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Embriologia e morfogenesi. — *Effects of Bonellin on fertilization of the eggs of the sea urchin, Sphaerechinus gralaris* (*). Nota di
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RIASSUNTO. — Sono state studiate le alterazioni strutturali nelle uova del riccio di mare, *Sphaerechinus granularis*, provocate dalla *bonellina*, il pigmento verde della *Bonellia viridis*. La *bonellina* altera la struttura della membrana cellulare e di quella nucleare e inibisce la divisione cellulare, lasciando inalterata la divisione del nucleo. Viene discussa l'ipotesi che l'alterazione della superficie cellulare possa interferire con l'organizzazione dei microfilamenti e quindi con la formazione del solco di divisione.

INTRODUCTION

In 1931 Baltzer showed that aqueous extracts of the proboscis of the sea worm, *Bonellia viridis*, were able to change the undifferentiated larvae into males. Besides the effects on the genital apparatus, masculinization is accompanied by the selective inhibition of the differentiation of certain cell lines (e.g. the blood system) whereas other organs, such as the nervous system and the digestive tract, are relatively well developed. The most striking effect, however, is a general inhibition of the growth of the male larvae resulting in animals which are several orders of magnitude smaller than the females (1–3 mm long as compared to the one meter long female). Hence, one may argue that one of the major effects—although possibly not the primary one—of the masculinizing factor(s) is on cell division. In order to experimentally test this hypothesis and in view of the culturing of the larvae of *Bonellia in vitro*, we have decided to use the sea urchin embryo as a test system. Lallier (1955) and Nigrelli *et al.* (1967) demonstrated that these extracts exhibit a strong inhibitory effect on the development of sea urchin eggs. Pelter (1978) recently demonstrated that bonellin, the major constituent of the aqueous extracts of the proboscis of *Bonellia*, has a chlorine structure which is unrelated to chlorophyll (Pelter *et al.*, 1976) and is responsible for the masculinization of larvae of the worm.

In a previous paper (Cariello *et al.*, 1978) we showed that the major pigment of the integument of the animal body is a bonellin derivative which we have named neobonellin, while in the case of the proboscis bonellin itself is

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the major pigment. We have also found that bonellin is at least twice as effective as neobonellin in inhibiting cleavage and development of sea urchin embryos.

It sould be borne in mind that bonellin is mostly concentrated on the proboscis, where the larvae become preferentially attached.

In this paper we report the results of an investigation of the fine structural cellular alteration in bonellin-treated eggs of the sea urchin *Sphaerechinus granularis*. We show that besides interfering with cleavage, bonellin also interferes with the cortical components of the egg (oolemma and cortical granules). Bonellin also appears to damage the organisation of the nuclear membrane. Other cell structures, notably the mitochondria, are not affected by this treatment. Moreover the oxygen consumption of the eggs is essentially identical to that of the controls.

MATERIALS AND METHODS

The isolation of bonellin dimethylester was performed according to the method previously described (Cariello *et al.* (1978)). Bonellin was obtained by hydrolysis of the dimethyl-ester for 40 h with 6 N HCl at room temperature and then extracted with diethyl ether. (Purity 100 % in TLC with different eluents). Oxygen consumption was measured polarographically with a Gilson oxygraph. Eggs of *Sphaerechinus granularis* were collected from a single female for each experiment. Samples containing about 5000 eggs/ml were treated for 10 min. with 0.5 μg/ml of bonellin (9.5×10^{-7}M) in sea water containing less than 0.01 % of Tween 80 (necessary to dissolve bonellin in sea water) at room temperature and under controlled illumination; they were washed twice and resuspended in sea water. Controls (5000 eggs/ml) were treated in the same manner except for the presence of bonellin.

Five min. after insemination the eggs were washed and resuspended in water containing sulfanilamide to prevent bacterial growth. Control eggs were treated in the same manner except for the incubation in bonellin.

For light microscope analysis samples were collected and observed *in vivo* and fixed overnight in Carnoy. The eggs were then transferred to 75 % acetic acid and stained with 1 % orcein in 75 % acetic acid. For electron microscopy the eggs were fixed in 1 % glutaraldehyde in 77 % sea water and post-fixed in 1 % OsO_4, also in 77 % sea water, and the material was embedded in Spurr resin (TAAB). Sections were cut with a Reichert OmU4 ultramicrotome and examined with a Philips 200 electron microscope.

