AN ULTRACYTOCHEMICAL STUDY OF THE RESPIRATORY POTENCY, INTEGRITY, AND FATE OF THE SEA URCHIN SPERM MITOCHONDRIA DURING EARLY EMBRYOGENESIS

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ABSTRACT

Cytochrome oxidase activity via cytochrome *c*, as demonstrated by the diaminobenzidine procedure, has been employed in this electron microscope cytochemical study to determine the respiratory potency, integrity and fate of the *Arbacia* sperm mitochondrion at fertilization and during early embryogenesis. The sperm mitochondrion remained intact and was intensely positive for cytochrome oxidase activity both during and after penetration into the egg. The mitochondrion remained highly reactive throughout zygote formation, up to the eight-cell stage. The sperm mitochondrion formed many projections and buds in the cytoplasm of immature oocytes, monospermic and polyspermic eggs, and in blastomeres. At all stages of early embryogenesis, close juxtaposition and structural contacts were observed between the highly reactive sperm mitochondrion and the less reactive egg mitochondria.

The results suggest that following fertilization the mitochondrion of the sea urchin spermatozoon retains some degree of metabolic autonomy within the ooplasm. The structural integrity of the paternal mitochondrion is maintained along with a functional respiratory enzyme system (cytochrome c-a_3). The hypothesis that the fertilizing sperm mitochondrion may have some relevance to sea urchin development is discussed.

Deposition of the haploid paternal genome into the egg of complimentary echinoderm species is the consummation of fertilization. A related series of intricate morphogenetic and metabolic events assure karyogamy, ooplasmic activation, and differentiation of the zygote (Runnström, 1933; Monroy and Tyler, 1967; Ohnishi and Sugiyama, 1963; Epel, 1964 *a* and *b*; Piatigorsky and Whiteley, 1965; Fry and Gross, 1970 *a* and *b*; Lundblad, 1954; Stearns, 1974).

The respiratory rate of the sea urchin egg is accelerated at fertilization (Ohnishi and Sugiyama, 1963; Epel, 1964 *b*). This is consistent with the suggestion that metabolically quiescent egg mitochondria become activated with respect to oxidative phosphorylation (Monroy and Tyler, 1967).

The intact sea urchin sperm mitochondrion is deposited into the egg at fertilization (Longo and Anderson, 1968; Anderson, 1968). Its degree of autonomy in the ooplasm, and its influence on metabolic activation of the ooplasm and on embryogenesis and cytoplasmic inheritance are unanswered questions at this time. In this electron microscope cytochemical study, a mitochondrial

enzyme marker is employed to determine the integrity, respiratory potency, and fate of the sea urchin sperm mitochondrion during fertilization and early embryogenesis.

Since sea urchin sperm respiration depends upon the catalytic activity of the cytochrome system (Mann, 1967; Afzelius and Mohri, 1966), the degeneration of membrane would seemingly include swelling, the leakage of vital constituents (e.g., weakly bound cytochrome *c*), ATP, and other cofactors, and an upset in ion-exchange reactions. It is our contention therefore that senescence or disruption of mitochondrial metabolic activity would most likely be marked by a disruption of the respiratory chain sequence and the inability to visualize cytochrome oxidase staining via cytochrome *c* with the diaminobenzidine (DAB) procedure (Anderson et al., 1975).

MATERIALS AND METHODS

Mature male and female *Arbacia punctulata* were used in these experiments. Eggs and sperm were obtained by injecting 0.5 ml of 0.55 M KCl into the perivisceral cavity.

To obtain monospermic fertilization, a drop of "dry" sperm was added to 2 ml of egg suspension in filtered sea water. Eggs were maintained at 22°-24°C and examined at intervals from 3 to 120 min after insemination.

To induce polyspermy, eggs were incubated either for 5 min in 0.5% nicotine in sea water (Colwin et al., 1957) or for 10 min in 0.5% trypsin in sea water (Tyler and Tyler, 1966). The treated eggs were then washed several times in filtered sea water, inseminated with "dry" sperm and examined at intervals from 10 to 120 min.

Samples were fixed for 30 min in a cold (4°C) cacodylate-buffered formaldehyde-glutaraldehyde mixture (Karnovsky, 1965) in sea water, pH 7.3. The cells were rinsed in several changes of 0.1 M sodium cacodylate in sea water and then postfixed in 2% osmium tetroxide in sea water. They were then dehydrated in an ethanol/sea water series and embedded in Epon.

