Parthenogenetic activation of rhesus monkey follicular oocytes *in vitro*

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SUMMARY

Rhesus monkey follicular oocytes obtained from gonadotropin-injected animals were treated with hyaluronidase and cultured in Whitten's medium. Of 74 oocytes beyond germinal vesicle stage, 57 were activated. Twenty-one of the activated oocytes cleaved one to three times. Behavior in culture and the values of relative Feulgen-DNA content in nuclei of six normally cleaved 6- to 10-cell parthenogenones showed that four resulted from the activation of primary oocytes, one from a secondary oocyte, and one from a primary polyploid oocyte. One parthenogenone with four pronuclei, and two with a single pronucleus (without extrusion of the first and second polar bodies in either case) were also observed.

INTRODUCTION

In recent years, study of the experimental parthenogenesis of the mammalian egg has led to some understanding of the mechanisms involved (reviews: Tarkowski, 1971; Graham, 1974; recent papers: Kaufman & Sachs, 1975, 1976; Kaufman, Huberman & Sachs, 1975; Bałakier & Tarkowski, 1976). Parthenogenesis of the mouse egg has been studied in detail, and this model is particularly useful in elucidating various aspects of fertilization and differentiation. Comparison of data obtained from mouse systems with that from other species, especially primates, can provide important information about the universality of the mechanisms of parthenogenetic activation.

Reports on spontaneous parthenogenetic activation of primate eggs are

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limited. Saglik (1938) reported a 2-cell egg in a gibbon's ovary, presumably the consequence of spontaneous parthenogenesis. Two other reports of cleavage eggs or even a blastocyst in human ovarian follicles (Krafka, 1939; Shettles, 1957) are somewhat questionable, since nuclei are not conspicuous in photographs of the blastomeres or their presence was not stated. It has been recently shown that benign ovarian teratomas in humans originate from parthenogenetically activated ovarian oocytes (Linder, McCaw & Hecht, 1975). Comparison of normal host tissue and teratoma cells using chromosomal banding patterns and isozyme diversity indicate that tumors originate from oocytes after the first meiotic division.

The only report of attempted parthenogenetic activation of primate ova is by Reimann & Miller (1939), who described the polar body extrusion and the beginning of cleavage in a human tubal ovum activated *in vitro* by pricking.

We report here the activation of monkey ovarian oocytes and their subsequent development in a chemically defined medium.

MATERIALS AND METHODS

Experimental animals

Three adult and five juvenile (prepubertal) female rhesus monkeys caged without males were hormonally stimulated using a modification of the technique described by Batta & Brackett (1974). Animals were each given a single daily intramuscular injection of 100 i.u. pregnant mare serum (PMS) (Pregnyl, Organon) for 4 days, then 200 i.u. of PMS for 4 days. Twenty-four hours later a single intramuscular injection of 4000 i.u. human chorionic gonadotropin (A.P.L., Ayerst, or Antuitrin 'S', Parke-Davis) was administered. The injection schedule was initiated on the 3rd day of the menstrual cycle for adult rhesus monkeys, and randomly for the prepubertal animals.

Oocyte recovery

Laparotomies were performed 36 h after administration of human chorionic gonadotropin. The animals were anesthetized with phencyclidine hydrochloride (Sernylan, Bio-Ceutic Laboratories, Inc.), and their ovaries exposed by mid-ventral incision. Strict aseptic conditions were maintained. Oocytes in their cumulus masses were recovered from visible follicles by gentle aspiration into a tuberculin syringe containing 0·1 cc culture medium. Follicles were punctured from the side using a 25-gauge needle inserted bevel down. Aspirates from four or five follicles were placed in a 60 mm plastic Petri dish containing 4 ml of culture medium and, with a dissecting microscope, were searched for oocytes. At this step in the procedure, the pH of the medium was maintained either by covering the contents of the Petri dish with paraffin oil, or by replacing the sodium bicarbonate with 25 mM of N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (Hepes, Sigma Chemical Co., St Louis, Mo.). Oocytes not found

separated from cumulus masses were released by vigorous shaking with a fine needle in 4 ml of medium to which 1 or 2 ml of hyaluronidase solution were added (400 NF/ml, Sigma Chemical Co.).

