

MITOCHONDRIAL BIOGENESIS DURING DIFFERENTIATION OF *ARTEMIA SALINA* CYSTS

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ABSTRACT

Mitochondria isolated from cysts of *Artemia salina* (brine shrimp) were found to be devoid of cristae and to possess a low respiratory capability. Hydration of the cysts induces marked biochemical and morphological changes in the mitochondria. Their biogenesis proceeds in two stages. The first stage is completed within 1 h and is characterized by a rapid increase in the respiratory capability of the mitochondria, their cytochrome oxidase, cytochrome *b*, cytochrome *c* and perhaps some morphological changes. In the second stage there is an increase in the protein-synthesizing capacity of the mitochondria as well as striking changes in mitochondrial morphology leading to the formation of cristae.

INTRODUCTION

The crustacean *Artemia salina* (brine shrimp) can reproduce in two ways: either the fertilized egg in the female ovisac directly evolves into nauplius larvae, or, during the dry season, it stops development at the stage of the gastrula, encysts, and is released in this form (Finamore and Clegg, 1969). These cysts may be desiccated by natural drying or osmotically by high environmental salinity after which they enter a state of dormancy called cryptobiosis. It seems that desiccation of cysts activates the gastrula which, when rehydrated, will undergo further differentiation to the pre-nauplius stage; this latter process taking place without cell division in about 15 h. Further incubation of the encysted prenauplii induces hatching and gives rise to a free-swimming nauplius larva. At this stage cell division is resumed (Nakanishi et al., 1962; Finamore and Clegg, 1969).

The biogenesis of mitochondria has been extensively studied in protists (Linnane and Haslam, 1970; Watson et al., 1971; Plattner et al., 1971). However, much less information is available on animal mitochondria. In this report we show that during rehydration of the *A. salina* cysts at 30°C,

profound ultrastructural and biochemical changes occur in the mitochondria which make this system a useful model for the biogenesis of animal organelles.

MATERIAL AND METHODS

Brine shrimp eggs were purchased from Sternco Industries (Longlife Aquarium Products, New York). Cysts were washed by floatation in cold saturated NaCl, followed by washing with cold water. The cysts were then incubated in the cold or with agitation at 30°C in artificial sea water (Dawson, 1959) containing, per liter, 24.7 g NaCl, 0.79 g KCl, 6.3 MgSO₄·7H₂O, 4.6 g MgCl₂·6H₂O, 1.0 g CaCl₂, and 0.2 g NaHCO₃ supplemented with 0.1 g dehydrostreptomycin and 100,000 U penicillin G. For the study of nauplii, incubation was carried out for 24 h; swimming nauplii were then allowed to settle and were isolated by suction.

Isolation of Mitochondria

After incubation, cysts or nauplii were washed twice with buffered sucrose solution (0.44 M sucrose, 3 mM Na₂ EDTA-Tris and 5 mM Tris-HCl, pH 7.5), suspended in 5–10 vol of buffered sucrose solution and

homogenized with 10 strokes of a loose-fitting (for cysts) or tight-fitting (for nauplii), motor-driven Potter-Elvehjem homogenizer. All these steps as well as further operations were carried out at 4°C. The suspension was then filtered through Miracloth (Calbiochem, San Diego, Calif.) and centrifuged twice for 10 min at 1,500 *g*. Mitochondria were pelleted by centrifuging for 10 min at 15,000 *g* and washed once with the buffered sucrose solution. 1 g dry weight of cysts yields a soluble extract (supernate obtained after 10 min at 1,500 *g*) containing 85 mg of cytoplasmic proteins and 1.9 mg of mitochondrial proteins.

Electron Microscopy of Mitochondrial Fraction

The electron microscopy work has been done by Dr. Yehuda Marikovsky with the technical assistance of Mr. S. Himmelhoch from the Department of Biological Ultrastructure, Weizmann Institute of Science. Mitochondrial pellets were fixed in 2% glutaraldehyde containing 0.25 M sucrose and 0.05 M phosphate buffer (pH 7.5) for 2 h at 0°C. They were washed twice with the sucrose-phosphate solution and postfixed for 1 h with 2% Os₂O₄ containing 0.25 M sucrose and 0.05 M Na phosphate buffer, pH 7.5. The fixed pellets were dehydrated and embedded in Epon 812. Thin sections were cut in a Danon-Yeda ultramicrotome and double stained with uranyl and lead citrate. Micrographs were taken in a JEM-7 electron microscope at 80 kV. Other samples were negatively stained with phosphotungstic acid.