Thin sections were stained with alcoholic uranyl acetate and lead citrate (Venable and Coggeshall, 1965) and examined with Hitachi HU-11C and Siemens 101 Elmiskop electron microscopes.

Demonstration of Cytochrome Oxide Activity via Cytochrome C

Following formaldehyde - glutaraldehyde fixation, eggs were rinsed for at least 8 h in several changes of 0.1 M cacodylate buffer in sea water, and incubated for 1–3 h at 37°C in 0.1% 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Co., St. Louis, Mo.) in Tris-HCl buffer, pH 7.2, according to Anderson et al. (1975). The cells were then washed in several changes of Tris-HCl buffer and postfixed in osmium tetroxide in sea water. Subsequent to alcohol-water dehydration the cells were embedded in Epon.

Sections that were unstained or lightly stained with lead citrate were examined for cytochrome oxidase activity.

Control specimens were pretreated with 0.1 M KCN solution before incubation in complete DAB medium. In some experiments DAB was omitted from the incubation medium.

OBSERVATIONS

The Sea Urchin Spermatozoon

Sea urchin sperm are classified as the "primitive" type because of their simple morphologic form, comprised of a rounded head, membranous mitochondrial complex next to the nucleus, and an elongate flagellum. Our results suggest that fresh and aged sea urchin sperm are structurally intact and possess an intact cytochrome system. The reaction product for cytochrome oxidase is restricted to the internal membranes (cristae) of sperm mitochondria.

Egg Mitochondria

Mitochondria of unfertilized and fertilized sea urchin eggs are reactive for cytochrome oxidase activity with the DAB procedure. The enzymatic reaction proceeded rapidly using endogenous mitochondrial substrates, and exogenous cytochrome *c* was not added to the incubation medium. Cytochrome oxidase activity was inhibited after incubation in KCN solution. In the unfertilized egg the mitochondria existed primarily in clusters, frequently associated with lipid droplets in the cortex of the egg. Reactive mitochondria were dispersed in the cytoplasm of fertilized eggs and zygotes.

Sperm Penetration and Incorporation: General Features

The details of sperm penetration and incorporation are similar to those described for sea urchins (Franklin, 1965; Anderson, 1968; Longo and Anderson, 1968; Longo and Anderson, 1970 *a* and *b;* Longo and Schuel, 1973 *a* and *b*) and for other species (*Hydroides* and *Saccoglossus;* [Colwin and Colwin, 1967]; *Mytilus;* [Longo and Anderson, 1969]; *Urechis;* [Tyler, 1965]; *Spisula;* [Longo and Anderson, 1970 *c*]). The mechanism of sperm penetration into the cytoplasm of the immature oocyte, ripened eggs made polyspermic with nicotine or trypsin, and monospermic eggs is identical. Gamete membrane fusion is followed by the

incorporation of several sperm in fertilization cones of immature oocytes and polyspermic eggs, and the incorporation of a single sperm in control monospermic eggs. In the latter case, formation of a fertilization cone is complete within 2–5 min after insemination, and zygotes cleave and develop in synchrony throughout the eight-cell stage. In polyspermic eggs, the protracted cortical reaction allowed sperm penetration for a period between 10 to 20 min postinsemination and subsequent development was asynchronous. Inseminated immature oocytes were markedly polyspermic and failed to differentiate.

Fate of the Mitochondrial Complex During Fertilization and Early Zygote Development

The mitochondrial complex, flagellum, and nucleus of the fertilizing spermatozoon are consistently incorporated into the egg cytoplasm during fertilization of *Arbacia*. Residual plasma membrane separates from the sperm components which are subsequently exposed to the cytoplasm. The sperm structures are transported as a unit through the egg cytoplasmic cone to the cortical organelle-rich zone. Subsequently, the sperm components separate and the pronuclei differentiate (Figs. 1–3).

Mitochondria of the fertilizing spermatozoa stain intensely positive for cytochrome oxidase activity (Figs. 1 and 2). They are easily recognized and distinguished from the egg mitochondria because of their great size, and more intense reactivity for cytochrome oxidase. Once beneath the fertilization cone, the sperm components separate and the reactive sperm mitochondrial complex is visualized next to yolk platelets and egg mitochondria at varying distances from the sperm pronucleus (Fig. 3). Before and during the phases of pronuclear fusion, sperm mitochondria may be observed either near the site of sperm penetration, at sites between or next to the male and female pronuclei, or at random sites in the medullary or cortical regions of the zygote cytoplasm.

