Formation of Mitochondria in Yeast Cells

I. Development of Mitochondrial Membranes in Anaerobically Grown Yeast Cells

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SUMMARY

Biochemical analyses of mitochondrial marker substances, especially both cardiolipin and oligomycin-sensitive ATPase [EC 3.6.1.3], as well as electron microscopic observations were carried out to elucidate the process of mitochondrial development in anaerobic yeast cells. Cardiolipin was found to be localized at mitochondria in anaerobic cells. Its cellular content was a little higher at stationary phase than at exponential phase in glucose-grown cells and further increased in galactose-grown cells. The lipid content of the mitochondrial preparation obtained from the glucose-grown stationary cells was nearly as high as that of the galactose-grown cells. It was also comparable to that of aerobic stationary cells where mitochondria are fully developed. Both cellular and mitochondrial levels of oligomycin-sensitive ATPase activity were also found to rise greatly in galactose-grown anaerobic cells, although not in the stationary cells grown anaerobically on glucose. These high levels of the mitochondrial markers indicate a developmental change in mitochondrial structure even in anaerobically grown cells, which lack mitochondrial cytochromes. In the process of aerobic adaptation, formation of respiratory system was observed to be achieved much faster in galactose-grown cells than in glucose-grown cells, and not to be inhibited by chloramphenicol and high concentrations of glucose in the former cells, indicating also the developmental change in mitochondrial structure in anaerobic cells. The developmental change was also corroborated by electron microscopic observations that revealed the occurrence of two types of mitochondria in anaerobic cells. One of them was found in glucose-repressed cells and characterized by numerous electron dense granules in the matrix.

In contrast, the other type found in glucose-derepressed cells had the electron lucent matrix. No crista membrane was recognized in both types of mitochondria in anaerobic cells, although the infoldings of the inner membrane, which partition the matrix into two parts and therefore are called "septum membranes", frequently appeared in the stationary cells. On the basis of these results, the process of the mitochondrial development in yeast cells is discussed.

Occurrence of mitochondrial profiles in anaerobically grown yeast cells has been demonstrated by several workers. The profiles have been called either "promitochondria" (1) or "mitochondrial precursors" (2), since they lack mitochondrial respiration and mitochondrial cytochromes but on exposure of the cells to air become capable of respiring with the concomitant production of cytochromes. Besides anoxia, formation of mitochondrial respiratory system has been known to be repressed by high concentrations of glucose (3-5). In anaerobic culture, glucose has been used as a convenient carbon source and particular attention has not always been paied to the question whether the respiration-deficiency of the mitochondria in anaerobic cells is attributed to anoxia or to the glucose effect. Extensive studies on the nature of "promitochondria" has been carried out by Schatz's group who, however, has not directed special care to the glucose effect on the development of "promitochondria" in anaerobic yeast cells (1, 6, 7). Watson et al. have compared properties of "mitochondrial presursors" from anaerobic yeast cells grown under various conditions and they reported distinct biochemical changes between the precursors from

lipid-supplemented and lipid-depleted anaerobic cells but not between those from glucose- and galactose-grown cells except a minor morphological change (2). There are, however, some available reports suggesting exertion of the glucose effect on mitochondrial development in anaerobic yeast cells. Wallace et al. have described that number of mitochondria increases in anaerobic yeast cells when glucose is replaced by galactose (8). Further, in glucose-grown cells cellular content of cardiolipin has been reported to increase at stationary phase of growing (9). If the glucose effect on mitochondrial development is exerted even under anaerobiosis the most primitive mitochondria with simple structure and biochemical composition may be observable in glucose-grown anaerobic yeast cells, and therefore, for understanding the mechanism of mitochondrial genesis anaerobically grown yeast cells must represent a simple system to investigate.

In the present paper, we described that glucose-grown anaerobic yeast cells contain much smaller amounts of both cardiolipin and oligomycin-sensitive ATPase [EC 3.6.1.3] than galactose-grown cells do, indicating exertion of the glucose effect under anaerobiosis. Electron microscopic observations reveal two distinct types of mitochondrion occurring in glucose-repressed and -derepressed anaerobic yeast cells, respectively. Furthermore, the derepressed type of mitochondrion is shown to correspond to an advanced state in the process of mitochondrial development by the rapid formation of respiratory system during aerobic adaptation.

MATERIALS AND METHODS

Strain and Culture of Yeast Cells ---- A wild-type strain, JYD-56-G, of Saccharomyces cerevisiae, which can utilize galactose well, was isolated from an original strain, JYD-56 (10). Cultivations of yeast cells were performed aerobically and anaerobically as described previously (10). Concentrations of glucose were 2 % for aerobic and 4 % for anaerobic cultivations. However, when cells were to be harvested at exponential phase of growing, 4 % glucose was adopted even for both aerobic and anaerobic cultivations. Cells at an exponential phase were harvested at a population of 2-4 x 10^7 cells per ml, which was reached at about 14 hrs and 22 hrs after inoculation in glucose and galactose media, respectively. Cells at stationary phase were harvested when growth ceased. Population was 2-3 x 10^8 cells per ml, and the cultivation times were about 24 hrs and 32 hrs for glucose and galactose media, respectively.

Formation of Spheroplasts ---- To prepare spheroplasts, yeast cells were treated with bacterial glucanase for 30-60 min at 30° in a medium consisting either of 0.6 M sorbitol and 25 mM Tris-acetate buffer, pH 7.2 or of 0.5 M KCl and 10 mM citrate buffer, pH 5.8. The bacterial enzyme used in this experiment was prepared from the culture broth of Arthrobactoer species, YCWD-3, which was kindly supplied by Drs. Doi and Fukui, the Institute of Scientific and Industrial Research of Osaka University (11). The pretreatment with β-mercaptoethanol and EDTA was performed essentially as described by Duell et al. (12). The digestion of anaerobically grown yeast cells was carried out under nitrogen without the pretreatment.

Preparation of Subcellular Fractions ---- The spheroplast suspension was washed twice with a preparation medium consisting of 0.6 M sorbitol (Sigma), 25 mM Tris-acetate buffer, pH 7.2 and 1 mM EDTA. The washed spheroplast suspension was subjected to a 1 min homogenization on a CO_2-cooled Merkenshlager homogenizer (B. Braun Co., Germany) at the low speed with glass beads. The disrupted cell suspension was centrifuged at 600 x g for 10 min to remove unbroken cells. The cell homogenate thus obtained was continuously centrifuged at 2,500 x g for 8 min and 9,000 x g for 3 min. The fluffy layer was poured in the supernatant, and the pellet was suspended in the preparation medium and washed twice. The final pellet was used as mitochondrial fraction in the present investigation. The supernatant including the fluffy layer was centrifuged successively at 16,000 x g for 15 min and 105,000 x g for 60 min to give intermediate, microsome and supernatant fractions, respectively.

Analysis of Phospholipids ---- Direct treatment of intact yeast cells with organic solvent failed to extract phospholipids completely. Therefore, their cellular contents were obtained from analysis for spheroplasts. Lipid extraction from spheroplasts and subcellular fractions was carried out using chloroform and methanol mixture according to Bligh and Dyer (13). After concentration the extracts were chromatographed two-dimensionally on a precoated thin layer Silica gel plate (20 x 20 cm, Merck) to seperate individual phospho= lipid species. The solvent system used were the mixture of chloroform, methanol, water and acetic acid (65:25:4:1, by vol.)and that of chloroform, methanol and acetic acid (65:25:10, by vol.) for first and second developments, respectively. The identification of each phospholipid species was made by referring to chromatogram of

authentic compounds. Quantitative analysis of each phospholipid species was carried out by determining phosphorus of each spot either colorimetrically according to Bartlett (14), or radiometrically using [^{32}P]-phosphate. The radioisotope method was contrived to facilitated the analysis. In this method cells were grown in the presence of 0.2-1.0 mCi of [^{32}P]-phosphate in 1,000 ml of culture medium. After chromatography radioactivity of each spot on the plate was assayed in a scintillation counter using Bray's solution, in which 4 % of CAB-O-SIL gel powder (PACKARD) was added to avoid quenching by silica gel. In the usual cases about 80 % of the activity in the original extracts was recovered in each spot on the chromatogram. Content of phospholipid was expressed either as percentage of radioactivity on each spot to the total activity of phospholipids or as absolute quantity which was calculated by its percentage and the amount of original extracts obtained by the colorimetric determination. The radioisotope method is very convenient and enables to analyze very minor components. However, since there are various compounds of organic phosphates in culture medium consisting of yeast extracts and polypeptone and they may be metabolized at different rates, each phospholipid may not be always evenly labeled during cultivations. In order to clarify this point phospholipid compositions of a sample of aerobic stationary yeast cells were assayed by the colorimetric and radiometric methods and both results were cpmpared with each other. As shown in Table I, a little difference was found between values obtained by both methods but it is considered to be insignificant. In the following experiments, therefore, phospholipids were analyzed by the radioisotope method.

Assay of Cytochrome a and Respiration ---- Cellular contents of cytochrome a were assayed spectrophotometrically as described by Ishidate et al. (10). Rates of cellular respiration were assayed polarographically, and presented as the antimycin A-sensitive respiration, which was obtained by subtraction of that insensitive to the drug (15).

Determination of Enzyme Activity and Protein ---- Oligomycin-sensitive ATPase activity was assayed in cell homogenate and subcellular fractions after complete activation by treatment with 0.5 M Na_2SO_4 at 37° as described by Sone et al. (16). The concentrations of protein in subcellular fractions and spheroplasts were determined using bovine serum albumin as a standard, by the Lowry's and biuret methods, respectively (17, 18).

Preparation of Thin Sections and Electron Microscopy ---- Spheroplasts were fixed with the mixture of paraformaldehyde by OsO_4. After dehydrated with graded alcohol, the cells were embeded in Epon 812. The thin sections were observed with a Hitachi HU-11A electron microscope operating at 100 KV accelerating voltage.

Materials ---- Some of authentic phospholipid species used in the investigation were kindly supplied by Dr. K. Ishidate.

RESULTS

Phospholipid Composition and Cardiolipin Content in Anaerobic Yeast Cells ---- In order to examine changes in intracellular membrane structure, phospholipids were analyzed in anaerobically grown yeast

cells. Neither unique phospholipid species nor remarkable change in the composition of the lipid was observed among anaerobic cells grown under various conditions except small changes in cardiolipin content. (Table II). However, there was a distinct difference in the composition between aerobically and anaerobically grown yeast cells (Tables I and II). The changes in the content of phosphatidylcholine and phosphatidylethanolamine were rather large, but cannot represent structural change of a particular organelle, since these phospholipids are contained as main components in every kind of membrane in yeast cells. On the other hand, the decrease in cardiolipin content is remarkable and indicates immaturity or small quantity of mitochondria in anaerobic yeast cells, because the phospholipid is confined to mitochondrial inner membrane in mammalian cells and also found in mitochondria in aerobic yeast cells (9, 20). Furthermore, mitochondrial localization of the phospholipid in anaerobic yeast cells was shown by its nearly equal recovery percentage in each particulate fraction to that of oligomycin-sensitive ATPase upon subcellular fractionation (Table III).