Oxygen Consumption

Oxygen concentrations were measured polarographically using an oxygen electrode (Estabrook, 1967). Kinetic measurements were followed with the aid of a recorder for a period not exceeding 2 min either directly on 2.5 ml of cysts suspended in water or by addition of the filtered cysts to 2.5 ml of artificial sea water at 25°C. The nature of the suspending medium was not important, as the same respiration values were obtained in water or sea water.

Assay of Cytochrome Oxidase

Enzymatic activity (Smith and Conrad, 1956) was measured spectrophotometrically on aliquots of the 1,500 *g* supernatant, in a solution containing 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA at 25°C. The initial rate of oxidation was measured on a 25 mM solution of ferricytochrome C from horse heart (Type VI, Sigma Chemical Co., St. Louis, Mo.)

previously reduced by sodium hydrosulfite and filtered through a Sephadex G-15 (Pharmacia Fine Chemicals, Uppsala, Sweden) column.

Differential Spectra of Respiratory Pigments

Spectra were recorded according to Chance (1954) using a dual wavelength-split beam Aminco-Chance recording spectrophotometer (American Instrument Co. Inc., Silver Spring, Md.) from the Department of Biochemistry, University of Tel-Aviv. The cysts, incubated for different times at 30°C, were broken as described. After sedimentation of the debris, the mitochondria were pelleted and resuspended in 0.44 M sucrose containing 3 mM Na₂ EDTA-Tris and 5 mM Tris-HCl, pH 7.5. One sample was reduced with sodium dithionite, and its spectra were recorded versus a sample fully oxidized by sodium ferricyanide. The spectra were obtained after correction for the base line. The absorbance at 540 nm was taken as zero.

In Vitro Assay of Protein Synthesis with Whole Mitochondria

The assay was carried out as previously described (Schmitt, 1970), with 0.25 M sucrose instead of 0.5 M sorbitol and ATP being generated by phosphocreatine and phosphocreatine kinase. The specific radioactivity of the [¹⁴C]leucine was 312 mCi/mmol and the concentration of mitochondria was 3 mg/ml. Bacterial contamination was low and amounted to 10³–10⁴ cells/ml. At the end of the incubation, 0.1 ml aliquots were applied to Whatman 3 MM filter disks which were then heated for 30 min at 95°C in 7% trichloroacetic acid. Afterwards, the disks were washed three times with cold 7% trichloroacetic acid, ethanol, ethanol-ether (1:1), and finally ether. The dried filters were counted in a liquid scintillation counter.

In Vitro Assay of RNA Synthesis with Whole Mitochondria

The incorporation system was similar to that used by Reid and Parsons (1971) but omitting maleate and pyruvate. The concentration of [³H]UTP was 0.2 μM with a specific radioactivity of 14.8 Ci/mmol. Slightly better results were obtained when the assay was conducted in the presence of 0.25 M sucrose, provided the mitochondria were preswelled 1 h at 0°C in a 0.1 M phosphate buffer without sucrose. At the end of the incubation, 0.1-ml aliquots were applied onto filter paper disks and treated as described above except that the heating step was omitted.

RESULTS

Fig. 1 demonstrates that the major part of the respiratory capability of the cysts is gained within 1 h, after which the rate increases slowly. The undeveloped cysts, hydrated at 0°C (under conditions where little metabolic activity could take place), have a Q_{O_2} of about one-third that found for cysts

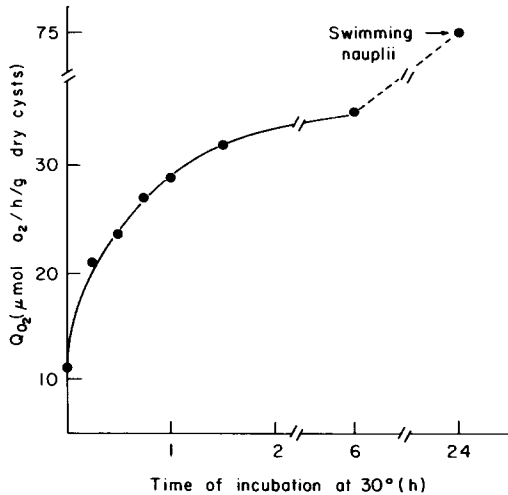


FIGURE 1 Oxygen consumption of the cysts after different times of incubation at 30°C.