Mitochondria of incorporated sperm are undiminished in the intensity of reactivity; hence the DAB staining of penetrating and penetrated sperm mitochondria is identical. Various mitochondrial profiles are seen. Frequently, intact mitochondria may be seen to surround ribosome-rich portions of the egg cytoplasm or to be juxtaposed to cisternae of the granular or smooth endoplasmic reticulum. With equal frequency, sperm mitochondria formed projections or buds. These mitochondrial projections are equally prominent in the cytoplasm of immature oocytes, monospermic and polyspermic eggs, and in blastomeres up to the four to eight-cell stage (Fig. 4).

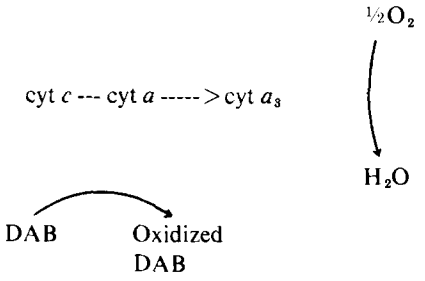
At all stages in early embryonic differentiation, sperm and egg mitochondria made physical contact (Figs. 4–8). In most cases the highly reactive sperm mitochondria were juxtaposed to several less reactive egg mitochondria (Figs. 5 and 6). Serial sections indicate that the outer regions of mitochondrial envelopes were apposed and perhaps confluent (Figs. 5 and 6).

When sperm mitochondria were recognized in the four to eight-cell stage blastomeres, they were always in contact with egg mitochondira. Both egg and sperm mitochondria were intensely positive for cytochrome oxidase activity (Figs. 7 and 8).