It has been demonstrated by earlier workers that the cellular content of cardiolipin, like cytochrome oxidase activity, is repressed in the presence of high concentrations of glucose (9). Table IV shows its cellular contents of various yeast cells. The glucose effect was clearly observed in aerobic cells. When cells were grown anaerobically on glucose there was a small but distinct rise in the content at the stationary phase where the sugar was exhausted in culture medium. The increase in the lipid content may not be attributed to glucose-derepression alone since every culture exhibited a definite increase at stationary phase. However, the lipid content of the exponential cells

grown on glucose is restrained at the lowest level, and the content of the stationary cells would reach a much higher level than the value presented in Table IV if carbon source were available after glucose-derepression under anaerobic conditions. In practice, cells grown anaerobically on galactose, which has been known not to repress synthesis of cytochrome oxidase in aerobic cells (4, 9), were found to contain a considerably large amount of the phospholipid even at the exponential phase. These results indicate that the glucose effect on cardiolipin content is exerted in anaerobic yeast cells.

Clearer indication of the change in mitochondrial structure after glucose-derepression is presented by a great increase in the cardiolipin content of mitochondria from anaerobic stationary cells. As shown in Table V, the content was found to be comparable to that of mitochondria from galactose-grown cells, while it was extremely low in the exponential cells grown anaerobically on glucose. The increases in both cellular and mitochondrial contents of cardiolipin may indicate changes in quantity, including number and size, as well as in structure of mitochondria in derepressed cells under anaerobic conditions.

Change in Oligomycin-sensitive ATPase Content in Anaerobic Yeast Cells ---- Changes in mitochondrial quantity and structure in anaerobic yeast cells were also examined by analyzing both cellular and mitochondrial levels of oligomycin-sensitive ATPase activity, which is associated with mitochondrial inner membrane. When grown anaerobically on galactose, the cellular activity of the enzyme reached the highest level, while it was remarkably lowered in the presence of high concentrations of glucose as well as chloramphenicol, an inhibitor of protein synthesis on mitochondrial 70 S ribosomes (Table VI). Since the soluble form of the ATPase, F_1, which lacks oligomycin-sensitivity,

has been demonstrated to be synthesized on cytoplasmic ribosomes in aerobic cells (21), these results indicate that integration of the enzyme in mitochondrial inner membrane requires some other proteins which are sy synthesized on mitochodnrial 70 S ribosomes even under anaerobic conditions as shown in aerobic cells by Tzagoloff et al. (22). Oligomycin-binding protein, a membranous protein subunit of the ATPase (23), must be included in these proteins. In anaerobic cells, however, this membranous protein does not seem to be readily synthesized after glucose-derepression, since, unlike in the case of cardiolipin, little rise in the level of the enzyme activity was observed at the stationary phase of glucose cultivation.

Specific activity of the enzyme was also estimated for mitochondrial preparations obtained from various yeast cells. As shown in Table VII, in each case the mitochondrial enzyme activity was proportional to the level of the activity in the original cells from which mitochondria were extracted, except in the case of aerobic exponential cells where the high mitochondrial activity exhibited a great contrast to the low cellular level. From these results it is inferred that great change in mitochondrial quantity does not occur after the transition of growing phase of anaerobic cultivation, while it does in aerobic cultivation.

Respiratory Activity and Cytochrome Content in Anaerobic Yeast Cells Grown on Galactose ---- The high contents of both cardiolipin and oligomycin-sensitive ATPase might arouse question whether mitochondria were integrated completely even under anaerobic conditions when grown on galactose medium. Actually, it has been discussed whether yeast cells grown anaerobically on galactose contain mitochondrial cytochromes and show active respiration, which is susceptible to antimycin-A (24, 25). In the present experiments, when

grown semi-anaerobically on galactose, yeast cells were found to be capable of respiring as well as to contain relatively large amount of mitochondrial cytochromes. However, neither mitochondrial cytochrome nor respiration was found in the cells grown under strictly anaerobic conditions. Content of cytochrome a, a representative mitochondrial cytochrome, in yeast cells grown under various conditions is shown in Table VIII.

Electron Microscopic Observations ---- From the changes in both contents of cardiolipin and oligomycin-sensitive ATPase it is inferred that mitochondrial development proceeds at an advanced stage in anaerobic yeast cells. With the intention of ascertaining this point fine structure of mitochondria in anaerobic cells was studied under an electron microscope and compared with that in aerobic cells. Aerobic stationary cells were observed to contain quite a few mitochondria which were diffusely spread in cytoplasma. Each of these mitochondria has several crista membranes projecting into its matrix (Figs. 1 and 2). These mitochondria were also observed frequently to possess another type of inner membrane partitioning the mitochondrial matrix as indicated by the arrow in Fig. 1. This type of membrane is clearly distinguished from cristae and referred to as "mitochondrial septum membrane" in this paper. In contrast to these profiles there is no indication of clear appearance of crista membranes in mitochondria of anaerobically grown yeast cells (Figs. 3, 4 and 5), but, like in the case of mitochondria in aerobic cells, "septum membranes"are observable at stationary phase (Fig. 4). The profile of mitochondrion shown in Fig. 3 was taken from the exponential cell grown anaerobically on glucose. It is interesting to compare the stricture of the mitochondrion with that of stationary cells shown in Fig. 5. The electron dense materials suspended in the mitochondrial matrix of Fig. 3 are hardly observable in the mitochondrion

of Fig. 5. Instead, a bundle of fine filaments suggesting DNA in its nature appears in the electron lucent matrix of the latter. As for the galactose-grown cells, mitochondria in the stationary cells were not morphologically distinct from those in glucose-grown stationary cells and, further, the electron dense materials were no longer observable even in those of the exponential cells. On the basis of these observations it is concluded that at least two types of mitochondrion corresponding to the glucose-repressed and -derepressed states occur in anaerobic yeast cells.

Aerobic Adaptation of Anaerobic Yeast Cells Grown under Various Conditions ---- In order to examine whether the conversion from the repressed-type of mitochondrion to the derepressed-type is a physiological process of mitochondrial development, kinetic analysis was carried out for the appearance of respiratory activity during aerobic adaptation of anaerobically precultivated cells. If the conversion is involved in the developmental process of mitochondria, then the cells grown on galactose become active in respiration much faster than those on glucose do. As shown in Fig. 6, a rapid formation of mitochondrial respiratory system was observed for the cells pre-cultivated anaerobically on galactose as compared with the glucose-precultivated cells. Furthermore, it is interesting that the formation of respiratory system was not repressed by glucose for initial two hours when galactose-precultivated cells were aerated. Similarly, the formation was inhibited only slightly by chloramphenicol but greatly by cycloheximide, a potent inhibitor of cytoplasmic protein synthesis (Fig. 7). The slight exertion of chloramphenicol inhibition is not attributed to the delayed penetration of the inhibitor into cells,

because, although experimental data are not presented here, the rapid formation of the respiratory system was also observed upon aeration of the galactose-precultivated cells which had been incubated anaerobically with the antibiotic for two hours in advance. The formation of respiratory system proceeded, however, only at the initial phase of adaptation and no longer after about two hours in the presence of chloramphenicol as well as high concentrations of glucose (Figs. 6 and 7). This may be explained by the blocking action of the inhibitors to the development of mitochondria which are newly generated in proliferating cells. These results suggest that proteins synthesized on mitochondrial ribosomesare required for the conversion from the repressed- to derepressed-type of mitochondria, which proceeds even under anaerobic conditions, and that the converted mitochondria are integrated by proteins produced in cytoplasma under aerobic conditions. The suggestion is also supported by the finding that when the anaerobic precultivation was carried out on galactose in the presence of chloramphenicol little respiratory activity appeared during aerobic adaptation (Fig. 7).

DISCUSSION

Occurrence of cardiolipin and oligomycin-sensitive ATPase in anaerobic yeast cells has been described by several workers (2, 6, 7, 9). By using these substances as markers of mitochondria, however, consistent results have not always been obtained among several studies on changes in mitochondrial quantity and structure in anaerobic yeast cells. Jakovcic *et al*. have reported that cellular content of cardiolipin increases at stationary phase not only under aerobic but also anaerobic conditions, although both the content and its increase are much smaller in the latter case (9). These results suggest that mitochondrial development proceeds but suffers the glucose-repression even under anaerobic conditions. Our results are consistent with this, and further, make the exertion of the glucose effect clearer by showing great increases in both the cardiolipin content and oligomycin-sensitive ATPase activity in anaerobic yeast cells grown on galactose instead of glucose. Contrary to our results, it has been reported that there is little difference between the enzyme activities in mitochondria from galactose- and glucose-grown cells (2). However, the values presented in the report do not seem to express real content of the enzyme in mitochondria, because the enzyme is present in a latent form *in situ* (16) and the values were obtained without an activating treatment.

Formation of mitochondrial respiratory system has been shown to require proteins produced in both mitochondria and cytoplasma (26-28). On an experiment of aerobic adaptation of glucose-grown cells, Groot *et al*. have described that respiratory system does not appear but mitochondrial protein synthesis takes place in the presence of

cycloheximide at the first phase of aeration, which is followed by the appearance of respiratory activity at the second phase where cycloheximide is replaced by chloramphenicol (26). From these results they have postulated that oxygen is required for the protein synthesis in mitochondria to achieve the formation of the respiratory system. The idea has been further advanced by the finding that three out of seven subunit proteins constituting cytochrome oxidase are synthesized in mitochondria (29, 30) and two out of the three subunits only under aerobic conditions (29). In the present investigation, however, when grown on galactose instead of glucose, mitochondrial development appears to proceed to an advanced stage even in anaerobic cells, as shown by the increases in the cardiolipin content and oligomycin-sensitive ATPase activity. Furthermore, from the stage the formation of the respiratory system is likely to be accomplished by some proteins synthesized under aerobic conditions rather in cytoplasma than in mitochondria, since the formation was blocked at initial phase of the aerobic adaptation neither by high concentrations of glucose nor chloramphenicol but by cycloheximide (Figs. 6 and 7).

Electron microscopic observations have been carried out to examine the occurrence as well as morphology of mitochondrial structures in anaerobic yeast cells by several workers (1, 2, 8). There is, however, considerable disagreement on the morphological description. Wallace et al. have demonstrated occurrence of mitochondrial structures in anaerobic yeast cells and observed increase in their number in galactose-grown cells (8). Plattner et al. have described the morphology of the "promitochondria" of which structures, including the presence of cristae, are identical with those of aerobic mitochondria (1). Contrary to this

it has been reported that anaerobic mitochondria lack cristae (31), and that there is a distinct difference between anaerobic "mitochondrial precursors" and aerobic mitochondria (2). In the present paper, mitochondria in anaerobic cells were shown to be surrounded with double layered membranes, like those in aerobic cells, but distinguished from the latter by the absence of crista membrane. In this respect, our observation is in agreement with the description by Swift et al. (31). At stationary phase, however, cells were observed frequently to contain the unique mitochondria partitioned by inner membranes, which have been shown to be an intermediate phase of mitochondrial division by Tandler et al. (32). The "septum membrane" is continuous to the inner membrane and appears very similar to crista membrane. Kanaseki has demonstrated that both septum and crista membranes are distinguished from each other by their thickness in a high resolution photogram (33). However, the "septum membrane" was not observed by Swift et al. (31) but might be taken for crista membrane by Plattner et al. (1). It may also be pointed out that possible formation of crista membrane occurs during cell preparation before fixation since the membrane was observed to be readily formed upon exposure of derepressed anaerobic cells to air at room temperature.