which were incubated for 1 h at 30°C. It should be noted, however, that the maximum level of respiration of the cysts attained remains very low. Muramatsu (1960) has previously observed that respiration of the cysts increases slowly after 3 h of incubation; however, he did not determine the respiratory changes that take place up to this period. It seems that after hatching at the nauplius stage, a further twofold increase in Q_{O_2} occurs yielding a final oxygen consumption of 70 $\mu\text{mol O}_2$ per h per starting g of dry cysts. Electron micrographs established that the low Q_{O_2} of the resting cysts is not due to damaged mitochondrial membranes; Fig. 2 shows a representative sample of negatively stained mitochondria from unincubated cysts. It is clear that membrane damage is minimal in this preparation.

We have also examined the fate of cytochrome oxidase, a typical mitochondrial enzyme localized in the inner membrane of the organelle. As shown in Fig. 3, the increase in the specific activity of cytochrome oxidase roughly parallels the increase in the respiratory rate. Using differential spectrophotometry (Fig. 4), the same conclusion is reached. The peak of cytochrome oxidase at 605 nm reaches a maximum value after incubation of the cysts for 1 h, while the contents of cytochrome

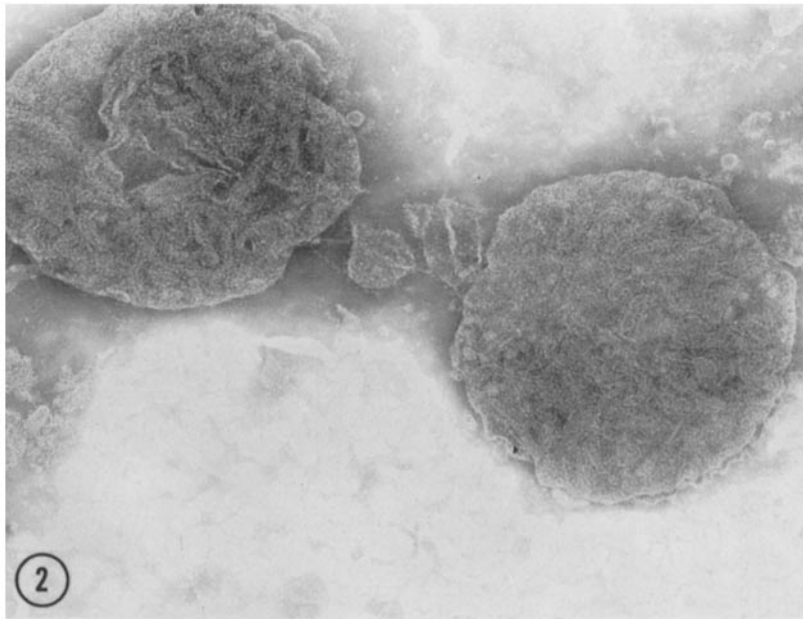


FIGURE 2 Electron micrograph of negatively stained mitochondria from unincubated hydrated cysts $\times 50,000$.

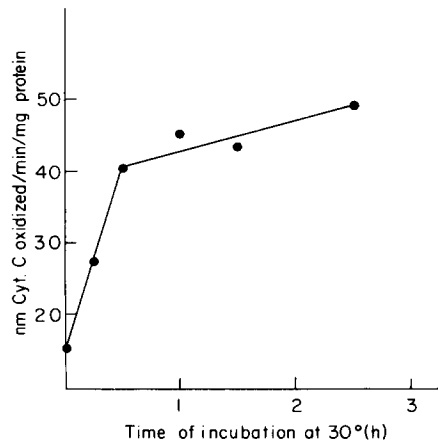


FIGURE 3 Cytochrome oxidase activity after incubation of the cysts at 30°C.

b (α -band at 564 nm) and cytochrome *c* (α -band at 553 nm) further increase until a plateau is attained around the 7th h of incubation. These results exclude the possibility that the rise in the respiratory rate is mainly due to an increase in the level of the respiratory substrates (at least until the nauplius stage). The amount of protein was roughly equal whether we started from cysts hydrated at 0°C or incubated for 6 h at 30°C. It should also be noted that the ratio of cytochrome *c* over cytochrome *b* increases during cyst incubation.