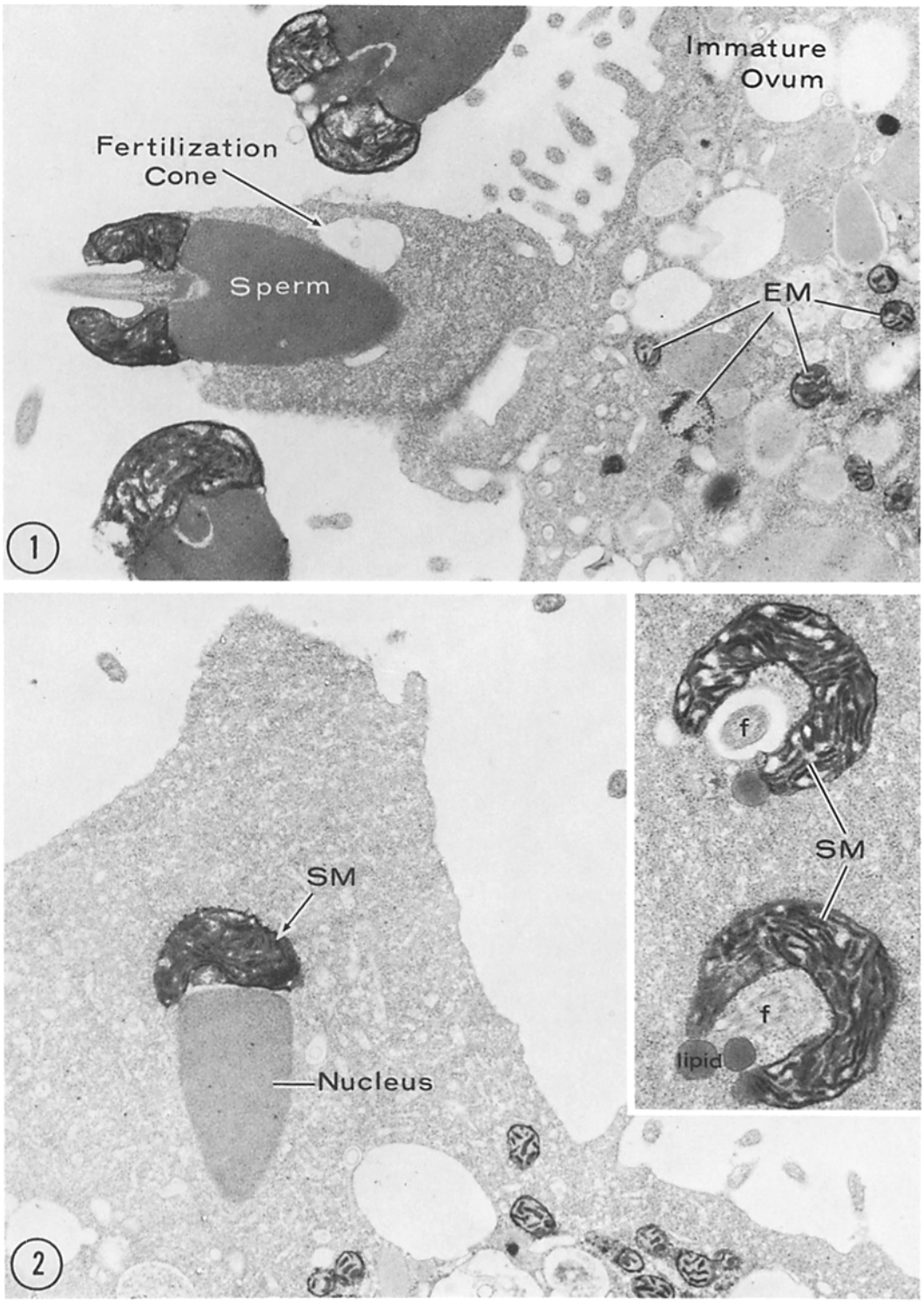
DISCUSSION

Rationale of the DAB Reaction

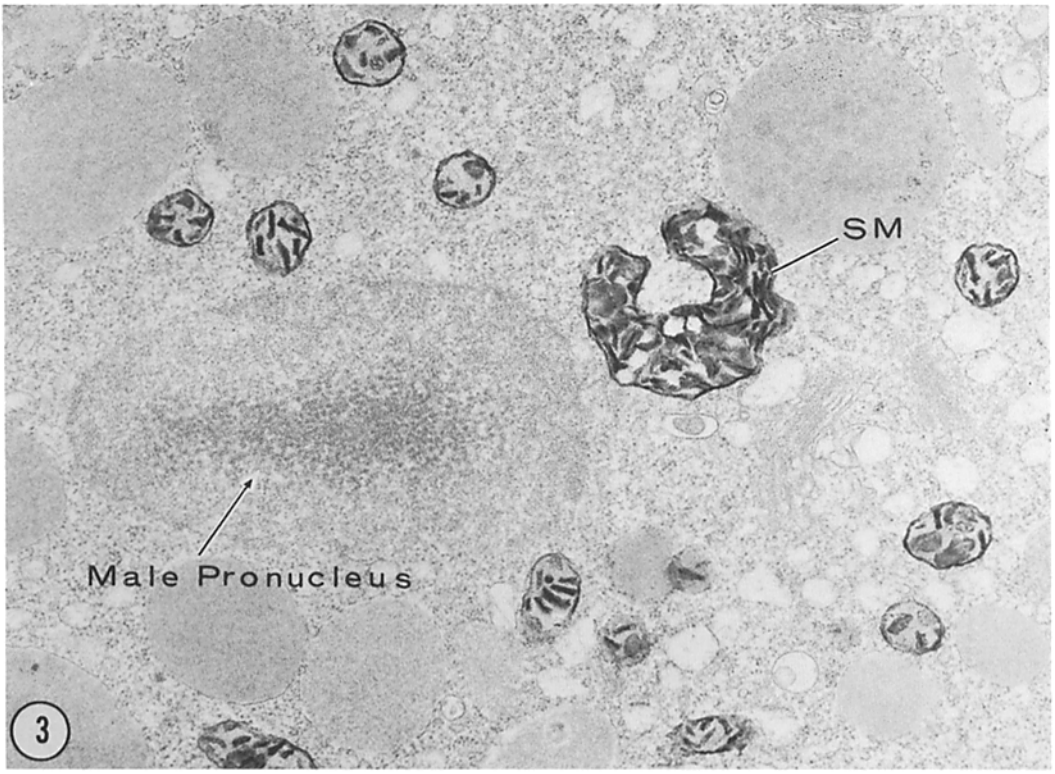
Tissue fractionation studies have established that cytochrome oxidase is a mitochondrial enzyme relegated to the internal membranes. It is also well-established that the DAB reaction may be used to demonstrate cytochrome oxidase activity via cytochrome *c* in the intact mitochondrion in tissue prepared for electron microscopy (Seligman et al., 1968; Novikoff and Goldfischer, 1969; Anderson, 1970; Anderson et al., 1975). Reactivity of this enzyme is inhibited by heat, potassium cyanide and sodium azide. Indeed, the reaction depends upon the integrity of the respiratory chain, with cytochrome *c* acting as hydrogen donor and oxygen at the end of the reaction acting as the electron acceptor. The entire terminal portion of the respiratory chain determines the DAB reaction:



If the reaction were only with cytochrome *c* it would be stoichiometric, producing insufficient amounts of reaction product for visalization. It should be emphasized, therefore, that the DAB reaction demonstrates the triplet (cytochrome *c*-



FIGURES 1 and 2 The incorporation of spermatozoa into fertilization cones of immature ova is shown in these micrographs. The large spermatozoan mitochondrial complex (*SM*) stains intensely positive for cytochrome oxidase activity and is distinguishable from the smaller, less positive egg mitochondria (*EM*). *Flagellum*, (*f*). Fig. 1; × 18,400. Fig. 2; × 17,600. Inset; × 20,000



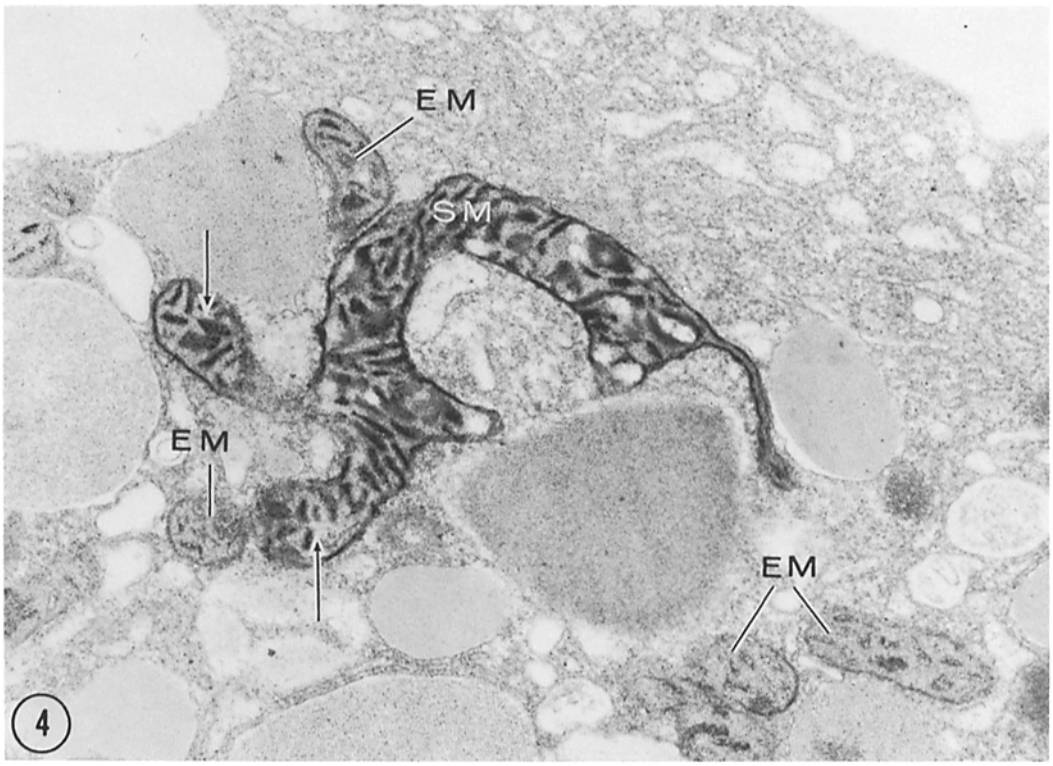
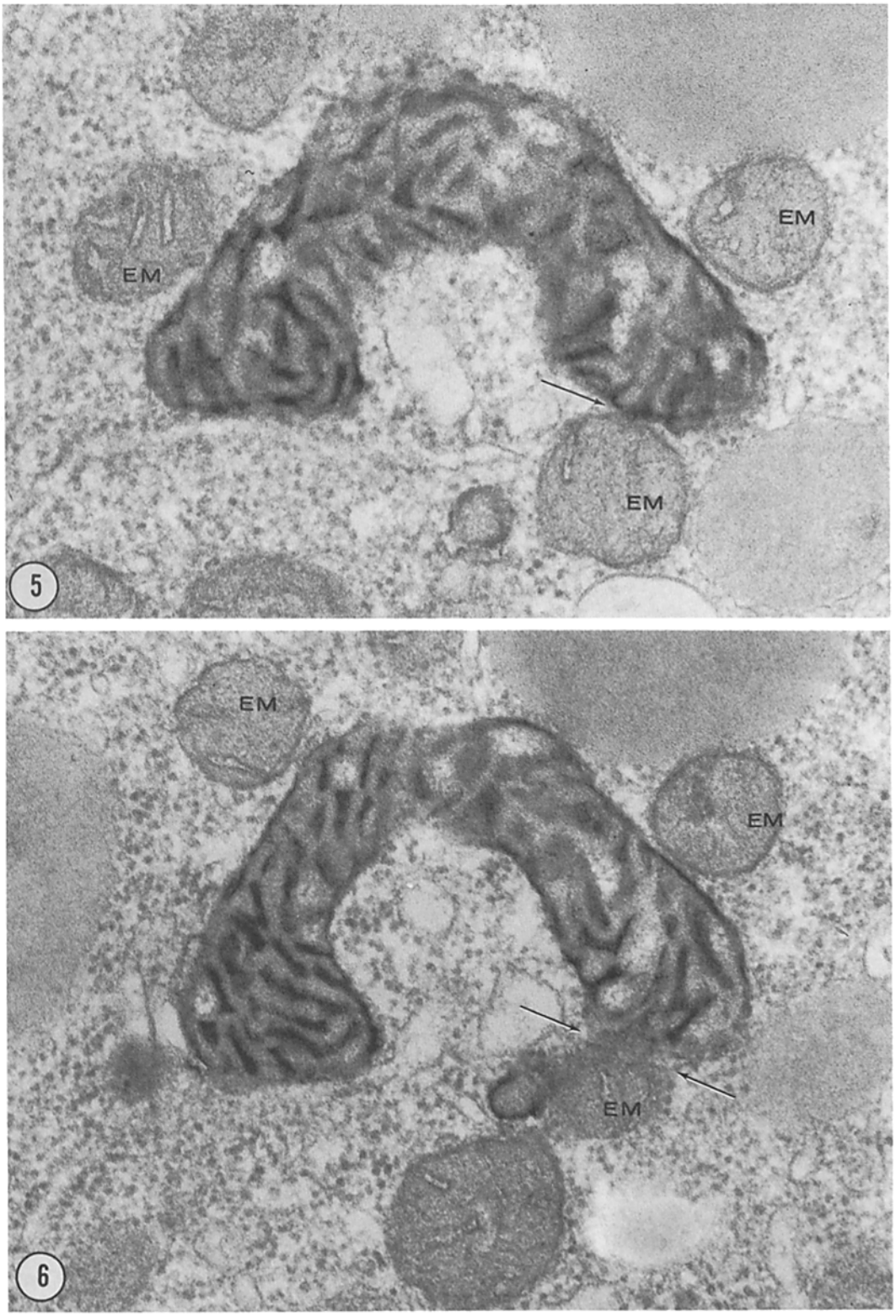
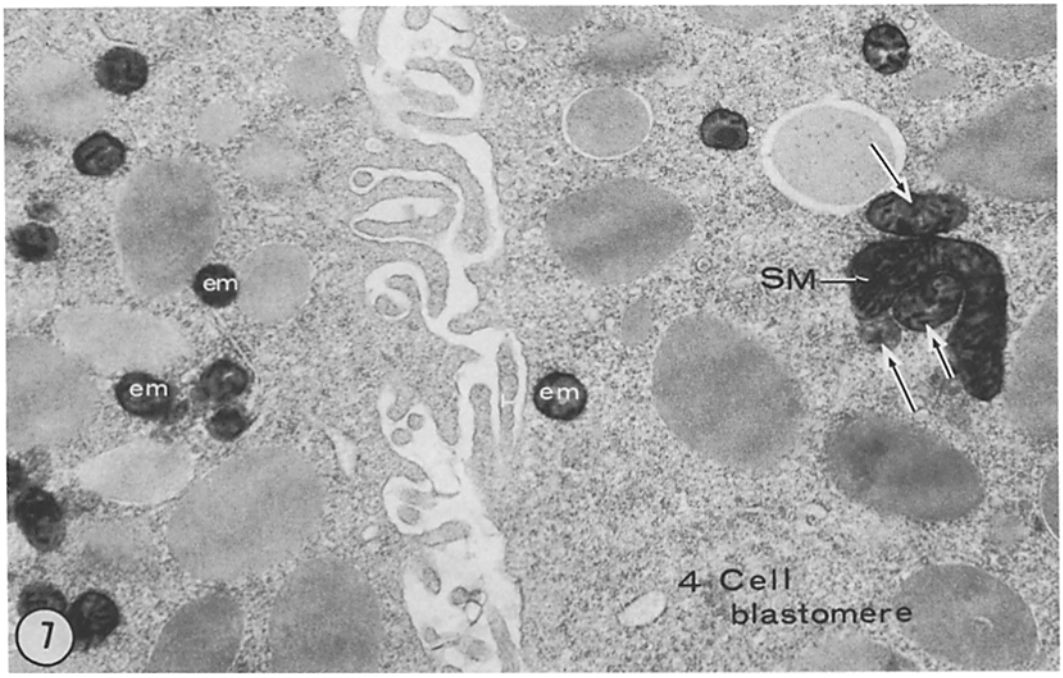