Oocyte culture

The culture medium used throughout these experiments was Whitten's medium (Whitten, 1971), modified (MWM) by decreasing the pyruvate concentration to 0·25 mM (Cross & Brinster, 1973) and the antibiotics to 80 units/ml for penicillin and 30 μg/ml for streptomycin sulfate, and by increasing the bovine serum albumin to 4 mg/ml (Biggers, 1971).

All collected oocytes were transferred to MWM containing 150 NF/ml of hyaluronidase and incubated for 20–30 min in 5 % CO_2 in air at 37 °C. Oocytes were then washed in MWM three times and exposed to hypotonic MWM (4/5 WM: 1/5 distilled water) for 1 h in the CO_2 incubator (Graham & Deussen, 1974). Finally, each oocyte was placed in a droplet (2–5 μl) of MWM in a 35 mm plastic Petri dish, covered with liquid paraffin oil, and incubated at 37 °C in an atmosphere of 5 % CO_2, 5 % O_2 and 90 % nitrogen. Oocytes from one of the animals were divided after hypotonic treatment into three groups; one was cultured in MWM, one in MWM with 0·04 mM (ethylene dinitrilo)-tetra-acetic acid, disodium salt (Na_2 EDTA) and one in MWM with 0·69 mM-Na_2 EDTA. At selected times, the dishes were removed and the oocytes were examined under bright field and phase-contrast microscopes.

At intervals some oocytes were fixed in 10 % formalin, mounted in egg white, and Feulgen-stained. Measurements of relative Feulgen-DNA (F-DNA) content were performed with a Vickers M 86 scanning cytophotometer at the wave length of 560 nm. Prints of rhesus monkey kidney cells were used as the standard for 2 C F-DNA.

<div style="text-align:center">RESULTS</div>

A total of 86 oocytes were obtained. Of these, only 11 were completely without corona cells after hyaluronidase treatment; the rest had a few cells to several layers of corona cells. No correlation could be made between the number of persisting corona cells and the fate of the eggs in culture. At the time of recovery, 12 oocytes contained a germinal vesicle, and 74 did not.

The ooplasm of freshly recovered oocytes appeared slightly granular but without vacuoles. The diameters of vitelluses of four oocytes without germinal vesicles from one juvenile monkey were 116–120 μm before hyaluronidase treatment; vitelluses of nine oocytes from another juvenile monkey measured 114–130 μm. When a germinal vesicle was present it was round, sharply outlined, and had a distinct single nucleolus. Oocytes were usually surrounded by a perivitelline space that contained small granules (Fig. 1). The perivitelline space was not observed in oocytes in the germinal vesicle stage. Polar bodies were not observed in any of the recovered oocytes.
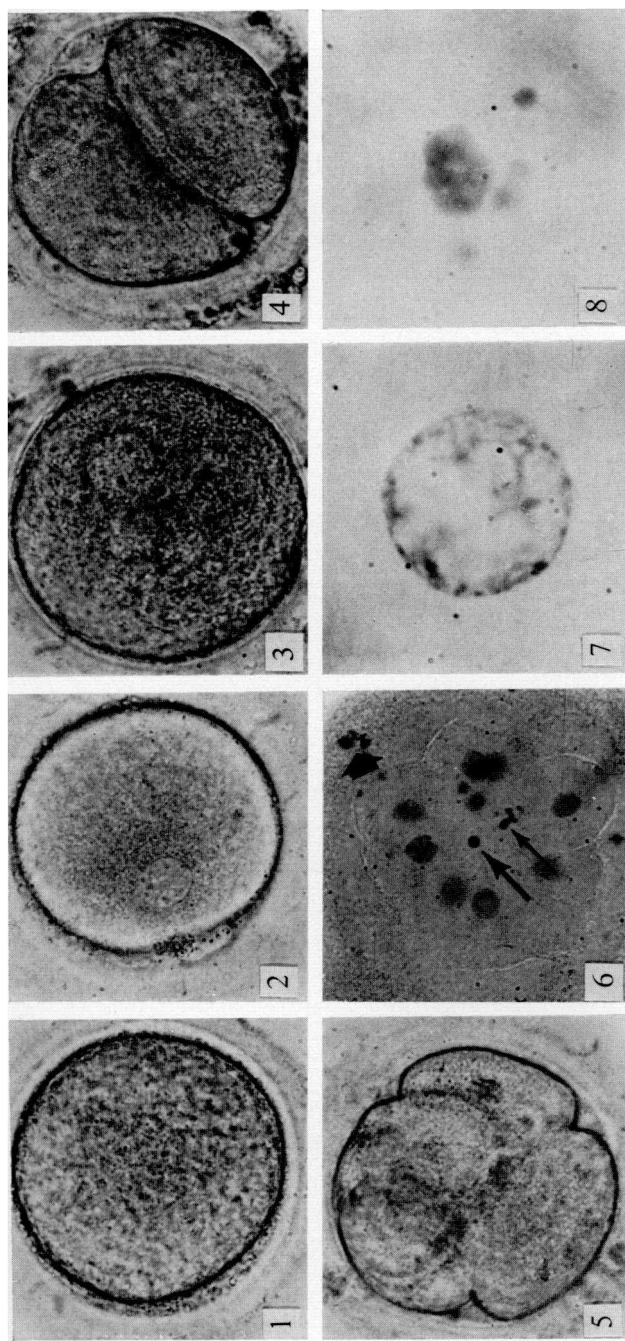
Fig. 1. Follicular oocyte after hyaluronidase treatment and hypotonic shock. Note semilunar perivitelline space with granules. × 320.