Morphological differences were also found between both mitochondria in glucose-repressed and -derepressed anaerobic cells. The repressed type of mitohhondrion is characterized morphologically by the existence of numerous electron dense granules in its matrix and biochemically by the low levels of both cardiolipin and oligomycin-sensitive ATPase activity. In contrast, the derepressed type of mitochondrion shows

disappearance of the electron dense granules and attains the high content of cardiolipin althougn the level of the ATPase activity is not always high. Since both cardiolipin and oligomycin-sensitive ATPase are essential components of respiration-competent mitochondrion the latter type is reasonably considered to represent an advanced state in the process of mitochondrial development in anaerobic cells.

The both types of mitochondria, with the exception of occurrence of crista membrane, are also found respectively in the glucose-repressed and -derepressed aerobic yeast cells (33), and the basic biochemical difference between aerobic and anaerobic mitochondria is shown by the presence of cytochrome system. It is, therefore, very likely that the mitochondrial cytochromes are confined to crista membrane which is differntiated rrom the inner membrane lying in parallel with the outer membrane, and that the differentiation of the inner membrane is readily performed in the presence of molecular oxygen from the advanced inter= mediate stage reached anaerobically.

To summarize the present observations, several unique configurations of mitochondria were shown in yeast cells grown under various conditions, and each of them was indicated to correspond to a distinct state in the process of mitochondrial development in yeast cells.

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Table I. Comparison between colorimetric and radiometric analyses of phospholipids in aerobic yeast cells. Details of the methods of phospholipid analysis are described in "MATERIALS AND METHODS". % PL represents the percentage to total phospholipids. Other abbreviations used in the Table are: CL, cardiolipin of diphosphatidylglycerol; PC, phosphatidylcholine; PI, phosphatidyl-inositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PA, phosphatidic acid; PG, phosphatidylglycerol; LPC, lyso-phosphatidylcholine; PL, phospholipid.

Phospholipid	Method	
	Colorimetry (% PL)	Radiometry (% PL)
CL	5.0	5.6
PC	37.0	40.1
PI + PS	19.4	17.0
PE	32.2	29.1
PA + PG	1.8	3.0
LPC + Other PL	4.6	5.2

Table II. Phospholipid compositions in various anaerobic yeast cells. Cells were grown anaerobically on glucose and galactose media and harvested at exponential and stationary phases. Each value indicated in the Table is a mean of 2 or 3 experiments. PLP represents total phospholipid phosphorus recovered in the extracts for each g of spheroplast protein. Other abbreviations are the same as those described in the Table I.

Phospholipid	Glucose		Galactose	
	Exponential (% PL)	Stationary (% PL)	Exponential (% PL)	Stationary (% PL)
CL	0.5	1.5	1.9	2.6
PC	42.7	45.8	50.1	49.8
PI + PS	25.1	19.5	22.0	22.1
PE.	18.3	16.2	17.4	16.2
PA	5.8	5.4	4.6	4.2
PG	0.0	0.5	0.3	0.3
LPC	3.7	5.5	2.5	2.8
Unidentified	3.9	5.6	1.2	2.0
PLP (μg P_i/g protein)	3,100	2,170	2,320	2,260

Table III. Distribution of cardiolipin in subcellular fractions from stationary cells grown anaerobically on galactose medium. Conditions and phase of cell growth are described in "MATERIALS AND METHODS".

Subcellular fraction	Cardiolipin			Oligomycin-sensitive ATPase	
	% PL	Specific content (nmoles P_i/mg protein)	Recovery (%)	Specific activity (μmoles P_i/min/mg proein)	Recovery (%)
Homogenate	2.3	2.6	100	0.13	100
Mitochondria	7.6	18.7	50.1	1.24	61.9
Intermediate	0.8	1.9	3.7	0.12	4.4
Microsome	0.2	0.3	1.8	0.01	1.6
Supernatant	0.0	0.0	0.0	0.00	0.0

Table IV. Cardiolipin contents in yeast cells grown under aerobic and anaerobic conditions. Each value indicated in the Table is a mean of 2 or 3 experiments.

Condition	Carbon source	Cardiolipin	
		Exponential	Stationary
		(nmoles P_i/mg protein)	
Aerobic	Glucose	1.45	3.44
"	Galactose	3.47	4.77
Anaerobic	Glucose	0.48	1.03
"	Galactose	1.36	1.82

Table V. Contents of cardiolipin in mitochondria from yeast cells grown under various conditions.

Condition	Carbon source	Cardiolipin	
		Exponential	Stationary
		(nmloes P_i/mg protein)	
Aerobic	Glucose	10.7	20.2
Anaerobic	Glucose	4.8	16.2
"	Galactose	19.2	18.7

Table VI. Oligomycin-sensitive ATPase activity in yeast cells grown under various conditions. CAP represents chloramphenicol, which was added in culture medium at a final concentration of 4 mg per ml.

Condition	Carbon source	Specific activity	
		Exponential	Stationary
		(μmoles P_i/min/mg protein)	
Aerobic	Glucose	0.07	0.27
Anaerobic	Glucose	0.01	0.02
"	Galactose	0.24	0.25
"	Galactose + CAP	0.01	-

Table VII. Oligomyin-sensitive ATPase activity in mitochondria from various yeast cells. CAP represents chloramphenicol and its concentration is as described in Table VI.

Condition	Carbon source	Specific activity	
		Exponential	Stationary
		(μmoles P_i/min/mg protein)	
Aerobic	Glucose	1.41	1.53
Anaerobic	Glucose	0.24	0.19
"	Galactose	1.36	1.24
"	Galactose + CAP	0.06	-

Table VIII. Content of cytochrome a in various cells.

Condition	Carbon source	Cytochrome a	
		Exponential (nmoles/10^{10}cells)	Stationary
Aerobic	Glucose	0.8	8.7
	Galactose	5.8	5.8
Semi-anaerobic	Glucose	0.3	1.3
	Galactose	5.3	5.7
Anaerobic	Glucose	0.0	0.0
	Galactose	trace	trace

Fig. 1. A profile of partitioned mitochondrion taken from the stationary cell grown aerobically on glucose. The inner membrane has two kinds of infoldings that project into the mitochondrial matrix. The incomplete transverse folds, the cristae mitochondriales, are of variable length, and the other folds indicated by the arrow, the"septum membranes", partition the mitochondrial matrix into two compartments. (x. 59,000)

Fig. 2. The profile of a mitochondrion taken from stationary cell grown aerobically on glucose. Besides the clear appearance of the cristae mitochondriales, a bundle of fine filaments is seen in the electron lucent matrix. (x. 59,000)

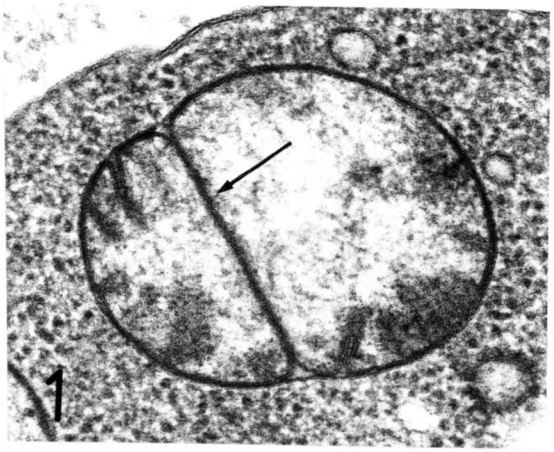
Fig. 3. A high power electron micrograph of a mitochondrion at a corner of the exponential cell grown anaerobically on glucose. No infolding of the inner membrane but a number of globular electron dense materials are seen in the matrix. (x. 100,000)

Fig. 4. The profile of a partitioned mitochondrion taken from the stationary cell grown anaerobically on glucose. No crista mitochondriale but the other kind of infolding of the inner membrane, the "septum membranes", is evident as seen in Fig. 1. (x. 53,000)

Fig. 5. The typical profile of a mitochondrion taken from stationary cell grown anaerobically on glucose. No infolding of the inner membrane is seen as in Fig. 3, but the electron lucent mitochondrial matrix makes a striking contrast to the electron dense matrix shown in Fig. 3. (x. 53,000)

Fig. 6. Respiratory adaptation of anaerobic yeast cells precultivated on glucose and galactose. Cells were grown in advance anaerobically to the stationary phase using glucose (2 %) or galactose (2 %) as a carbon source. After washing with cold water, cells were suspended in a fresh growing medium containing either sugar and aerated at 28°. At 1 hr intervals, aliquots were taken for the assay of respiratory activity. Sugars indicated at the left and right sides of arrows in the figure show carbon source present in the anaerobic precultivating and aerobic adaptive media, respectively. GAL and GLC in the figure represent galactose and glucose, respectively.

