functions continue to be operative in ρ^- cells, remains unimpaired. The mitochondrial system in petites thus deserves close scrutiny because the knowledge of what is retained may, by elimination, provide added insight to the nature of the mitochondrial gene products.

Furthermore it is an excellent model system for studying functions of mitochondria not associated with oxidative phosphorylation. In particular, the high level of ATPase in petite mitochondria, despite the apparent futility of its presence as a component of ATP production, throws into sharp relief the question of the role(s) of F_1 ATPase. There are roles which ATPase might fill, such as movements of protons, metal ions, or other small molecules which might proceed and serve the cell using ATP generated in the cytoplasm by the glycolytic pathway; for instance mitochondria from rat liver can bind Ca^{2+} using exogenous ATP.[92] We are currently investigating some of these possibilities.

The sensitivity of the biosynthesis of respiratory enzymes to high concentrations of glucose found in the wild type has been lost following the petite mutation. It is, therefore, possible that some mitochondrial gene product is involved in glucose repression. It is also possible that the repression system is shut off by the same mechanism that shuts off the biosynthesis of the respiratory system. We are currently trying to distinguish between these two alternatives.

In the instance described here, the bulk of the mitochondrial protein and lipid components must be synthesized and assembled in the total absence of a meaningful mitochondrial genome. Thus there do not seem to be any major problems involved in regulating the biosynthesis of petite mitochondria imposed by the petite mutation *per se*. This postulate is in good agreement with the results of our studies referred to earlier in which the expression of the mitochondrial genome was blocked by the use of CAP,[31,32] a drug that presumably inhibits mitochondrial protein synthesis at the level of the mitochondrial ribosome, and by the use of ethidium bromide, a drug that induces the petite mutation quantitatively in a population of cells.[31,59] In both cases only a few select mitochondrial functions were affected while all others continued to be expressed normally.

The results described here suggest several ways of considering mitochondria in mutant cells which may merit further exploration.

We assume that the particles are present in petite cells in order to perform certain functions which are operative even in the absence of oxidative phosphorylation. The possibilities are:

1. Only those proteins that function in some metabolically or structurally useful way are still present; this implies a regulatory system which represses the synthesis of those proteins specified by nuclear genes rendered non-functional in the absence of mitochondrial gene products.

2. All mitochondrial proteins encoded in the nucleus are synthesized on the endoplasmic reticulum not necessarily in normal amounts and are present in the mitochondrion (or more generally, in the cell), but those proteins whose function requires

the participation of a mitochondrial gene product are present in an inactive form. Previous findings suggest that apoproteins or derivatives of cytochromes $(a + a_3)$, b and c_1 are indeed present in the particles; cytochrome c is present as such. However, we have shown elsewhere the conversion of precursors of respiratory enzymes to an active form, and therefore by extrapolation, the activation of these postulated apoproteins does *not* appear to be a function encoded in mitochondrial DNA.[31,32] Furthermore heme biosynthesis and metabolism are operative in petite mutants since large amounts of at least two heme proteins, cytochrome c and catalase, are synthesized, and since ferrochelatase is present.[93]

3. Some combination of 1 and 2 above may best describe the situation.

Each of the above possibilities is susceptible to experimental analysis provided that sufficiently well-characterized preparations of mitochondrial membranes from respiration deficient cells are used routinely.

Evolutionary Origin of Mitochondria

In recent years a theory which postulates the origin of this organelle in a bacterial symbiont has been given widespread support and consideration in the literature.[60–64] The details and rationale of that model are well known and have been reviewed repeatedly so that they need be considered only briefly here.

Important steps in the establishment of this symbiosis are proposed to be the following—using Sagan's detailed description.[63]

1. Prokaryotic cells evolved an anaerobic metabolism in the earth's primordial reducing environment.
2. Photodissociation of water vapor in the atmosphere acted as the selective pressure that resulted in the evolution of porphyrin metabolism which ultimately led to porphyrin-containing proteins.
3. Photosynthesis in prokaryotes evolved—resulting in a gradual change in the atmosphere from a reducing to an oxidizing one.
4. Prokaryotic respiration evolved.
5. Prokaryotes which were photosynthetic adapted their porphyrin systems in order to respire in the dark. This step resulted in the immediate ancestors of the present-day blue-green algae.
6. An endosymbiosis involving both an aerobic and an anaerobic prokaryote was developed which later became obligatory, presumably by a series of consolidation steps at the level of DNA. The organisms are referred to by Sagan as "aerobic amitotic amoeboid organisms". She also proposes that in these cells all oxidative metabolism occurred within the symbiotic "promitochondrion", which only used its own "promitochondrial" gene products for its construction.
7. What then followed was the evolution of these primitive amoeboid organisms into eukaryotes involving a profound modification of the symbiotic relationship between the two genomes.