Fig. 2. 1-cell parthenogenone after 8 h in culture. Polar body is extruded (lower left) and pronucleus formed. This parthenogenone finally developed into the 8-cell stage. × 320.

Fig. 3. 1-cell parthenogenone with four pronuclei formed (one in the focus plane) after 56 h in culture. × 320.

Fig. 4. Rhesus parthenogenone at the beginning of first cleavage (70 h of culture). When examined 8 h later the cleavage was completed. Granular cells are still attached. × 320.

Fig. 5. 4-cell parthenogenone after 54 h in culture. × 320.

Fig. 6. Feulgen-stained 8-cell parthenogenone, fixed at 48th hour of culture. The picture taken with condensor lowered to visualize blastomere outlines. Nuclei of first (thin arrow) and second (thick arrow) polar bodies are projected on the center of the parthenogenone. Nuclei of several granular cells at the right upper corner (arrowhead). × 320.

Figs. 7 and 8. Nuclei of degenerating, supposedly 10-cell stage parthenogenone, with early (Fig. 7) and advanced (Fig. 8) signs of degeneration. × 1200.

The 74 oocytes obtained from three adult (nine oocytes) and five juvenile (65 oocytes) monkeys and found to have undergone germinal vesicle breakdown were followed in culture. Fifty-seven (six from adult and 51 from juvenile animals) showed signs of activation *in vitro* and 17 did not. An oocyte was considered to be activated *in vitro* when at least one of the following criteria was met: extrusion of one or more polar bodies, formation of one or more pronuclei, or cleavage (Figs. 2–5). Patterns of activation observed in the oocytes are summarized in Table 1. The most frequent patterns involved: (*a*) formation of a single pronucleus with an extrusion of a single polar body (PB + PR, 29 % of oocytes), (*b*) extrusion of a single polar body (PB, 18 % of oocytes), and (*c*) formation of two pronuclei (2 PR, 11 % of oocytes). The signs of oocyte activation appeared at the earliest, 8 h after eggs were cultured, and at the latest after 72 h of culture.

Twenty-one of the 57 oocytes activated were recognized as cleaved on the basis of their appearance in culture or after Feulgen staining (see later). Cleavage was most common among PB + PR oocytes (48 %) and 2 PR oocytes (25 %). Only one of 13 PB oocytes (8 %) cleaved.

Since in one case an activated oocyte was presumed to be fragmented but was found after Feulgen staining to be an 8-cell parthenogenone (Table 2, No. 1), the real number of cleaving parthenogenones might have been even higher (not all of the cultured oocytes could be used for staining). In seven cases, there was either abnormal cleavage (i.e. great differences in the size of resulting blastomeres or erroneous distribution of nuclear material between blastomeres) or fragmentation of 2-, 3- or 4-cell parthenogenones. However, in 13 cases cleavage was normal and resulted in the formation of nine 8- to 10-cell parthenogenones. The remaining four normally cleaved oocytes were arrested in the 2- or 4-cell stage.