Fig. 7. Effects of antibiotics on respiratory adaptation of anaerobic yeast cells grown galactose medium. Cells were grown in advance anaerobically in galactose medium to the stationary phase. After wash= ing with cold water, cells were suspended and aerated in the fresh growing medium containing 4 % glucose in the presence of either 4 mg per ml of chloramphenicol (●) or 0.2 mg per ml of cycloheximide (▲). The bottom plot (X) was obtained from aeration of cells in 4 % glucose without antibiotics, which were grown in advance anaerobically in the presence of chloramphenicol (4 mg/ml) instead. The upper most plot (△) showed in the figure was transfered from Fig. 6 as control.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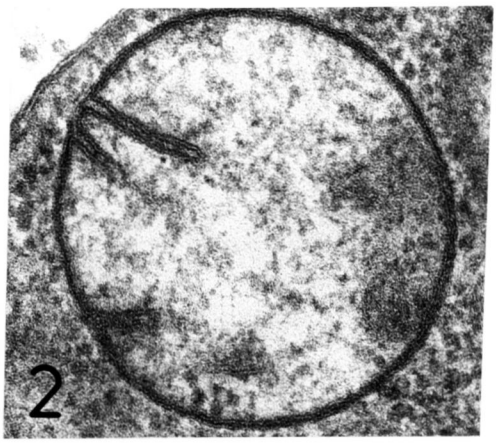
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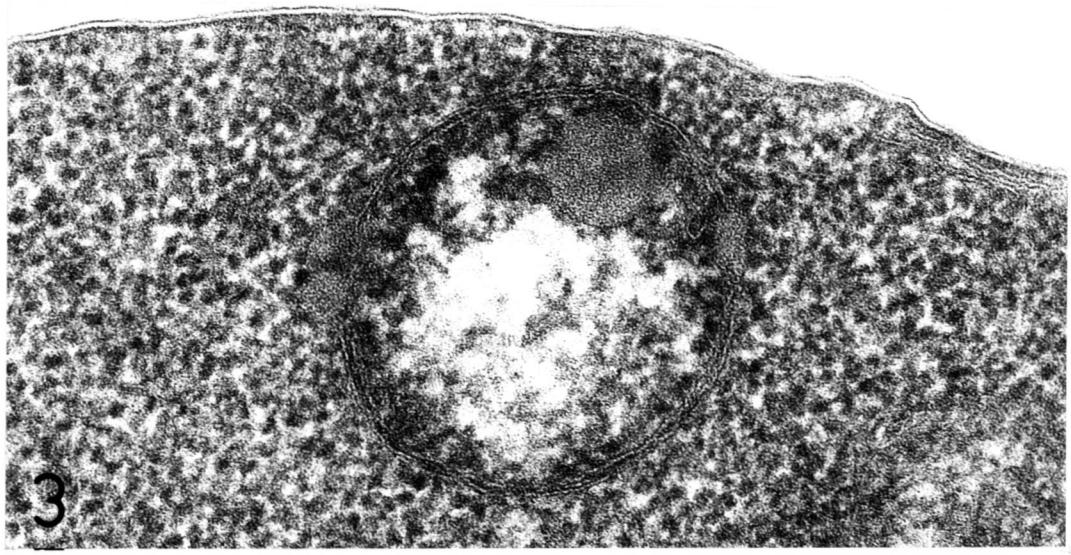
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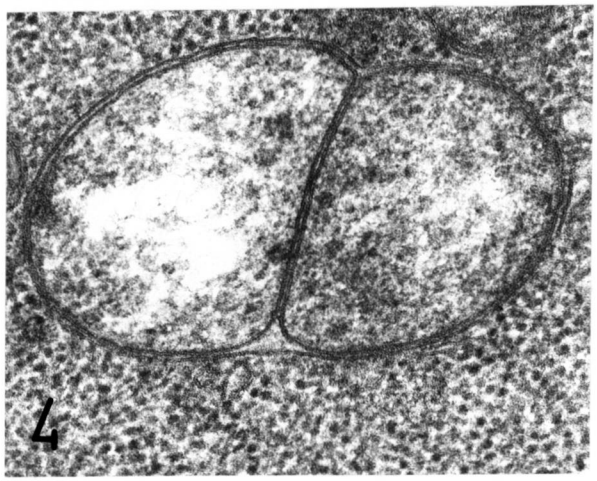
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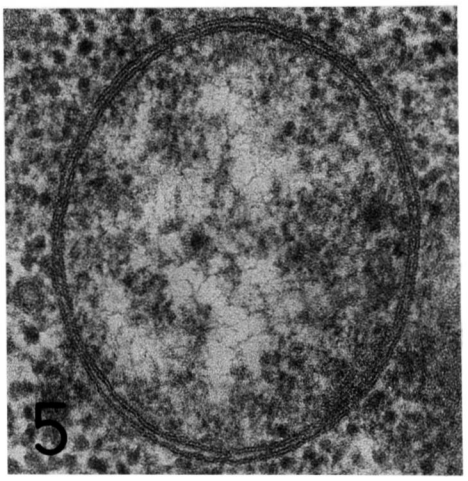
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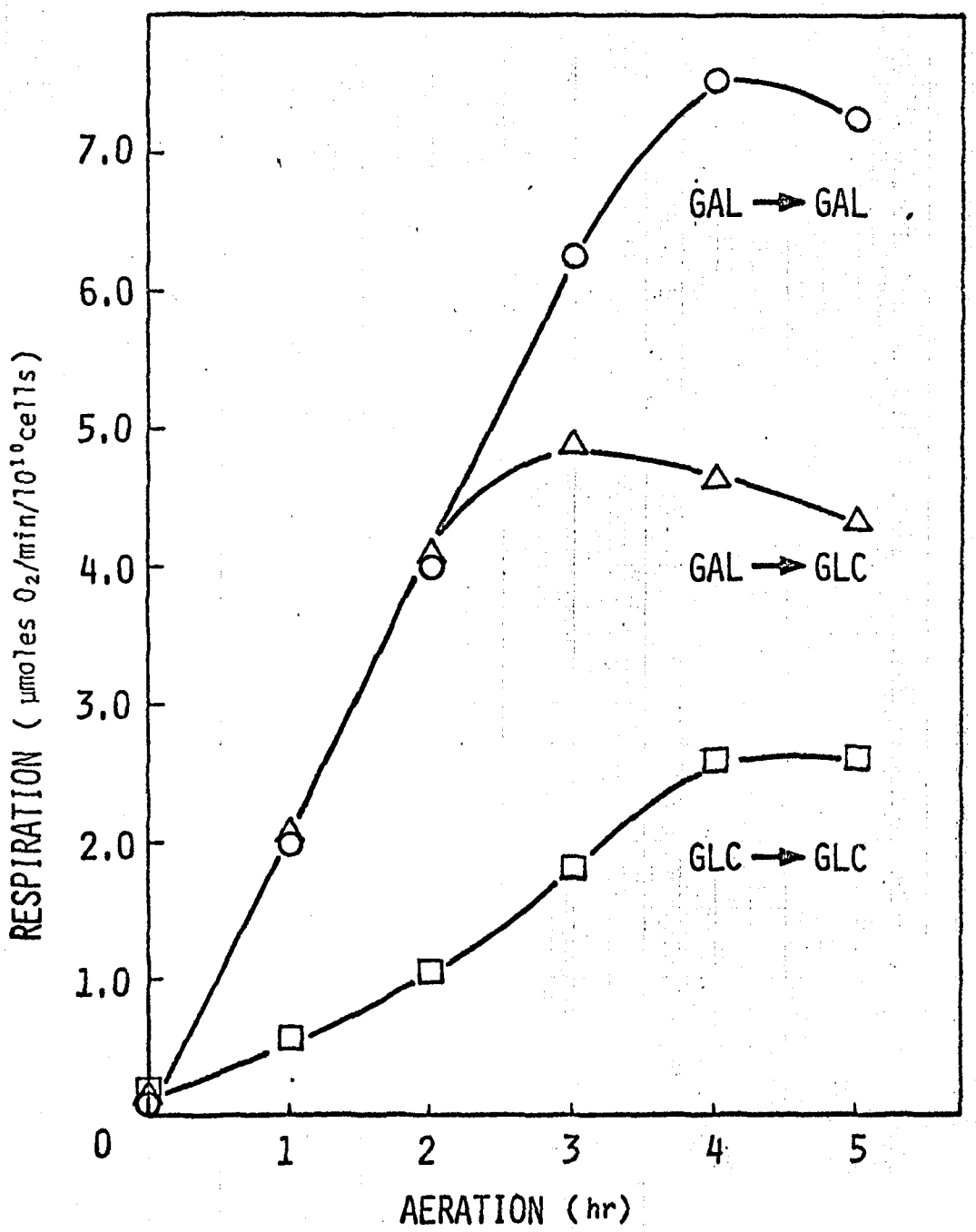
Fig. 6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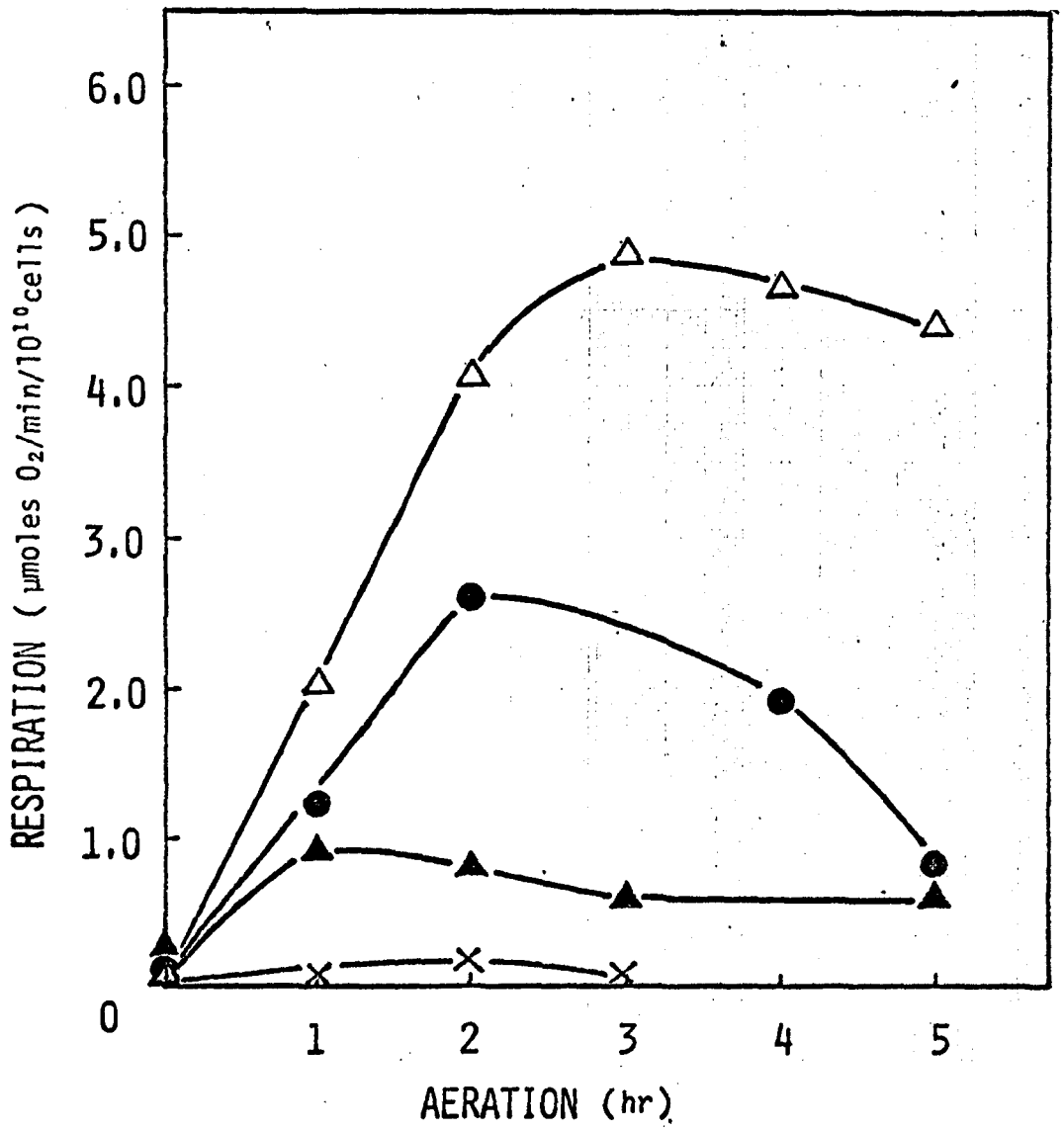
5.0
4.0
3.0
2.0
1.0
0
1
2
3
4
5
AERATION (hr)
GAL → GAL
GAL → GLC
GLC → GLC

Fig. 7


RESPIRATION (μmoles O2/min/10^10cells)
6.0
5.0
4.0
3.0
2.0
1.0
0
1
2
3
4
5
AERATION (hr)

Formation of Mitochondria in Yeast Cells

II. Two Types of Ribosomes Associated with Inner and Outer Membranes of Mitochondria

Isao Nagata

SUMMARY

The occurrence and content of intramitochondrial 70-S ribosomes were examined in various yeast cells by analysing r-RNA as well as electron microscopic observations with reference to synthesis of proteins comprising mitochondria. The ribosomes were found in all yeast cells examined except the cytoplasmic "petite" mutant yeast cells. Content of ribosome was affected neither in the glucose-repressed nor anaerobic cells. Electron microscopic observations also revealed that the cytoplasmic surface of the outer membranes of mitochondria are studded with large number of 80-S ribosomes. The adherent ribosomes were found to be able to synthesize protein on isolated mitochondria when supplemented with the soluble factors and ATP-generating system. The products of the protein synthesis on the ribosomes are discussed with reference to assembling of the inner membrane.

It is described in the preceeding paper that a developmental change in mitochondrial structure, except formation of cristae and cytochromes, proceeds in yeast cells beeing grown under anaerobic conditions, and that the change, like in the case of aerobic conditions, is blocked in the presence of high concentrations of glucose. The glucose effect has been assumed to be exerted on a step involved in the process of expression of the mitochondrial DNA, probably on the transcription step (1, 2). However, this may not exclude possibility of inhibition of intramitochondrial ribosome formation. The intramitochondrial ribosomes have been established to be sedimented at the 70-S value characteristic of prokaryotic cells, and therefore, distinguishable physicochemically as well as physiologically from cytoplasmic ribosomes sedimented at 80-S value (3, 4, 5). It has been demonstrated that r-RNA constituting the 70-S ribosomes are coded on mitochondrial DNA, and it is easily deleted spontaneously or by mutagen to yield the respiration-deficient "petite" mutant yeast (6, 7).