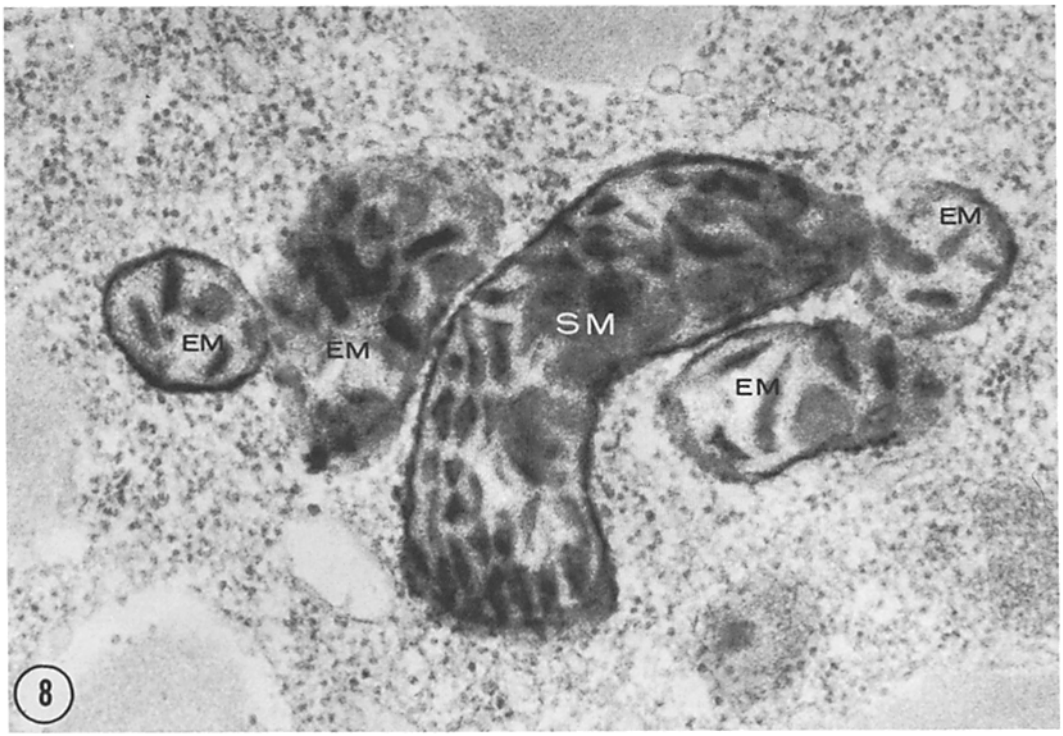
FIGURE 3 20 min after fertilization. At various stages in the differentiation of the male pronuclei, sperm (*SM*) and egg mitochondria may be located at random sites in the ooplasm. Both populations of mitochondria stain positively for cytochrome oxidase activity. × 21,600

FIGURE 4 The marked pleomorphism and budding of the sperm mitochondria (*SM*, arrows) are illustrated in an immature ooctye. The juxtaposition of sperm and egg mitochondria (*EM*) is also observed. × 36,200.