The morphology of mitochondria was investigated by means of electron microscopy. Care was taken to avoid artifacts due to lysis of mitochondrial preparations, damage to membrane, or loss of material during the purification stages. The mitochondria were purified by differential centrifugation and washed once by gentle homogenization. Figs. 5 A–D are electron micrographs of thin sections of mitochondria from cysts hydrated at 0°C, cysts incubated for 70 min or 7 h at 30°C and mitochondria isolated from nauplii. It is apparent that during the development of the cysts a striking change in mitochondrial morphology takes place. The mitochondria from unincubated cysts lack cristae and are poorly stained. After 1 h of incubation the mitochondria seem to show a somewhat more stained matrix, and perhaps some cristae start to develop. More pronounced changes are observed in the mitochondria isolated from cysts which were incubated for 7 h at 30°C. These mitochondria have definite cristae and a stained matrix while mitochondria isolated from nauplii perhaps possess even more cristae.

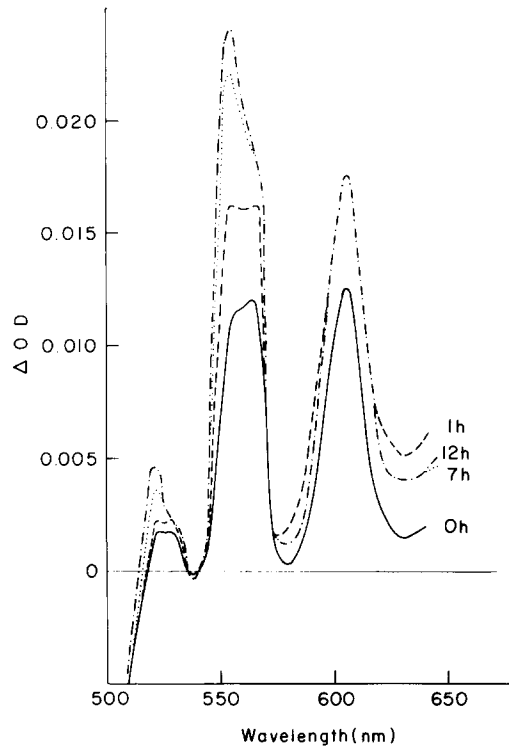


FIGURE 4 Differential spectra of cytochromes from a suspension of mitochondria (7 mg protein/ml) isolated from cysts incubated for: (—) 4 h at 0°C, (----) 3 h at 0°C and 1 h at 30°C, (·····) 7 h at 30°C, (-·-·-) 12 h at 30°C.

Mitochondria morphology during embryogenesis and muscle formation (Birt, 1971; Kleinow et al., 1971) have already been described in animal systems but this is the first time that such a striking transition has been observed. It is interesting to note that treatment of *L* cells with ethidium bromide leads to progressive mitochondrial changes (Soslau and Nass, 1971) resulting, finally, in mitochondria which are morphologically very similar to those observed in the unincubated *Artemia* cysts. Several mitochondria from unincubated cysts seem to possess a distinct middle membrane characteristic of dividing organelles (arrow in Fig. 5 A). It is possible that during cyst desiccation some mitochondria were caught in the middle of their division cycle.

What is the role of protein and RNA synthesis in the biogenesis of mitochondria? Since the cysts are quite impermeable, we examined the evolution of protein and RNA-synthesizing activity in isolated, whole mitochondria. As shown in Fig.