The present paper reports that the intramitochondrial 70-S ribosomes occur commonly in anaerobic yeast cells grown on glucose, denying the repressive effect on synthesis of r-RNA, and further that there are a great number of 80-S ribosomes bound to cytoplasmic surface of mitochondrial outer membrane in all kinds of yeast cells examined, including the "petite" mutant cells which completely lack the intramitochondrial 70-S ribosomes.

MATERIALS AND METHODS

Strains and Culture of Yeast Cells ---- JYD-56-G, a wild-type strain of Saccharomyces cerevisiae and JY-11, a cytoplasmic "petite" mutant, were used (8). Cultivations of yeast cells were performed aerobically and anaerobically as described in the preceeding paper.

Preparation of Growing Spheroplasts ---- In experiments for protein synthesis on 80-S ribosomes associated with mitochondria and for morphological observations of the ribosomes, spheroplasts were prepared essentially according to Kellems et al. (9). Spheroplasts, which were prepared as described in the preceeding paper, were washed with 1.0 M sorbitol and resuspended in the growth culture medium containing 0.1 % glucose and 0.5 M $MgSO_4$. The suspension was incubated at 30° for 1 hr and then kept in ice.

Preparation of Mitochondrial Membranes and Microsomes ---- Preparation of mitochondria and microsomes were carried out as described in the preceeding paper. When mitochondria binding 80-S ribosomes to their outer membrane were prepared, the growing spheroplasts after washed with 1 M sorbitol containing 5 mM $MgSO_4$ were disrupted and fractionated in a medium containing 0.6 M sorbitol, 10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 25 mM NH_4Cl_2 and 1 mM β-mercaptoethanol. Mitochondrial outer membranes were obtained according to Parson et al. (10) except that the final sucrose density gradient centrifugation was carried out at 15,000 rpm instead of 25,000 rpm, because of coagulating action of Mg^{++}.

Estimation of RNA Derived from Mitochondrial Ribosomes ---- Mitochondrial RNA was analyzed on a polyacrylamide gel electrophoresis after extraction. RNA was extracted from mitochondria with SDS-NaCl at 100° and treated with phenol according to Imamoto (11). The electrophoresis of the extracted RNA was carried out on 2 % polyacrylamide gel for 1-3 hrs at 20° using Loening's buffer system (12). The electrophoretic pattern of mitochondrial RNA was obtained by scanning in a TLC scanner (Shimadzu dual wavelength TLC scanner) after the gel was stained with 0.2 % acridine orange. For quantitative assay, mitochondrial RNA was obtained from the cells grown in the presence of 0.1 mCi of [^{3}H]-uridine or [^{14}C]-uracil in 1,000 ml of culture medium. After electrophoresis, the gel was sliced and resolved in 30 % H_2O_2 at 60°. Radioactivity of each gel slice (1 mm or 2 mm thick) was measured in a scintillation counter using Bray's solution. The amount of RNA of each band was calculated by its percentage of radioactivity to the total activity of RNA and the amount of original extracts obtained by the orcinol method (13).

Electron Microscopy ---- For negative staining, samples and 1 % uranylacetate were dropped on a colodion-coated copper grid successively. Excess stain was removed by filter paper. The sample was air-dried and coated with carbon. Preparation of ultra-thin sections and observation by electron microscope were done as described in the preceeding paper.

RESULTS

Electron Microscopic Observations of Intramitochondrial 70-S Ribosomes ---- It has been established that yeast mitochondria possess 70-S ribosomes distinguishable from cytoplasmic 80-S ribosomes (4, 5, 6). However, integral process of the intramitochondrial ribosomes has not yet been elucidated, and accordingly, it remains unsolved whether they are synthesized under the conditions which, for example anoxia and glucose repression, suppress mitochondrial formation.

In well developed mitochondria in aerobic yeast cells intra= mitochondrial ribosomes are readily observable in an electron= micrograph of ultra-thin section as polysomes bound to the inner membranes, both to cristae and septa (Fig. 1). The inner membrane-bound ribosomes are also clearly observed in mitochondria of anaerobic cells grown on galactose, where no crista membrane is formed though (Figs. 2 and 3). From electron microscopic observation, however, the occurrence of the intramitochondrial ribosomes are hardly recognized in the glucose-repressed cells of which mitochondria exhibit a number of globular electron-dense particles in the matrix as shown in the preceeding paper.

Mitochondrial Ribosomal RNA Content in Various Yeast Cells ---- Although electron microscopic observation failed to recognize the intramitochondrial ribosomes in the glucose-repressed cells, they were clearly detected and quantified on an polyacrylamide gel electrophoresis after phenol extraction from isolated mitochondria.

Fig. 4A shows an electrophoretic pattern of RNA extracted from a crude mitochondrial fraction which was prepared in the presence of 1 mM EDTA from aerobic yeast cells by single sedimentation at 10,000 x g. Three out of five distinct peaks exhibited in the pattern were identified with 28-S, 18-S and 5-S r-RNA's respectively derived from 80-S ribosomes by referring to that from a highly purified cytoplasmic ribosomal preparation (Fig. 4C). The rest of the bands were recognized to correspond to 23-S and 16-S r-RNA's respectively derived from 70-S intramitochondrial ribosomes, since, as described by Grivell *et al.* (14), these RNA's were confirmed by a sucrose density gradient centrifugation to be sedimented at 23-S and 16-S values respectively, although both the r-RNA components migrated more slowly on the electrophoresis than the respective r-RNA's from *E. coli* and even those from 80-S ribosomes. A large amount of the r-RNA derived from 80-S ribosomes found in the crude mitochondrial preparation were not attributed merely to contamination of microsomes or free ribosomes but, at least a part of them, to 80-S ribosomes bound to mitochondrial outer membrane as will be described later. Most of, but not all of, the r-RNA was able to be removed from mitochondria by a sucrose density gradient centrifugation after washing 4 times with EDTA-containing preparation medium (Fig. 4B). However, since the purification procedure took long time and was found to cause also a considerable losses of both 23-S and 16-S RNA's from intramitochondrial 70-S ribosomes, the crude mitochondrial preparation was used for determination of both species of r-RNA.

Fig. 5 shows an electrophoretic pattern of RNA extracted from mitochondria isolated from late exponential cells grown aerobically on glucose labeled with [^{3}H]-uridine. The amounts of both 23-S and 16-S r-RNA's were calculated from activities of the corresponding peaks in the pattern and data for various yeast cells are listed in Table I. Content ratio of 23-S to 16-S was approximately 2 for aerobic cells while considerably smaller for anaerobic cells. This is not explainable at present. However, no great change was found in the sum of both RNA contents for all cells examined except a little high value for aerobic exponential cells. These results indicate that the glucose effect on mitochondrial development is attributed rather to the repression of mitochondrial m-RNA synthesis like in the case of catabolite repression observed in E. coli (15, 16) than to that of r-RNA synthesis.

From the above results it follows that the intramitochondrial ribosomes are always found in yeast cells even when their mito= chondrial development is impaired by any adverse environments with an exception of respiration-deficient "petite" mutant yeast cells. In fact, electron microscopic observation never revealed the existence of the intramitochondrial ribosomes in the mutant cells (see Fig. 6). Polyacrylamide gel electrophoresis also exhibited complete lack of the r-RNA's derived from 70-S ribosomes but normal amounts of those from 80-S ribosomes bound probably to outer membrane (Fig. 7). These results are in good agreement with those reported by Kellems et al. (9), and lead to the conclusion that

in the "petite" mutant cells no protein synthesis takes place inside of mitochondria because of lack of 70-S ribosomes, and therefore, all proteins making up the mitochondrial structure with double layered membranes are the products of 80-S ribosomes in mutant cells (Fig. 7).

80-S Ribosomes Associated with Mitochondrial Outer Membranes ---- Cytoplasmic ribosomes have been observed under electron microscope to be aiighned along the surface of outer membranes of mitochondria in various yeast cells (For example, see Fig. 2). Their binding to the membranes, however, seemed very loose and a little different from that to rough surfaced endoplasmic reticulum and nuclear outer membranes, since some interstice was always observed between the membrane and each ribosome, which might be formed artificially. Recently, clear binding of cytoplasmic ribosomes to the outer membranes has been demonstrated by Kellems et al. using spheroplasts prepared from aerobic yeast cells in a growing medium containing 0.5 M $MgSO_4$ (9, 17, 18). Using the medium we confirmed the occurrence of bound ribosomes to outer membranes of mitochondria in various yeast cells including "petite" mutant yeast cells. The association appeared very similar to that of rough surfaced endoplasmic reticulum and was never found on vacuole and plasma membrane (Fig. 8, 9).

When mitochondria are prepared from spheroplast suspension according to routin method, most of, but not all of, the bound ribosomes to the outer membranes may be released in spheroplasts

or during disruption of them, while a small part of them remain bound. This is the reason why considerable amounts of r-RNA's derived from 80-S ribosomes were found even in a purified mitochondrial preparation. Upon further fractionation of the mitochondrial preparation in the presence of 5 mM $MgSO_4$ it was also found by main recovery of 28-S and 18-S r-RNA's in the outer membrane fraction that 80-S ribosomes are bound to the membranes (Fig. 10). The outer membrane-bound ribosomes were also observable to appear as polysomes both in negative staining of outer membrane and ultra-thin section preparations of isolated mitochondria when they were obtained in the presence of 5 mM $MgSO_4$ from spheroplasts treated in advance in the growing medium containing 0.5 M $MgSO_4$ (Figs. 11 and 12). From these observations the outer membrane-bound ribosomes are very likely to be active in protein synthesis. Actually, as shown in Fig. 13, the mitochondrial preparation from "petite" mutant cells, when supplemented the soluble factors and ATP-generating system, exhibited strong amino acid incorporating activity susceptible to cycloheximide but not to chloramphenicol. Proteins synthesized on the outer membrane-bound ribosomes may be expected to be constituents of mitochondria but this is an important problem to be solved.

DISCUSSION

It has been described by many workers that there occur 70-S type ribosomes inside of mitochondria in yeast cells (4, 15, 19). The ribosome consist of the large (50-S) and the small (30-S) subunits (3), and each subunit contains 23-S and 16-S RNA's respectively, but lacks 5-S RNA (19, 20). However, change in the content of the ribosome has not been studied under various growth conditions, although protein synthesizing activity in mitochondria has been known to be impaired a great deal by environmental factors. In the present investigation the intra= mitochondrial r-RNA content was found to be affected greatly neither by anoxia nor by high concentrations of glucose. Accordingly, glucose may block the transcription step of mito= chondrial DNA, like in the case of catabolite repression observed in bacterial system (16). However, it cannot exclude the possibility that the inhibitory action is exerted on the translation step, since no difference in amino acid incorporating rate in vitro was reported between isolated mitochondria from glucose-repressed and -derepressed cells (2).

Neither intramitochondrial ribosome nor the r-RNA was found in the cytoplasmic "petite" mutant cells. This is in good agreement with the report by Wintersberger (20) and accounted for reasonably by the fact that the r-RNA is coded on mitochondrial DNA and the "petite" mutant results from more than 50 % deletion of the DNA (7, 21). This is also the case in "poky" mutant of

Neurospora in which 16-S RNA content is found 1/10 of 23-S RNA (22).