The evidence which has been interpreted as supporting the above scheme, or an alternative one which ascribes the origin of mitochondria to the differentiation of single members within a colony of a prokaryotic organism, has been exhaustively reviewed

recently by Nass.[64] In general it consists of a long list of similarities between *present-day* mitochondria and *present-day* bacteria.

There is, however, another, perhaps equally plausible, model which has been almost totally overlooked (see ref. 65 for a brief mention). That model postulates a mitochondrial genome, originally episomal in nature and related to the nuclear genome in a way perhaps analogous to the gene amplification observed in the developing oocytes of certain amphibians.

This analogy is to the now well characterized formation of multiple micronucleoli—containing DNA specifically coding for rRNA—that takes place during oogenesis in amphibians such as *Xenopus* (see ref. 66 for a concise review). This type of gene amplification appears to be a mechanism by which an organism may ensure active biosynthesis of selected gene products of particular usefulness to the cell within a particular organelle under a particular set of conditions as would be the case, in our example, for more efficient utilization of an aerobic environment.

Alternate mechanisms for amplifying genetic information, such as diploidy, polyploidy and gene duplication on the chromosome are known at the current stage in evolution and they also may have played some role in the evolution of the cytoplasmic mitochondrial genome. Each may have been tried, modified or discarded in turn in the course of evolutionary history.

Differing markedly with the reasoning of Sagan and others, we suggest that the amplification of mitochondrial genes may constitute a relatively recent modification; that is, it may have even occurred subsequent to the evolution of a primitive eukaryotic cell, and certainly required aerobic metabolism and prior or concomitant enzyme packaging within a specialized intracellular pro-organelle.

This gene amplification and sequestration model of mitochondrial origin postulates that the genetic information now resident in the DNA of these particles had its origin in the chromosome of the same cell; it allows for the continued presence in the nucleus of genes related to those in the mitochondria at least in some of the cells existing today. But the mutability and genetic and metabolic independence of mitochondrial DNA as well as the nature of the information it contains suggests that this, as suggested, is neither precise nor extensive, or else that it cannot, ordinarily, be expressed. The selective advantage of having multiple copies of genes for certain mitochondrial proteins is clear; such a situation would provide for the possibility of extrachromosomal recombinational events yet ensure greater protection against mutational modification of a functional respiratory apparatus; it would simplify the complex problem of performing and regulating the assembly of an organelle from insoluble subunits which need otherwise be synthesized in diverse regions of the cell. Gene amplification and sequestration may thus have modified an existing genetic apparatus which may already have included multiple copies for crucial membrane proteins, making it selectively more favorable—rather than paving the way for the evolution of the organelle. We know that the presence and replication of nucleic acids within an organelle is neither necessary nor sufficient for the evolution of that organelle (e.g. microbodies, lysosomes, endoplasmic reticulum). Furthermore, even where the presence of such DNA is certain it is now known that by no means all genes required for the complete specification of the organelle have been sequestered in this way.[28,31,32,45,59,67,68] The model also raises the possibility that the genes actually sequestered may differ from species to species.

It would be of the greatest interest to determine whether any mitochondrial genes* are still present in the nucleus; if they are, then the gene amplification analogy becomes stronger and the necessity for amplifying only one or a few of the many mitochondrial proteins would pose an intriguing question. If, however, no mitochondrial genes are present in the nucleus at this point in evolution the problem becomes much more complex; we may, then, be studying a system far removed on the evolutionary time scale from the original gene amplification event and which may now no longer resemble it in its characteristics even though it began in that way. Taking this more extreme view, one might postulate that the gene amplification mechanism sequestered a considerable amount of information in the organelles but during eons of evolution much of that information was lost from the mitochondrial genome—through mutation, deletion or exchange with and replacement in the nuclear DNA—so that only a small fraction of the original information has remained within the organelle.

In order to support a gene amplification model for the origin of mitochondrial extra-chromosomal information we must take a position on the body of evidence with which proponents of the symbiotic model have been able to gain a large following.