RESULTS

In the bonellin-treated eggs the fertilization membrane is lower than in the controls (Pl. I) and there are fewer surface microvilli. On the contrary, mitochondria and the Golgi complex are unaltered (Pl. II). The nuclear membrane is wrinkled with numerous discontinuities, while the mitochondrial membranes and cristae appear undamaged (Pl. III).

Observations with the light microscope show that in the bonellin-treated eggs the fusion of the pronuclei takes place synchronously with the control eggs. However, the nucleus appears to be swollen in the treated eggs (Pl. IV, *a*, *b* and *c*). When the zygote nucleus enters mitosis the spindle does not differ from the controls, nor is there any detectable difference in the progress of mitosis. Nevertheless no cleavage furrow is formed (Pl. IV, *d e* and *f*).

At the time of the second cleavage, each of the two nuclei of the bonellin-treated eggs divides (and spindles have a normal appearance) but again no cell division takes place. This results in an egg with four nuclei (Pl. IV, *h*, *i*, *l* and *m*). Asynchronous mitoses leading to a single cell with three nuclei have occasionally been observed (Pl. V, *a* and *b*). Similar asynchrony occurs also in later stages.

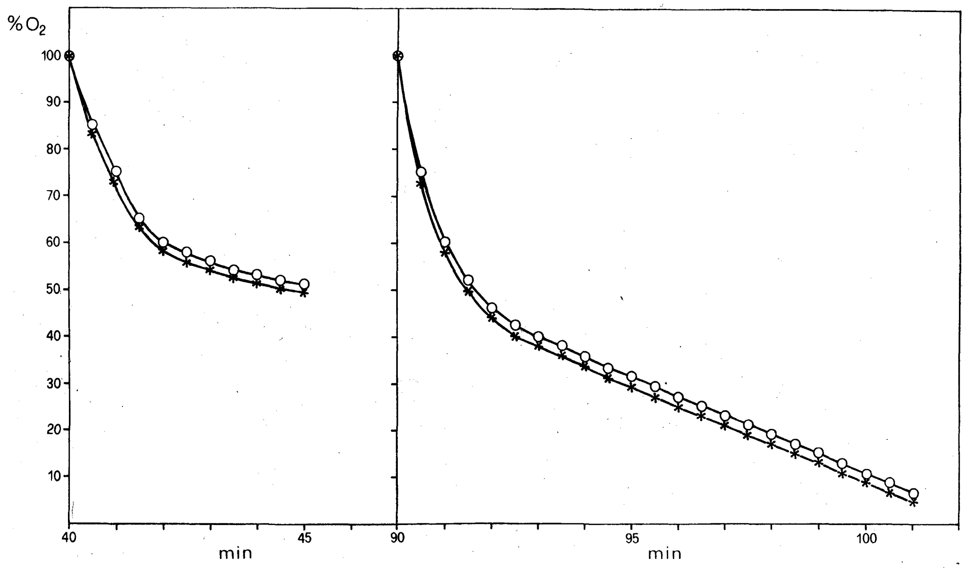

Fig. 1. – Oxygen consumption of fertilized eggs in the presence and absence of bonellin. These data are a single determination from one of ten similar experiments. o—o no bonellin; *—* 9.5×10^{-7} M bonellin.

Finally, it should be mentioned that in the treated eggs the nuclear divisions are always delayed with respect to the controls. The eggs can survive several hours.

Oxygen consumption.

Oxygen consumption is not affected by bonellin treatment. Fig. 1 shows the curve of O_2 consumption of eggs 40 and 90 min. after fertilization with and without bonellin. Also oxygen consumption of the unfertilized eggs is not affected by bonellin.

DISCUSSION

Our previous observations (Cariello *et al.*, 1978) showed that bonellin interferes with cleavage and development of the sea urchin egg. The present work shows that bonellin appears specifically to damage the cortical structures of the egg and the organization of the nuclear membrane. The effect on the cortical structures is shown by the alteration of the organization of the cortical granules and in particular by those images suggesting that some of them open. This results in an altered fertilization reaction, i.e. incomplete elevation of the fertilization membrane and reduced number of surface microvilli. In spite of these alterations, the polyspermy-preventing reaction does not seem to be impaired. Indeed, polyspermic eggs have never been observed.