It is very interesting that the cytoplasmic surface of the outer membranes of mitochondria bears large numbers of adherent 80-S ribosomes. Although the close association of ribosomes with the outer membranes has never been observed in other organisms, this may not be of contiguity but physiologically significant. A number of protein constituents of the inner membranes including both hydrophobic intrinsic and hydrophylic proteins, have been known to be produced on 80-S ribosomes in yeast cells (23). The proteins brought under the category, such as each subunit of F_1-ATPase (24) and three out of six subunits of cytochrome oxidase (25), seem very likely to be synthesized on the ribosomes of the outer membrane so that nascent peptides of these proteins can reach the inner membranes directly through the outer membranes.

ACKNOWLEDGEMENTS

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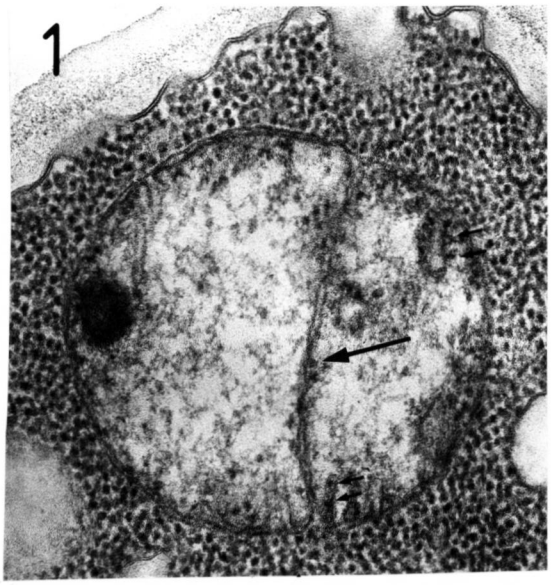
Table I Ribosomal RNA contents in mitochondria from various yeast cells. RNA was extracted from each [^{3}H]-uridine-labeled cells indicated in the Table and was analyzed by polyacrylamide gel electrophoresis according to "MATERIALS AND METHODS". Each RNA content was estimated according to the method described in "MATERIALS AND METHODS".

Cells			RNA content (μg/mg protein)	
			23-S	16-S
+ O_2	Glucose	Exponential	10.1	5.1
	Glucose	Late Exponential	7.6	3.4
	Glucose	Stationary	5.2	3.2
- O_2	Glucose	Exponential	5.9	4.2
	Galactose	Exponential	7.4	4.8

Fig. 1



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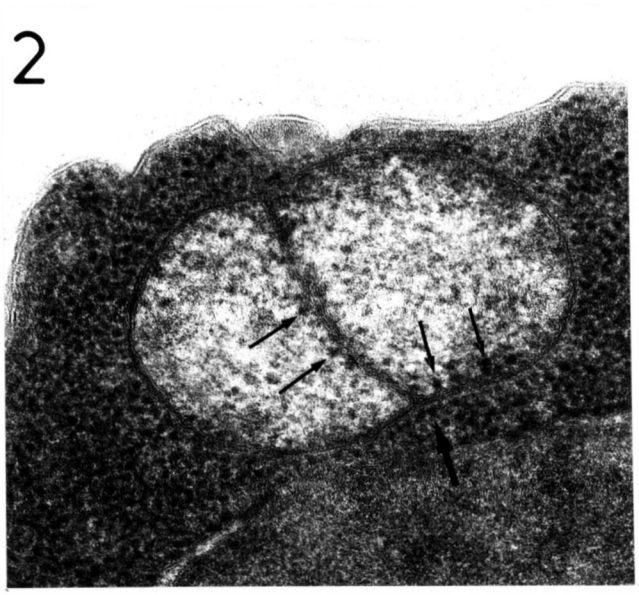

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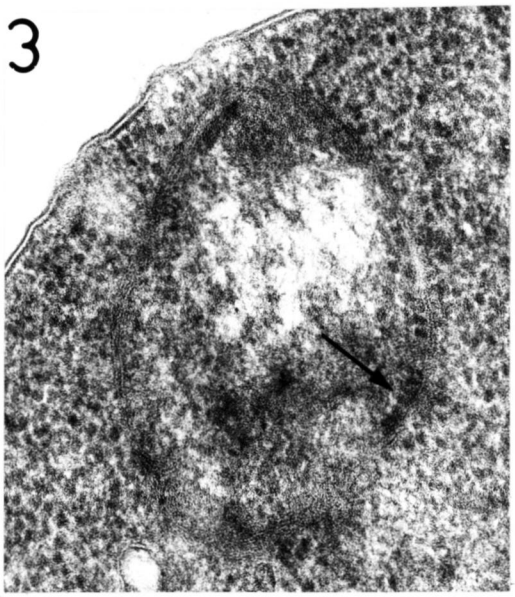

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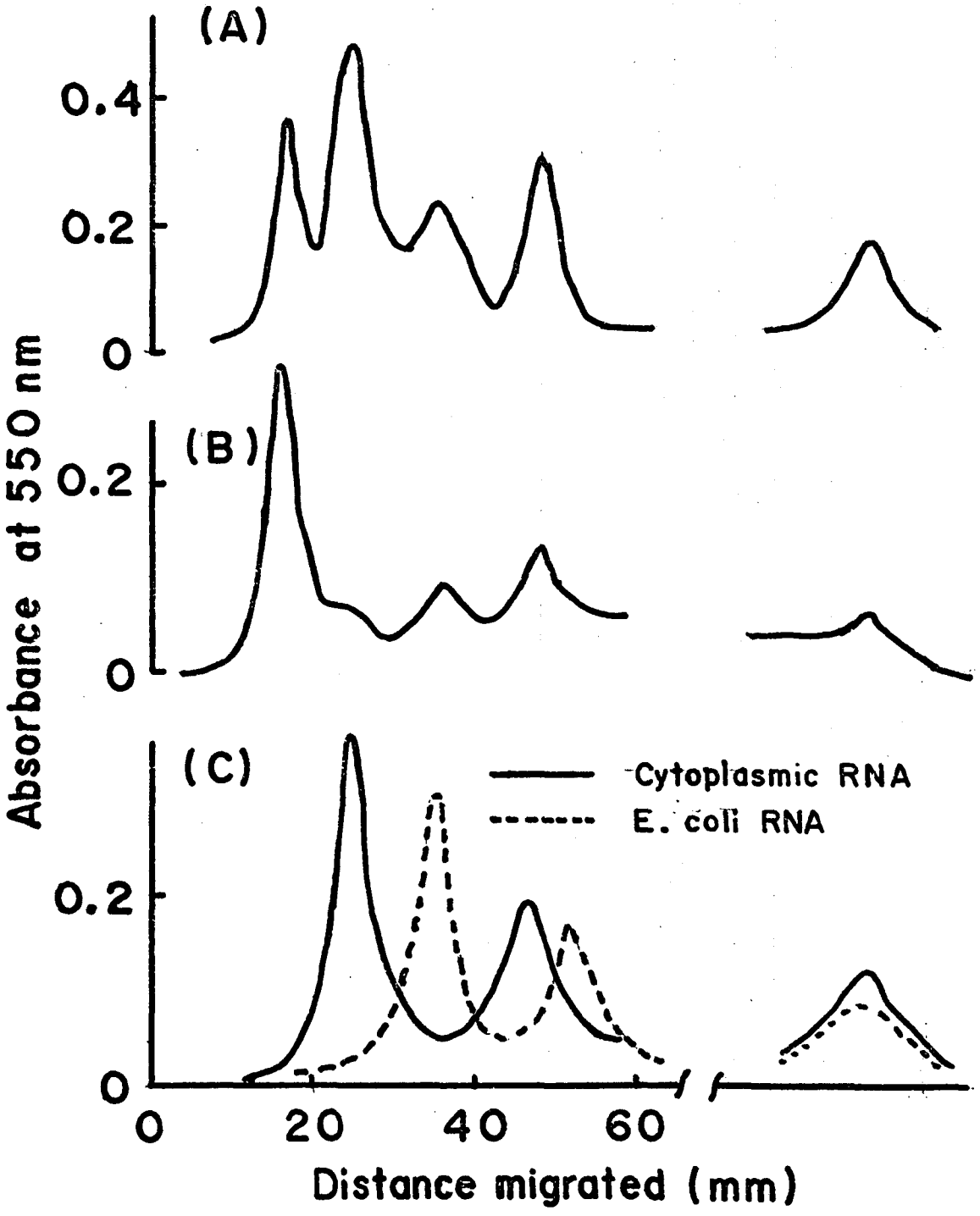
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0.2
0
(B)
0.2
0
(C)
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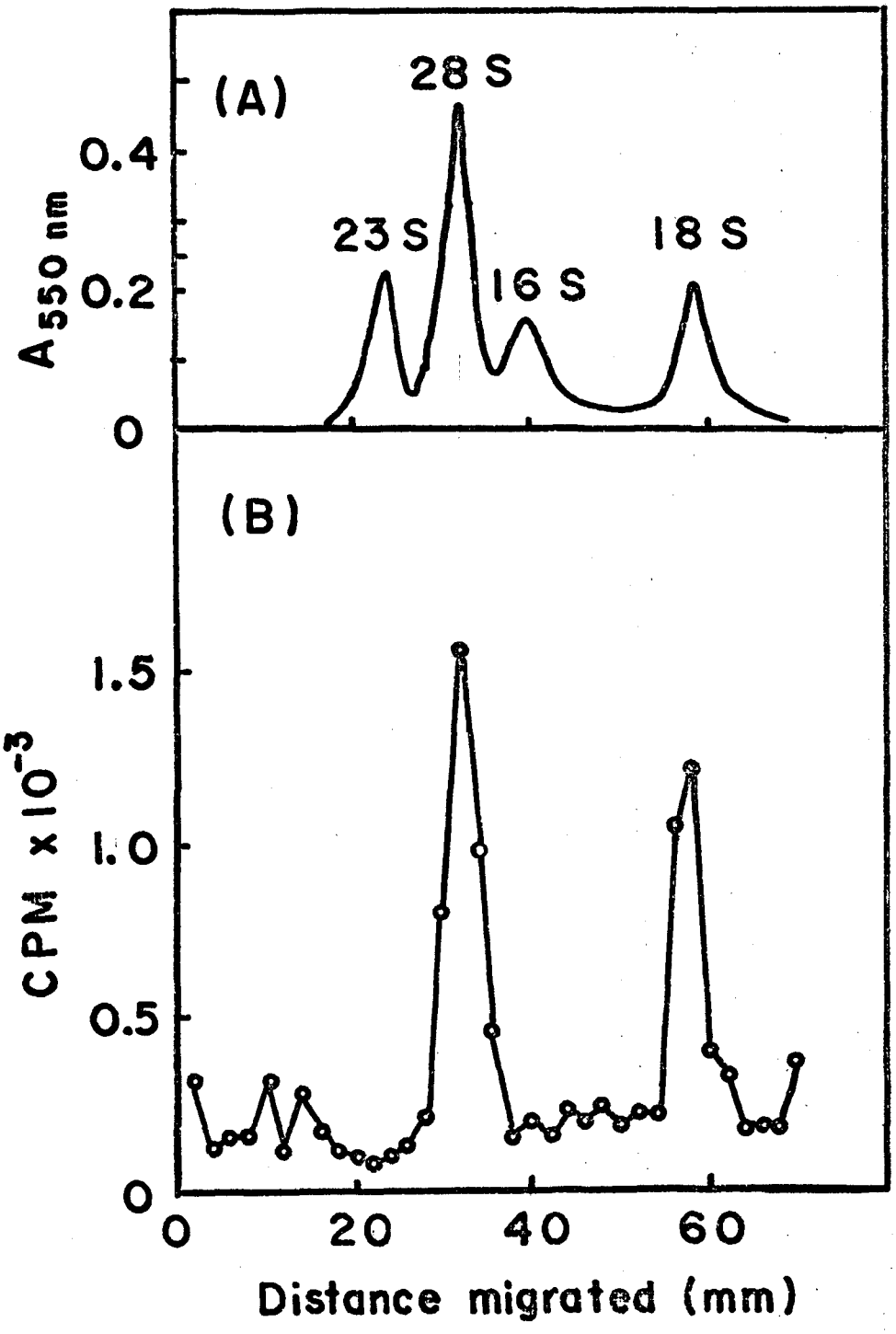
Cytoplasmic RNA
E. coli RNA
0
20
40
60
Distance migrated (mm)

Figure 5.