Much of their data falls into the category of circumstantial evidence; there are a number of properties of mitochondria that bear a strong, but perhaps superficial resemblance to bacterial systems. For instance, the fact that certain inhibitors of bacterial protein synthesis are effective in inhibiting the biosynthesis of a certain class of mitochondrial proteins[45,69,70] does not prove that the inhibitor acts in the same manner in the two systems, nor need it imply any evolutionary relationship. If all the genes for mitochondrial ribosomal RNA are in fact, mitochondrial, then they have become separated from the cistrons coding for the other ribosomes of the cell and evolved independently and under a different set of selective pressures regardless of the model selected so that the basic difference in antibiotic sensitivity between the two systems is neither surprising nor enlightening. Furthermore recent evidence shows that sensitivity of mitochondrial protein synthesizing systems to a variety of antibiotics is quite variable.[71,72] In fact striking differences between different species[73] and even strains of yeast[74] have been reported. Furthermore, although the rat liver mitochondrial protein synthesizing system is sensitive to CAP it is unaffected by erythromycin and lincomycin.[72]

In addition the analogy between mitochondrial and bacterial ribosomes breaks down on a key point: their base composition. One of the most characteristic and distinguishing features of bacterial ribosomes is the relative invariance of their base composition at $\sim 53\%$ $(G + C)$ regardless of wide variations in that of their DNA.[75–78] Although information concerning these parameters in mitochondria is still rather fragmentary it is clear that their ribosomal RNA can vary from $\sim 25\%$ $(G + C)$ (yeast[79]) or $\sim 35\%$ $(G + C)$ (*Neurospora*[80,81]) all the way to $\sim 58\%$ in cauliflower[82] and that this variation rather accurately mirrors that of the responsible mitochondrial DNA.

Implicit to this variability of base composition of these rRNA species is a variability in base sequence. This is especially likely in view of the findings of Pinder *et al.*[83] which show that closely related organisms have similar polynucleotide fingerprints of rRNA while unrelated organisms have dissimilar fingerprints. Such dissimilarity may be anticipated with mitochondrial rRNA species as well. Since ribosomes are known to be wholly self-assembling structures *in vitro* and probably *in vivo* as well, there must be

* "Mitochondrial genes" must be clearly distinguished from "genes for mitochondrial proteins".

a close homology and fit between ribosomal RNA species and ribosomal proteins.[84,85] Therefore mit-ribosomal proteins must vary also. It has been shown that mit-ribosomal proteins *do differ* from the cytoplasmic ribosomal proteins in *Neurospora*; furthermore, evidence was presented which shows that the synthesis of mit-ribosomal proteins is cyclo sensitive and CAP insensitive.[86] However, Davey *et al.*[87] report that certain mit-ribosomal proteins in yeast are specified by mitochondrial genes but are translated on cytoplasmic ribosomes.

Although a definitive assessment of these varied and often conflicting data is not possible, it is safe to conclude that part but probably not all of the protein synthesis machinery is not specified by mitochondrial genes. Provided that the molecular basis of mitochondrial antibiotic resistance is in agreement with findings in bacterial systems then it is likely that some but not all of the mitochondrial ribosomal proteins are mitochondrial gene products.

Implicit in the symbiotic model is the assumption that a benevolent parasite invaded the primordial cell, which was characterized by a respiratory system which would fare poorly in the presence of the components brought in by the invader. On the other hand, the gene amplification model assumes that the respiratory system and its subcellular localization in the mitochondrion arose during the sequential evolutionary development of the cell and that the system as we know it today represents an efficient emergent modification of a basic pattern which was already inherent in the primordial cell. This point is strengthened by the fact that mitochondrial genes do not specify their own polymerase or the majority proteins of the mitochondrial inner membrane.[45] Furthermore agents known to be highly efficient at mutagenizing the mitochondrial DNA in yeast (e.g. acriflavine, UV) are known to be able to cure episomes in bacteria.[88,89]

As possible confirmatory evidence for this postulate we may cite the observations by Dr. S. Watson and his collaborators[90,91] who have demonstrated the elaboration by the plasma membrane of extensive, highly organized and cristae-like membranous intracellular organelles in a number of autotrophic marine bacteria under conditions of severe environmental stress. It may therefore be fruitful to search in organisms of this kind for multiple genes of a chromosomal or perhaps even an episomal nature capable of coding for one or more key membrane proteins.

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