In addition, the organization of the nuclear membrane appears to be affected by bonellin. This is primarily shown by the numerous discontinuities observed in the nuclear double membrane. Such an alteration may probably account for the greater swelling of the zygote nucleus observed in the case of bonellin-treated eggs.

The normal appearance of the mitochondrial membrane and the mitochondrial cristae is in sharp contrast with the alteration of the nuc'ear membrane in treated eggs. This, together with the fact that O_2 consumption in the bonellin-treated eggs is not affected, suggests that mitochondrial function is not grossly altered.

The most conspicuous effect of bonellin is the suppression of cleavage while nuclear division is permitted to proceed. Observations both *in vivo* and whole mount preparations, and on thin sections at the electron microscope show that the mitotic apparatus is not grossly affected. Assuming that the main target of bonellin is the egg surface, there are two possible interpretations.

The first is that the surface alteration of the egg interferes with the assembly of microfilaments which is responsible for the formation of the cleavage furrow. The alternative interpretation takes into account the hypothesis of Sakai (1968) who suggests that the egg cortex receives a signal from the mitotic apparatus to divide. In the present case, since the mitotic apparatus is unaltered, the absence of furrow formation can be ascribed to the altered membrane structure.

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EXPLANATIONS OF PLATES I–V

Plate I.

Zygote surface showing the fertilization membrane (FM) with perivitelline space (PVS) and microvilli (MV) five min., post-insemination in the control (*a*) ×44160 and in an egg treated with bonellin (*b*) ×57960.

Plate II.

Section of the zygote's surface showing mitochondria (M), Golgi complex (GC) and fertilization membrane (FM) 1 hr after insemination in the controls (*a*) ×44160 and in the treated eggs (*b*) ×25760.

Plate III.

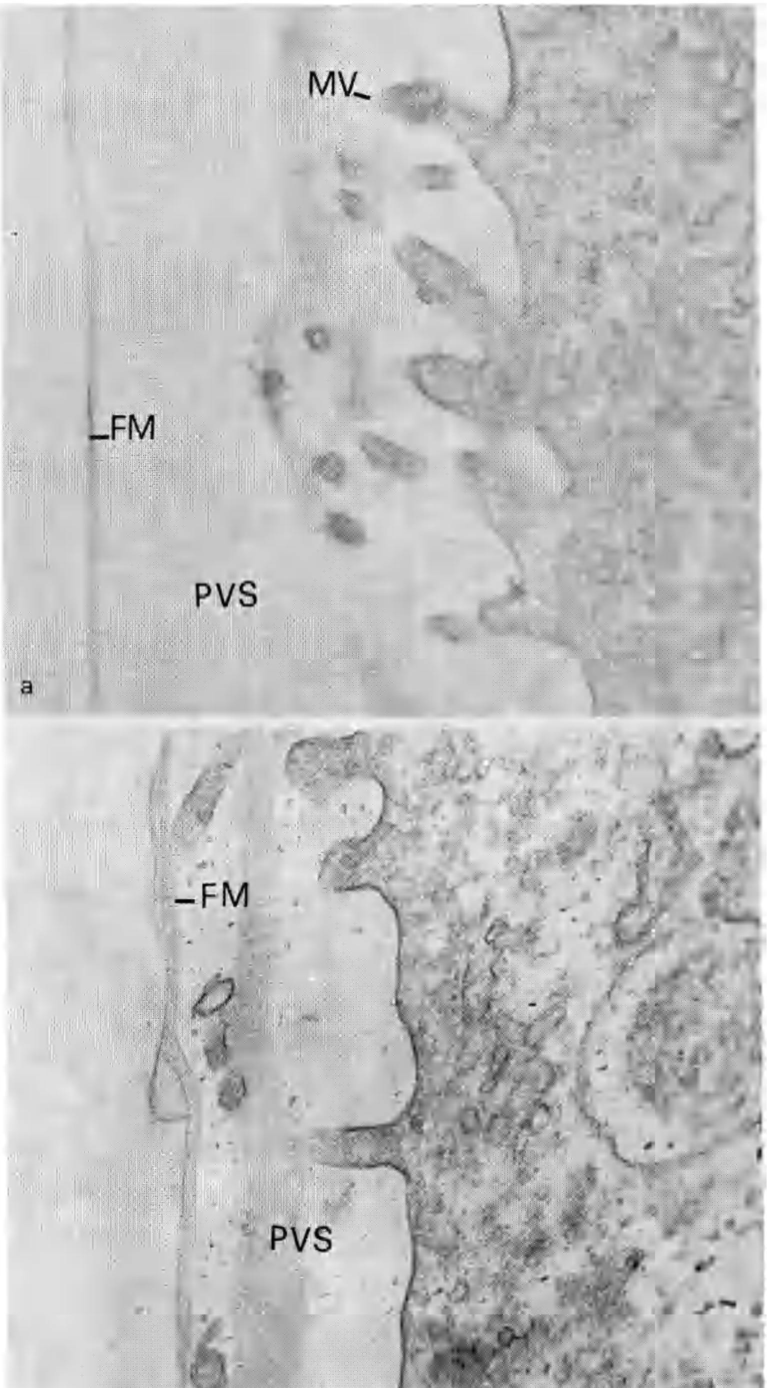
Eggs 1 min after fertilization. *a*) membrane (NM) of the nucleus (NC) in a control. ×41000; *b*) nuclear membrane (NM) and mitochondria (M) in bonellin-treated eggs ×44160.