(A)
28 S
23 S
16 S
18 S
0.4
0.2
0
$A_{550\,nm}$
(B)
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1.0
0.5
0
CPM x 10^{-3}
0
20
40
60
Distance migrated (mm)

Fig. 6

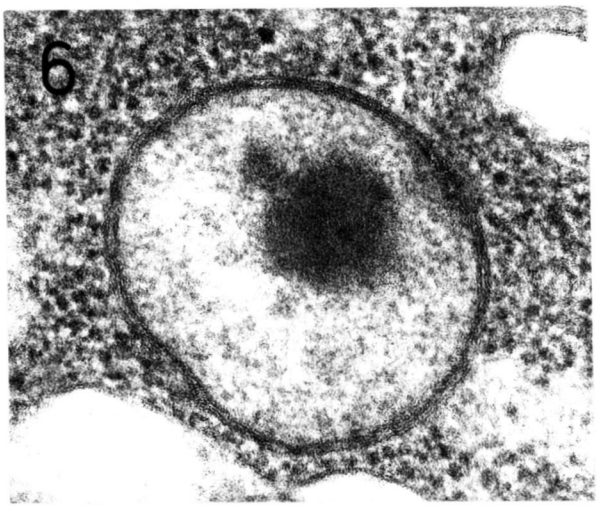
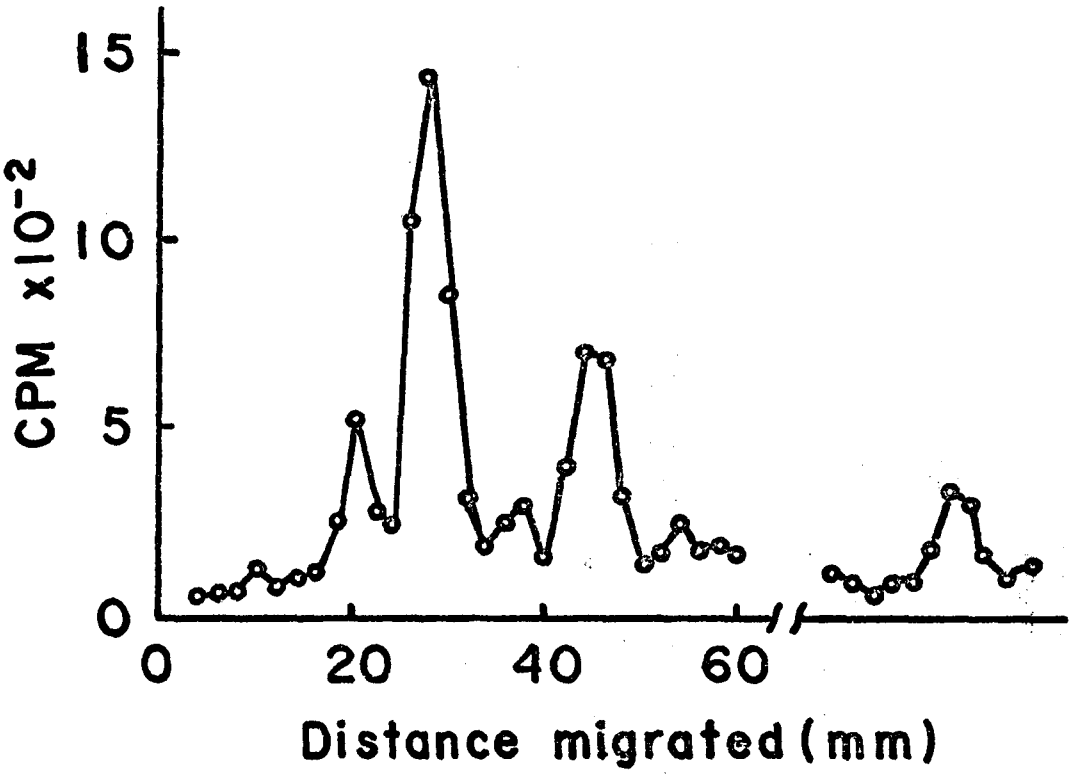


Figure 7



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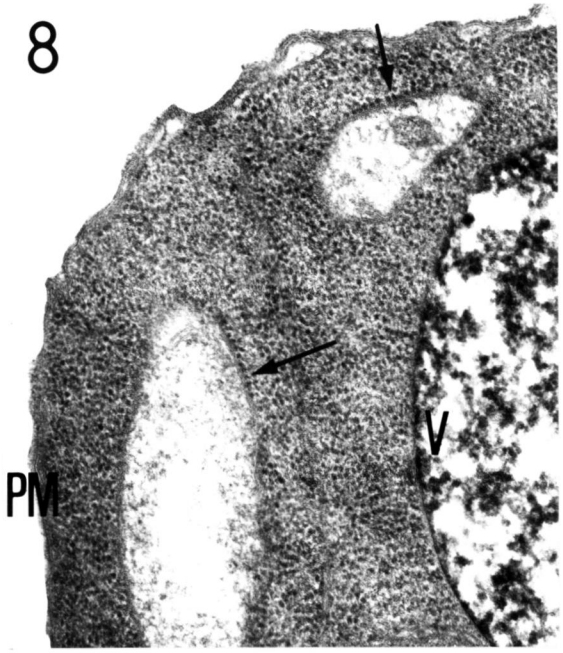

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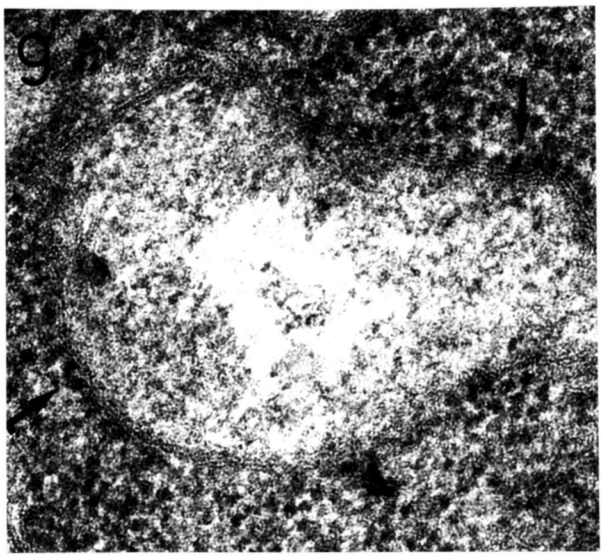
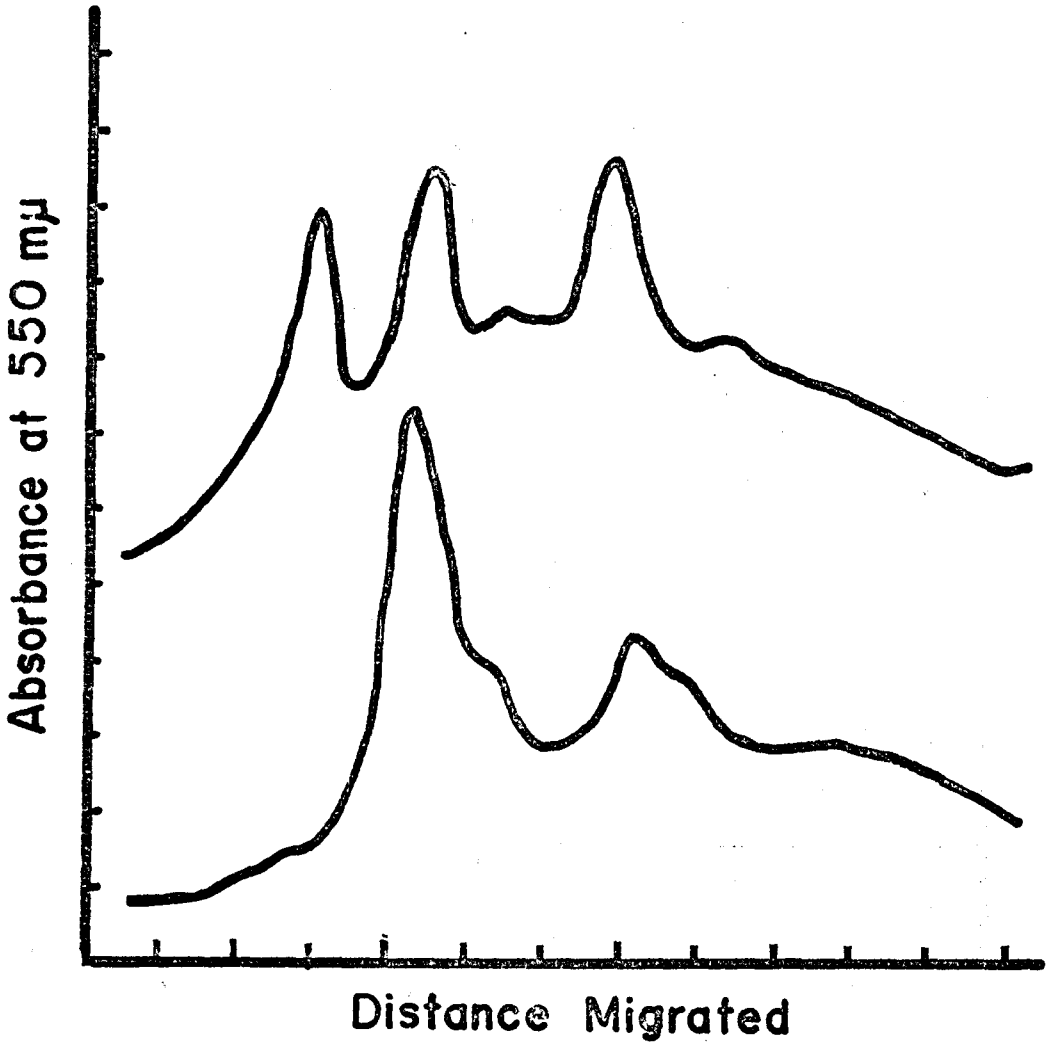


Fig. 10



(9)