FIGURES 5 and 6 Serial sections through a sperm-egg mitochondrial complex in a nicotine-induced polyspermic egg are illustrated in these micrographs. The envelopes of the intensely reactive sperm mitochondria and less reactive egg mitochondria (*EM*) appear contiguous (arrows). × 60,000





FIGURES 7 and 8 Blastomeres of a 4-cell embryo are illustrated in this micrograph (Fig. 7). A recognizable sperm mitochondrion in one blastomere stains intensely for cytochrome oxidase activity and is surrounded by egg mitochondria (arrows). A serial section through the sperm-egg mitochondrial complex is illustrated at higher magnification (Fig. 8). Sperm mitochondria, (*SM*). Egg mitochondria, (*EM* and *em*). Fig. 7; × 16,500. Fig. 8; × 62,000

a_3) and that the absence or inhibition of any one component of the chain inactivates the reaction and prevents staining. If incorporated sperm mitochondria were to degenerate during early embryogenesis then no cytochrome oxidase staining would be expected. On the basis of these ultracytochemical criteria, therefore, incorporated sperm mitochondria are considered viable.

The Possible Significance of the Sperm Mitochondria During Embryogenesis

Fertilization involves the transmission of male heritable characters to the egg. The amalgamation of haploid paternal and maternal genes results in the establishment of the diploid somatic state and assures continuity of the cell line between generations. Ample evidence supports the expression of both nuclear and cytoplasmic genes during embryogenesis. Albeit maternal expression is more prominent by virtue of the fact that the ooplasm plays host to sperm components. The mitochondria of sperm and egg are of major interest in this regard since they are now well-established carriers of genetic information, capable of self-replication and some autonomy with respect to the nuclear genes (Piko et al., 1967; Nass et al., 1965). Sperm of many species contribute mitochondria to the ooplasm of fertilization. Since the importation of sperm mitochondria in some species is nonexistent (Ursprung and Schabtach, 1965), their overall developmental contribution is in question at this time. In species where one or multiple mitochondria are transmitted into the ooplasm, it is conceivable that both sperm and egg mitochondria may function to support the respiratory metabolism of the early embryo. Because of the large reserve of female mitochondria, however, it would be expected that the maternal contribution would exceed the paternal contribution. Moreover, since sperm mitochondria seem to be relegated to one blastomere, the transmission of most mitochondria to most progeny cells may be entirely through the mother. Indeed, this is true for species where the sperm mitochondrion is excluded at fertilization.

For sperm mitochondria to be important during the early embryogenic state, it is essential to establish a link between sperm mitochondria and metabolic activation of the ooplasm and/or biogenesis of mitochondria, including synthesis of DNA and expression of sperm mitochondrion gene products. Indeed, functionality of the sperm mitochondrion cannot be excluded since important molecular events, particularly template activity, may be occurring at levels below detection with current molecular and cytochemical techniques, or else are masked by events of the egg mitochondria during embryogenesis. There is a great dearth of information concerning the existence of DNA in sperm mitochondria, its function during embryogenesis, and the degree of interplay and dependence of sperm mitochondria with egg mitochondria.

Our studies indicate that a certain degree of metabolic autonomy may be ascribed to the sperm mitochondrion within the ooplasm. Change in shape but no drastic change in structural integrity follows transmission into the ooplasm. Its structural integrity is maintained along with the functional integrity of its respiratory enzyme system. It remains a potent source of cytochrome oxidase activity. The juxtaposition of the sperm and egg mitochondrial systems is indeed transient. But this transient contact may be all that is needed for the transference of key substances between membranes of the contiguous organelles.

The bulk of the evidence suggests that sperm mitochondria are impotent with respect to DNA synthesis and function in the ooplasm. The strongest data in support of this view come from molecular hybridization experiments in frogs that tend to show that the embryonic mitochondrial DNA is derived from the egg rather than the sperm (Dawid, 1972). Dawid's results further emphasize the need to determine the degree of function of the sperm mitochondrion and the degree of interplay between paternal and maternal mitochondria during early embryogenesis. For our part, our results can only suggest that sperm mitochondria within the ooplasm are metabolically very active with respect to their ability to respire. In addition, sperm mitochondria make structural contacts with egg mitochondria and may show relevance not only in the biogenesis of new mitochondria, but perhaps also in other important metabolic functions during sea urchin embryogenesis.

The authors thank Dr. Frank Longo (University of Tennessee Medical Center), Dr. Hans Ris (University of Wisconsin) and Dr. Hewson Swift (University of Chicago) for reading this manuscript.

This research was supported, in part, by Grant M73-109 from The Population Council, Rockefeller Foundation, United States Public Health Service Grant HD-07110, and the University of Chicago Cancer Center

Grant CA 14599-10. Dr. Perotti is a Fulbright-Hays Fellow from the Instituto di Anatomia, Universitá di Milano, Italy.

Received for publication 25 February 1975, and in revised form 25 April 1975.

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