Plate IV.

Nucleous of zygote 40 min after fertilization. *a*) and *b*) controls ×448; eggs treated with bonellin: *c*) ×448; *d*) anaphase of the first division. ×448; *g*) anaphase of the second division ×448; *l*) interphase after the second division ×448; *m*) interphase after third division ×560.

Plate V.

Bonellin-treated egg. *a*) abnormal anaphase after the first division ×448; *b*) interphase after the second abnormal division ×448; Control eggs *c* and *d* ×560.


MV
FM
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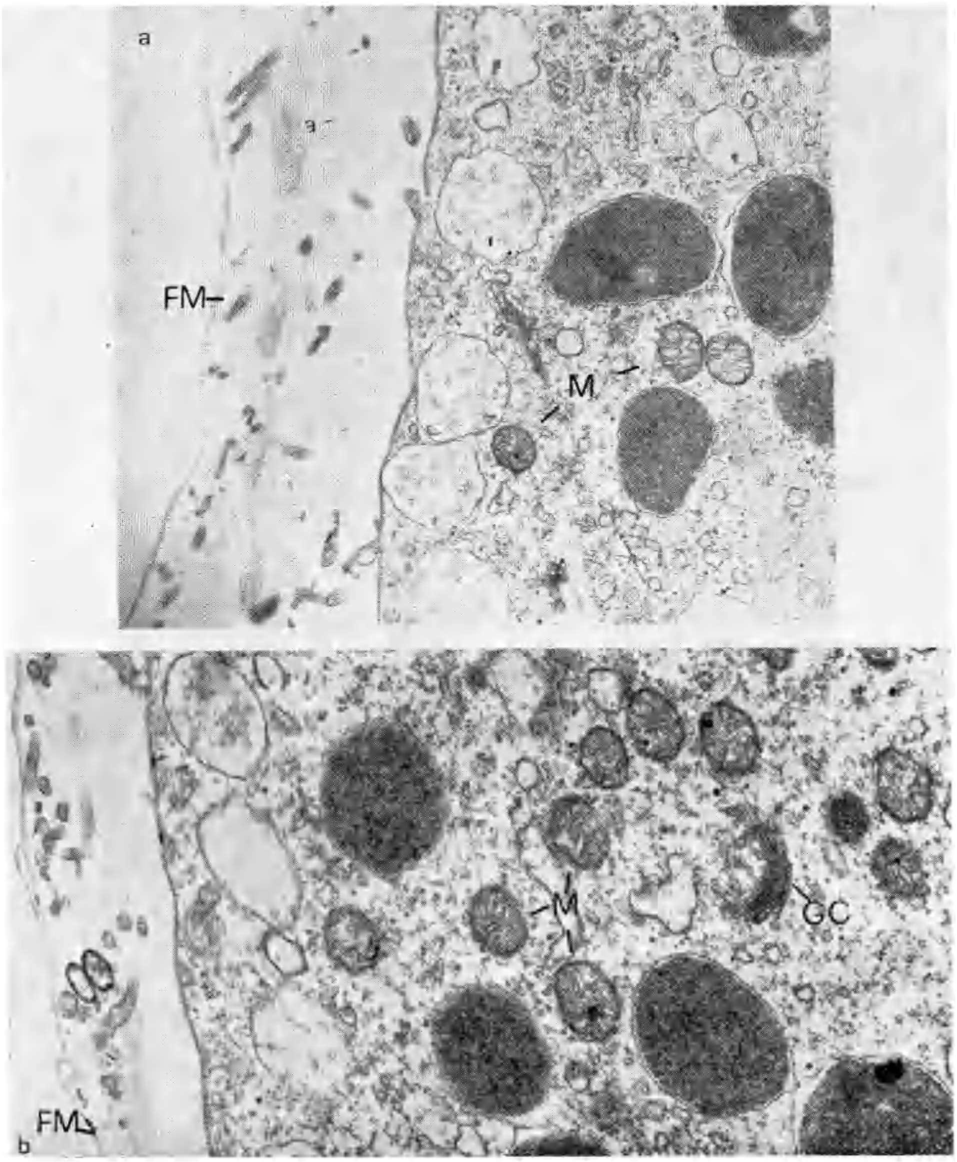
a
FM
PVS
b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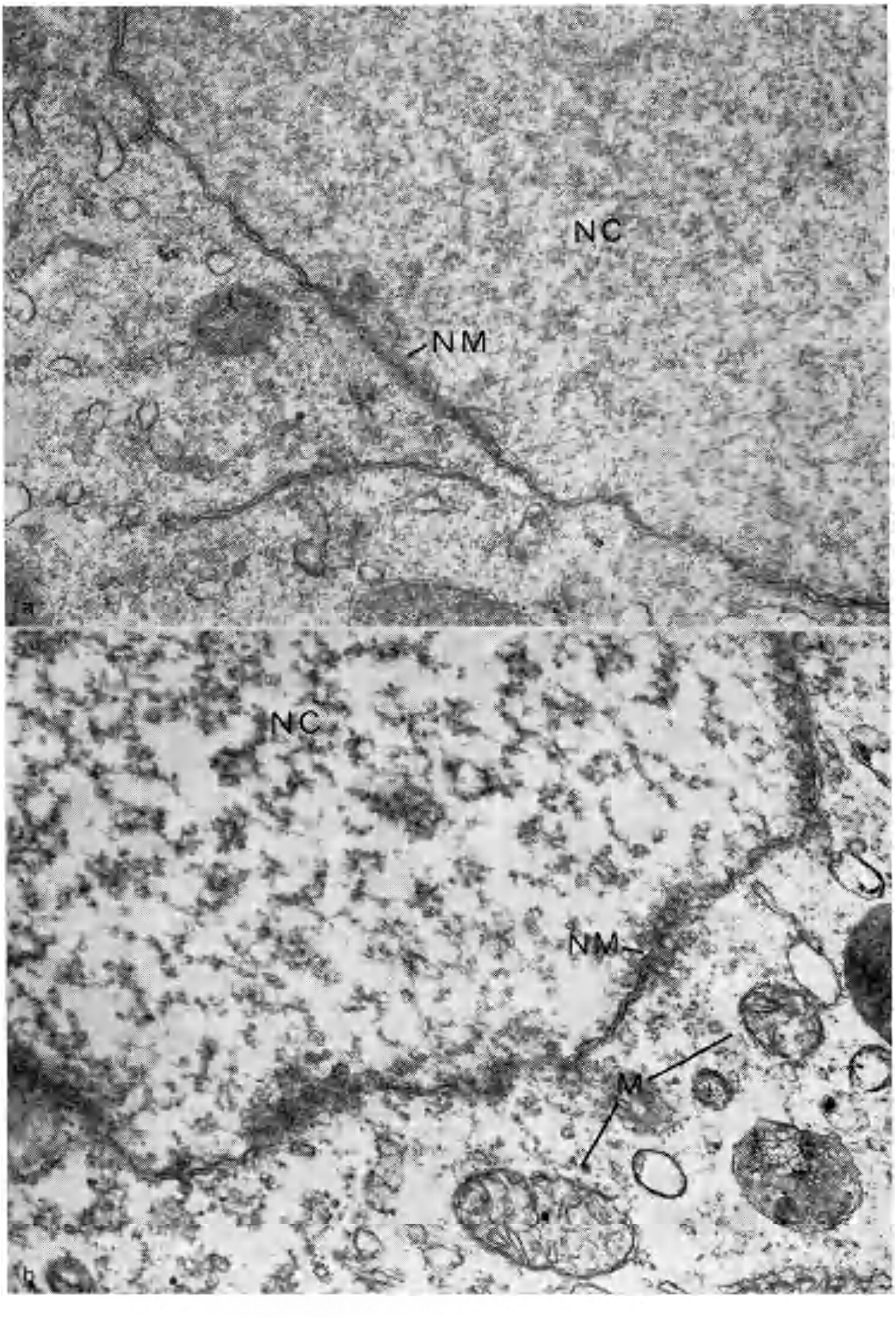