Fig. 11

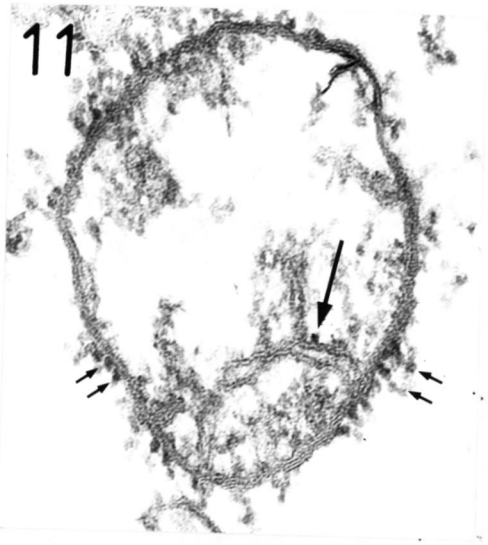


Fig. 12

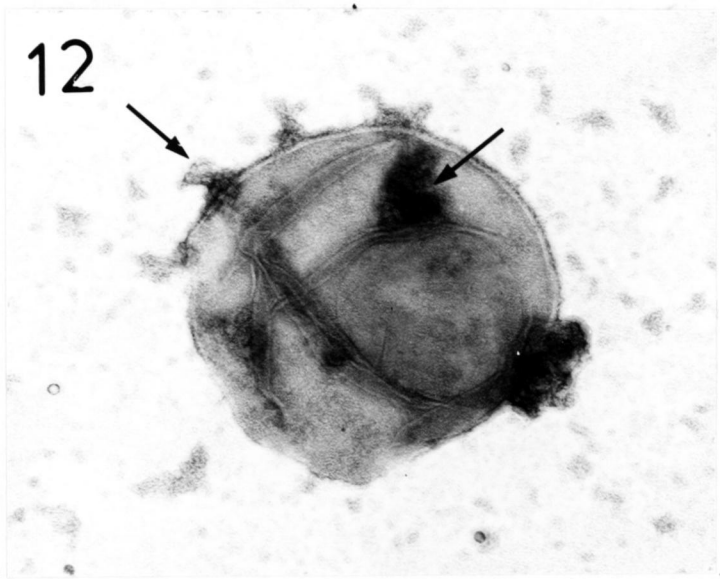


Figure 13.

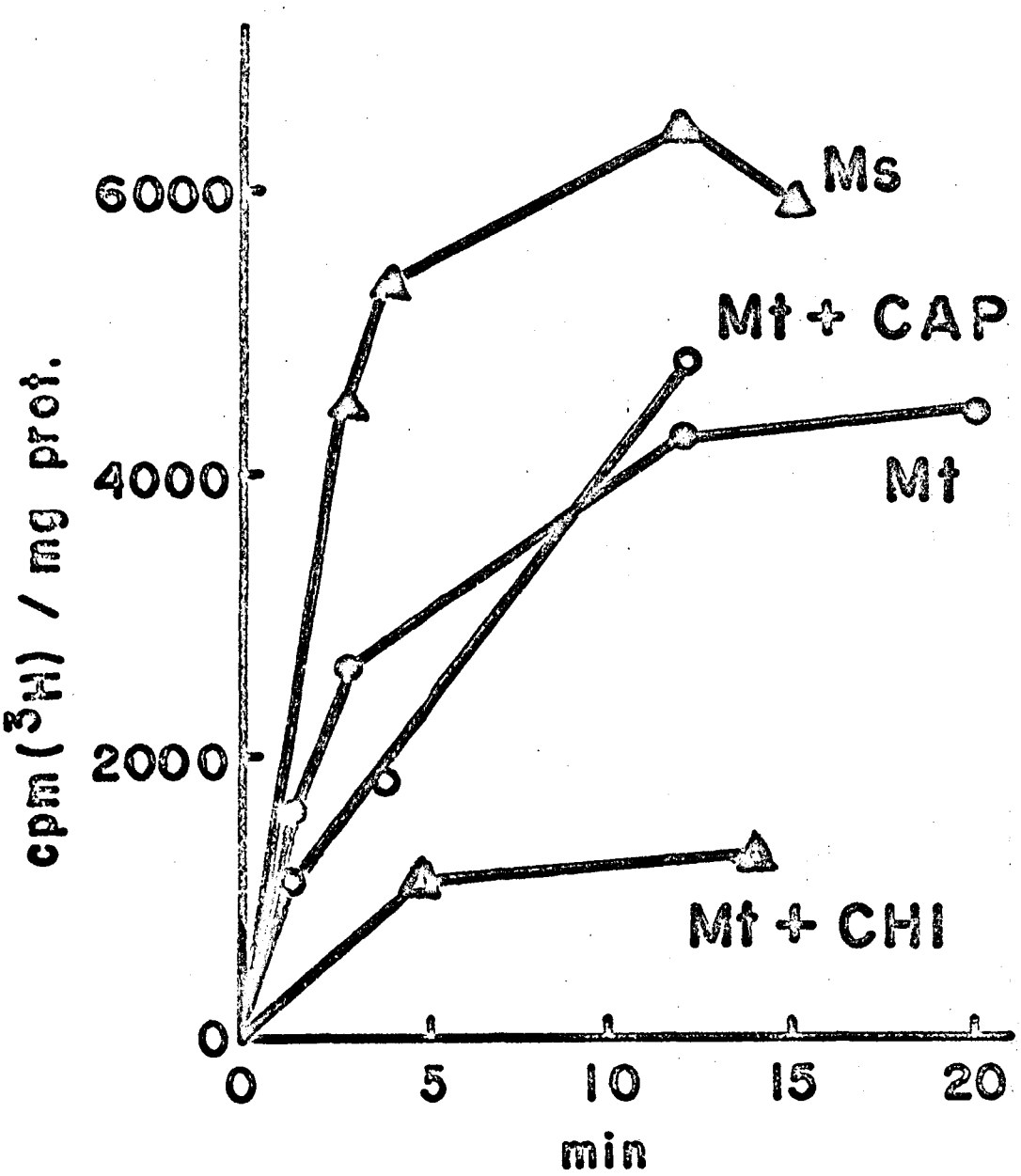


Fig. 1 Electron microscopic profile of a mitochondrion in a cell at early stationary phase grown aerobically on glucose. Short and long arrows in the figure show 70-S ribosomes bound to cristae and septum membranes, respectively.

Fig. 2 Electron microscopic profile of mitochondrion taken from stationary cells grown anaerobically on glucose. 70-S ribosomes can be seen to bind to the mitochondrial inner membranes and septum membrane (⟶). Cytoplasmic 80-S ribosomes are also observed to associate with mitochondrial outer membranes (➡).

Fig. 3 Electron microscopic profile of mitochondrial 70-S ribosomes in an anaerobic cell at stationary phase grown on glucose. Arrows in the figure show polysome-like 70-S ribosomes aligned along mitochondrial inner membranes.

Fig. 4 Polyacrylamide gel electrophoretic pattern of RNA extracted from crude and washed mitochondria. Mitochondria were prepared in the preparation medium containing 1 mM EDTA (graph A) and then, further purified by sucrose density gradient centrifugation (36% - 52 %) after four time washing with the same preparation medium (graph B). Solid and dotted lines represent RNA's from purified yeast 80-S ribosomes and E. coli ribosomes, respectively (graph C).

Fig. 5 Polyacrylamide gel electrophoretic pattern of RNA extracted from isolated mitochondria. Wild-type yeast cells were grown aerobically on glucose to late exponential phase in the presence of [^{3}H]-uridine. RNA was extracted from isolated mitochondria and gel electrophoresis was carried out as described in "MATERIALS AND METHODS".

Fig. 6 An electron micrograph of a mitochondrion taken from a ρ^- "petite" mutant cell at early stationary phase grown on glucose. In the matrix, which is surrounded by double layered membranes, electron dense materials exist but 70-S ribosomes are never seen.

Fig. 7 Polyacrylamide gel electrophoresis of [^{3}H]-labeled mitochondrial RNA from ρ^- "petite" mutant cells. RNA of ρ^- "petite" mutant cells were labeled and extracted as described in "MATERIALS AND METHODS". Gel electrophoresis of the [^{3}H]-labeled RNA was carried out together with none-labeled mitochondrial RNA from wild-type cells.

(A) The profile was obtained by scanning with 550 nm beam after staining with acrydine orange.

(B) Radioactivity was measured for each 2 mm gel slice.

Fig. 8 Electron microscopic profiles of 80-S ribosome attachment to the mitochondrial outer membranes. Spheroplasts prepared from aerobic cells grown on glucose were incubated semianaerobically in the growing culture medium containing 0.5 M $MgSO_4$ as described in "MATERIALS AND METHODS". After 1 hr incubation, cycloheximide was added (100 μg/ml) and immediately the spheroplasts were fixed. Arrows in the figure indicate 80-S ribosomes bound to outer membranes of mitochondria. Neither vacuole (V) nor plasma membrane (PM) attaches these ribosomes.

Fig. 9 Electron microscopic profiles at high magnification of 80-S ribosome on mitochondrial outer membrane. The electron micrograph was taken from the same sample used in Fig. 8. Arrows indicate the 80-S ribosomes.

Fig. 10 Polyacrylamide gel electrophoretic pattern of RNA from outer membranes of mitochondria. Mitochondria were prepared in the preparation medium containing 5 mM $MgSO_4$ from aerobic early stationary phase grown on glucose. From the mitochondrial preparation the outer membranes were obtained by hypotonic treatment and subsequent sucrose density gradient centrifugation in the medium containing 5 mM $MgSO_4$. Upper curve: total mitochondria, lower curve: outer membranes.

Fig. 11 Electron microscopic profiles of 70-S and 80-S ribosome attachment to a isolated mitochondrion. Mitochondria were prepared from the growing spheroplasts described in Fig. 8. Short and long arrows in the figure show 80-S ribosomes bound to the outer membrane and 70-S ribosomes bound to cristae membrane, respectively.

Fig. 12 Electron microscopic profiles of 80-S ribosomes on the surface of isolated outer membranes of mitochondria. Observation by negative staining method was done as described in "MATERIALS AND METHODS". Outer membrane attached with 80-S ribosomes were prepared as described in the legend of Fig. 10. Arrows show clusters of polysome consisted with more than 10 ribosomes on the outer membrane.

Fig. 13 In vitro amino acid incorporation by 80-S ribosomes bound to mitochondria from ρ^- "petite" mutant cells. Reactions were carried out essentially according to Kellems's method(9). The reaction mixture contained 155 μmoles NH_4Cl_2, 10 μmoles magnesium acetate, 21 μ moles Tris-Cl, pH 7.4, 1 μmoles dithiothreitol, 0.4 mmoles sorbitol, 0.1 μmoles mercaptoethanol, 1 μmoles ATP, 0.5 μmoles GTP, 0.1 μmoles each unlabeled amino acid minus leucine, 10 μmoles phosphoenolpyruvate, 11 μg pyruvate kinase, pH 5 fraction(1.5 mg protein)and mitochondria (1.52 mg protein) or microsomes(0.92 mg protein)in a final volume of 1.0 ml. If added, concentrations of chloramphenicol(CAP) and cycloheximide(CHI) were 100 μg/ ml and 50 μg/ ml, respectively. After preincubation for 5 min at 30°, 10 μCi of [^{3}H]-leucine (50 Ci/mmole) was added and at the indicated time 0.1 ml was

transfered into 1.0 ml of cold 5 % TCA containing 0.5 mg of unlabeled leucine. Each sample was treated for 10 min in boiling water and filtered on Watman GF/C glass fiber filter paper, rinsed with 5 % TCA containing 0.5 mg per ml of unlabeled leucine followed by a rinse with 95 % ethanol. Samples were dried and counted. Mitochondria and microsomes were prepared in the preparation medium containing 5 mM $MgCl_2$ from the spheroplasts incubated in the growing medium containing 0.5 M $MgSO_4$ as described in "MATERIALS AND METHODS".