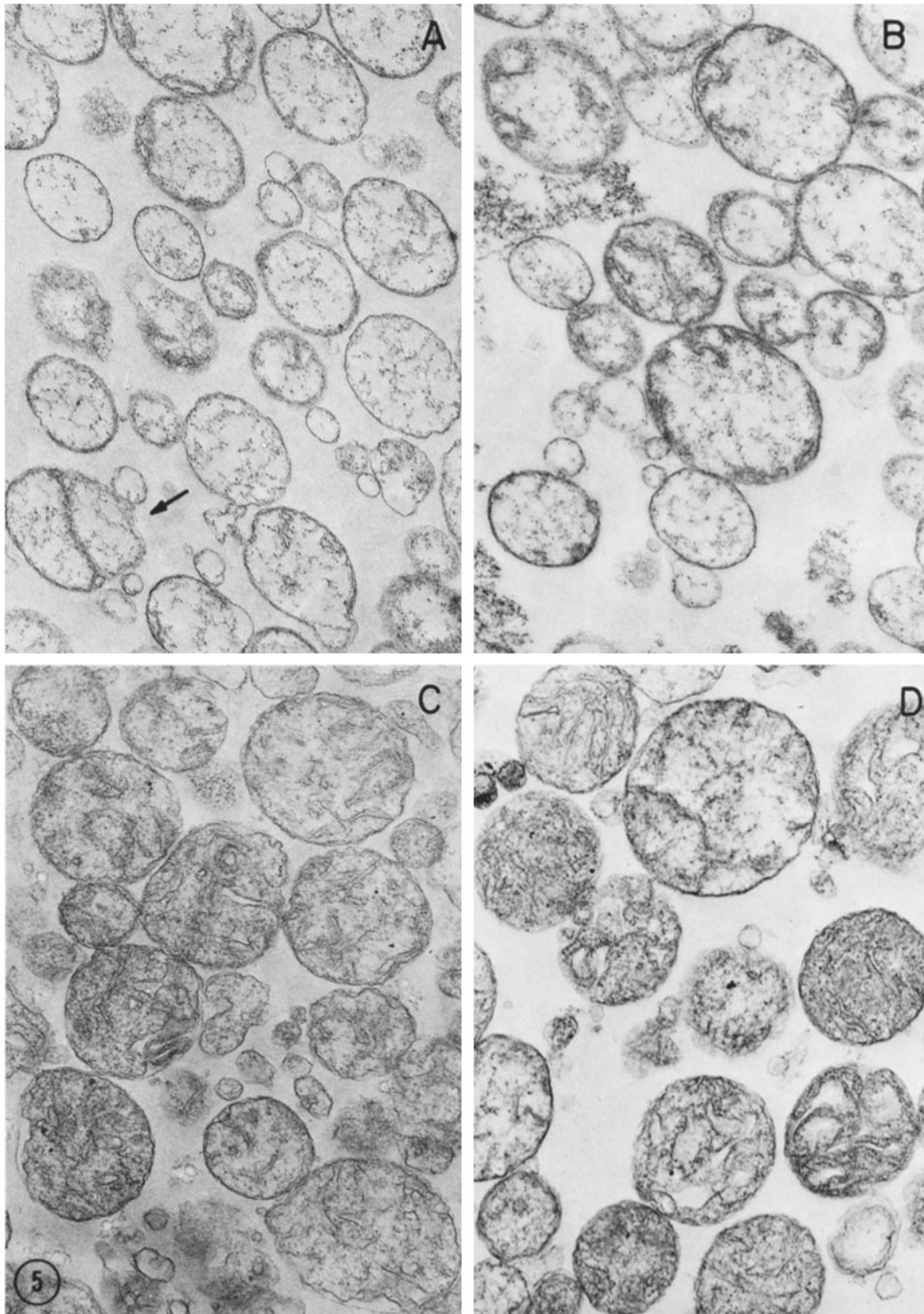


FIGURE 5 Electron micrographs of mitochondrial fraction from cysts: (A) hydrated at 0°C for 3 h $\times 25,000$, (B) hydrated at 0°C, then incubated 70 min at 30°C $\times 25,000$, (C) incubated for 7 h at 30°C ($\times 20,000$, (D) incubated for 24 h at 30°C (swimming nauplii) $\times 30,000$.