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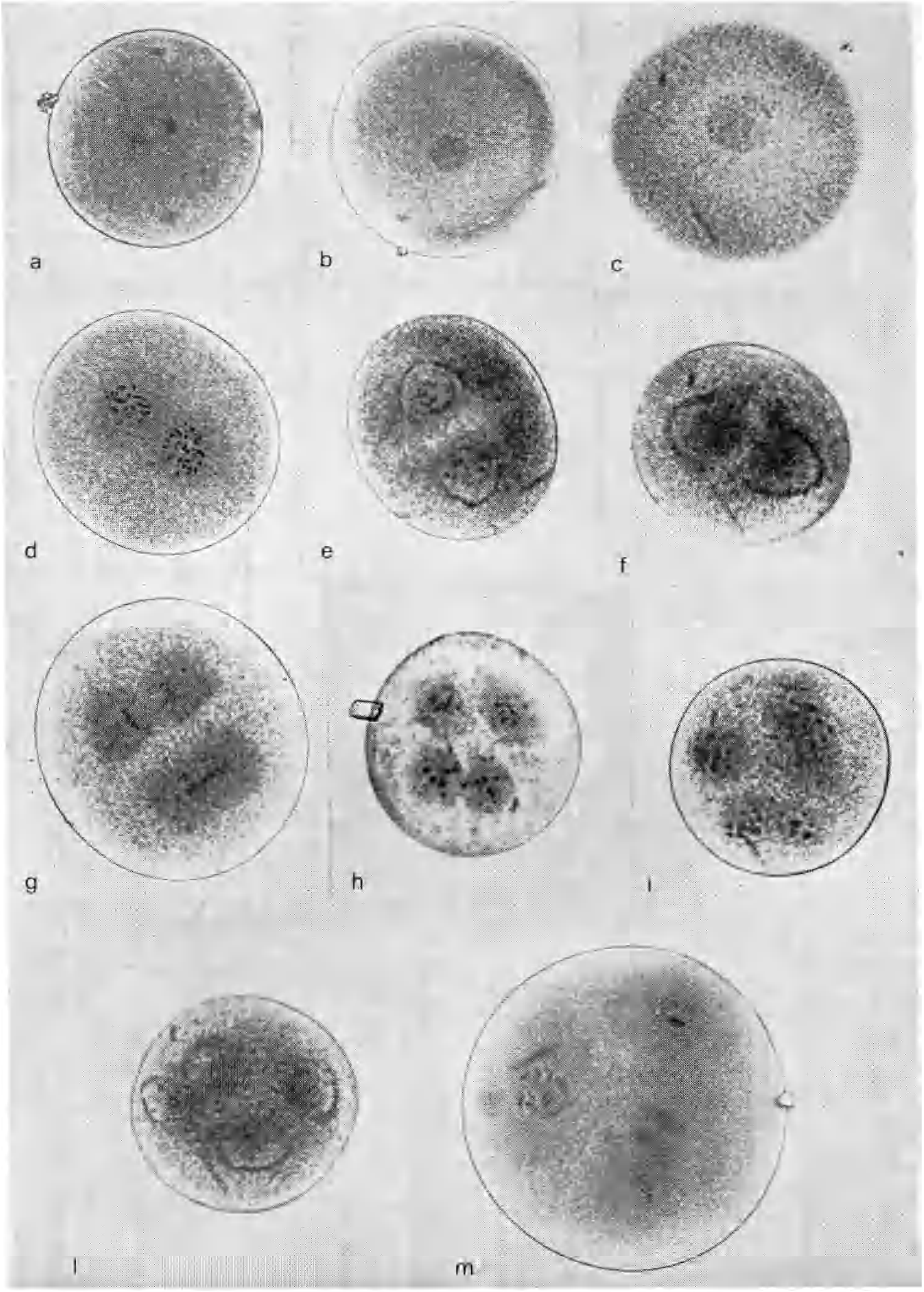
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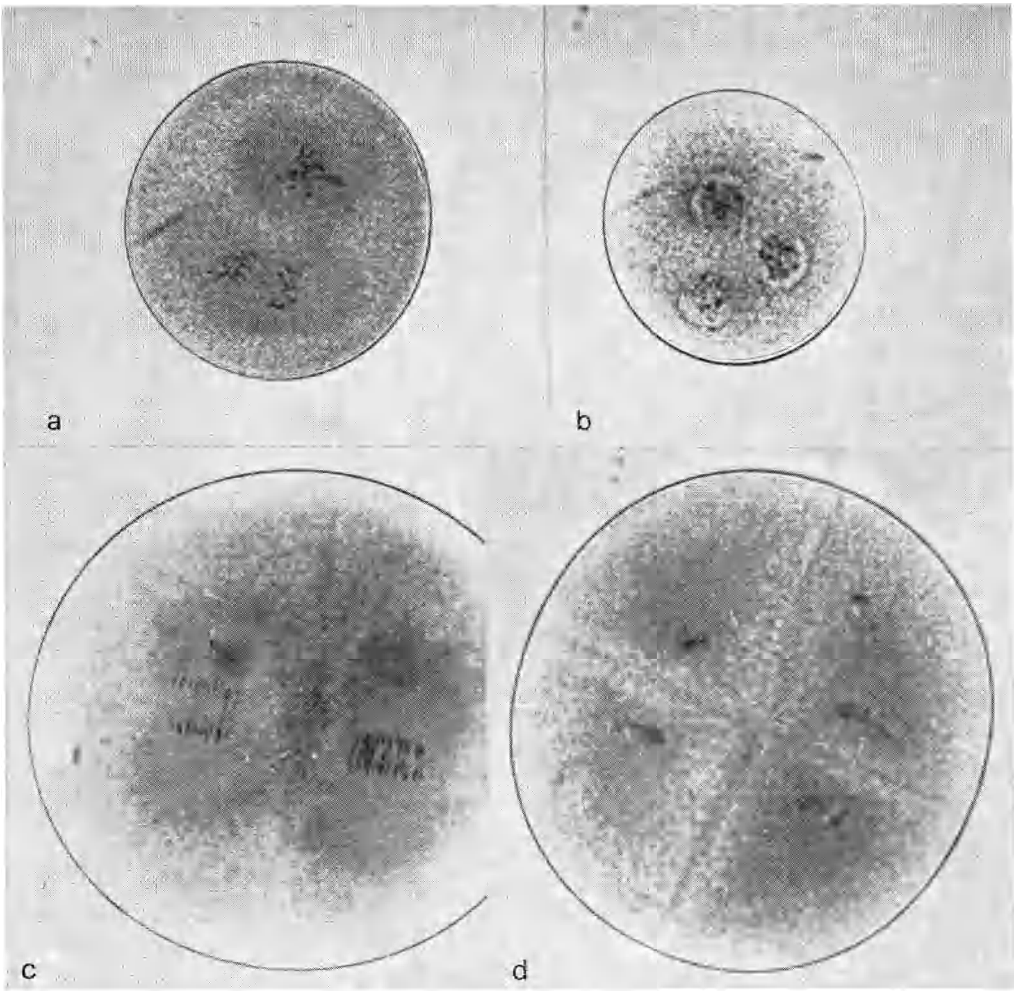
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