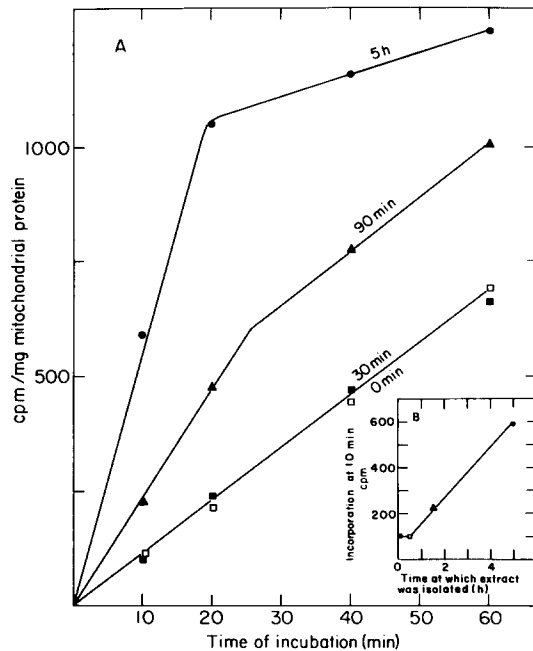


FIGURE 6 (A) Time course of in vitro incorporation of [¹⁴C]leucine into protein by mitochondria isolated from cysts incubated for different times at 30°C. (□) 0 min, (■) 30 min, (▲) 90 min, (●) 5 h. (B) Incorporation at 10 min as a function of incubation time of the cysts at 30°C. (Data taken from Fig. 6 A).

6 A, the protein-synthesizing activity of the mitochondria increases with increasing times of incubation of the cyst at 30°C. The protein-synthesizing activity was not inhibited by cycloheximide (100 μg/ml), while chloramphenicol (200 μg/ml) caused over 98% inhibition. These results indicate that mitochondrial protein synthesis was measured in these experiments. As shown in Fig. 6 B there is a lag of about 30 min before the increased protein synthetic activity is observed. As the main part of the respiratory capability of the cyst is gained during the first hour of incubation at 30°C (Fig. 1), it may be concluded that the initial rise in respiration is most probably due to assembly of precursor molecules present in the mitochondria. Indeed, in yeast where respiratory adaptation requires mainly *de novo* protein synthesis, 4 h of incubation are needed to obtain full respiratory capability (Ephrussi, 1950; Slonimski, 1956). The increased rate of protein synthesis in *Artemia* mitochondria does not seem to be due to changes in the intra-respiratory chain-linked synthesis of ATP, since

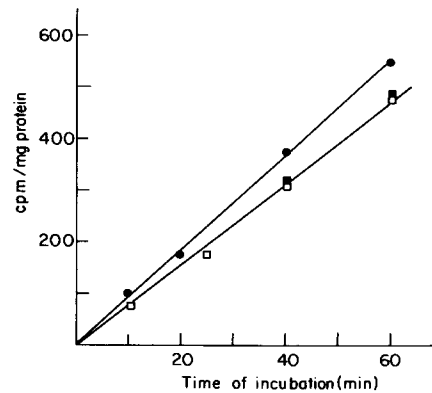


FIGURE 7 Time course of in vitro incorporation of [³H]UTP into RNA by mitochondria isolated from cysts incubated for different times at 30°C. (□) 0 min, (■) 25 min, (●) 4 h.

omitting the ATP regenerating system from the assay resulted in very low levels of amino acid incorporation. Our experiments, however, do not exclude an increase in the translocation of external ATP through the mitochondrial membrane.

Fig. 7 shows the time-course of [³H]UTP incorporation into RNA. Very small differences in the rate of incorporation were found for the different stages of development of the cyst. Whether this result reflects the *in vivo* situation is, however, difficult to ascertain. In vitro incorporation of nucleic acid precursors into mitochondrial RNA can proceed only with damaged mitochondria (Neubert and Helge, 1965; Saccone et al., 1967); under these conditions some loss of specificity could occur. Moreover, contamination by nuclear RNA polymerases is probable. From direct colorimetric measurements, we have found that there is about a twofold increase in mitochondrial RNA and DNA content within the first 3 h of incubation. Nuclear RNA synthesis also seems to increase under these conditions as does protein synthesis on cytoplasmic ribosomes (Clegg and Golub, 1969).

DISCUSSION

The results presented in this report show the developing cyst of *A. salina* to be an excellent system for studying the biogenesis of animal mitochondria. It seems likely that in these cysts mitochondrial biogenesis proceeds in two stages. The first stage is characterized by a rapid increase in the respiratory capability of the mitochondria and perhaps some morphological changes; while in the second stage there is an increase in the protein-synthesizing

capacity of the mitochondria as well as striking changes in mitochondrial morphology leading to the formation of cristae. It may well be that the early increase in respiratory activity is due to the assembly of precursor molecules. Such an assembly could involve an increase in the inner membrane surface area, thus causing a rise in the respiration rates. An alternative would be that the attachment of respiratory apoenzymes to specific sites on the mitochondrial membrane might cause their activation.

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