In an attempt to improve the efficiency of the development of monkey parthenogenones, mature oocytes from one of the juvenile animals were divided into three groups, each of which was cultured in a different medium (see Materials and Methods). Of nine oocytes cultured in plain MWM, only one developed into an 8-cell parthenogenone. Of 11 oocytes cultured in MWM with 0·04 mM-Na_2 EDTA, four developed into 6- to presumably 10-cell parthenogenones (Table 2, Nos. 5–8), and of 11 oocytes cultured in the presence of 0·69 mM-Na_2 EDTA two formed 8- or 9-cell parthenogenones (Table 2, Nos. 9, 10).

Among eggs obtained from one of the juvenile animals, 12 oocytes in the germinal vesicle stage were found. Of this number only one oocyte underwent germinal vesicle breakdown in culture and subsequently extruded a polar body after 44 h of culture, another oocyte formed a pronucleus, and ten remained at the germinal vesicle stage.

Of the 17 oocytes that were not activated, nine remained unchanged while followed in culture for from 32 h to 7 days. These arrested oocytes looked normal, even on the 7th day of culture, but their ooplasm was slightly more granular

Table 1. *Pattern of activation and further fate in culture of rhesus monkey ovarian oocytes stimulated with hyaluronidase*

Further fate in culture	Not activated	Pattern of activation (numbers of oocytes are shown)								
		PB	2PB	PB+PR	2PB+PR	PB+2PR	PR	2PR	4PR	Unknown
Arrested or degenerated before the first cleavage	17	12	1	11	2	1	2	6	1	
Cleaved		1		10	1	1		2		6

Table includes all oocytes with undergone germinal vesicle breakdown cultured during described experiments. PB, polar body extruded; PR, pronucleus formed.

Table 2. *Cytophotometric measurements of rhesus parthenogenones*

Medium and time of culture	Curriculum and morphology in culture	Identification number	No. of nuclei	Feulgen-DNA content (C)		
				PB I	PB II	Measurable nuclei
MWM, 48 h	PB → fragmentation	No. 1	8	1·7	1·0	1·8, 1·9, 2·0, 2·1, 2·1, 2·1
MWM, 44 h	PB+PR → abnormal cleavage (3 large blastomeres, 1 large in fragments, 2 small)	No. 2	3	×	×	5·4ᵃ, 1·6ᵇ, 1·6ᵇ
	PB+PR → degenerated 7-cell	No. 3	8ᶜ	×	×	1·2, 1·2, 1·5, 1·7, 1·9, 2·0, 2·1, 2·5
	PB+PR → degenerated 2-cell	No. 4	2ᵈ	1·4	×	10·7
MWM with 0·04 mM-Na₂ EDTA, 101 h	PB+PR → 8-cell → 10-cell	No. 5	12ᵉ	1·7	—	1·2, 1·2, 1·3, 1·9, 2·0, 2·1, 2·2, 2·8, 2·8, 2·9, 3·2, 3·9
	PB+PR → 7-cell	No. 6	8ᶠ	1·1	—	3·0, 3·3, 3·4, 3·9, 4·1
	PB+PR → fragmentation	No. 7	6ᵍ	2·1	—	3·0, 3·2, 3·4, 3·8, 6·4, 6·7
	PB+PR → degenerated 8-cell	No. 8	8	1·0	—	2·7, 3·2, 3·9, 3·4, 3·5, 3·8, 3·8
MWM with 0·69 mM-Na₂ EDTA, 44 h	PB+PR → 5-cell	No. 9	8	×	×	1·4, 1·9, 1·9, 1·9, 2·0, 2·7
	PB+PR → 8-cell	No. 10	9	×	×	1·2ʰ, 1·5ʰ, 2·7, 2·8, 2·8, 3·0, 3·4ⁱ, 3·5, 3·9

MWM, Whitten's medium; ×, possible loss after zona removal; PB, polar body extruded; −, polar body absent; PR, pronucleus formed.

ᵃ Large nucleus with slight chromatin clumping, nucleoli visible.
ᵇ Condensed, irregular fragments.
ᶜ Regular blastomeres, very small subnucleus close to each of first four nuclei.
ᵈ Two large nuclei in apposition, with many nucleoli, overlapped by fragments of the third nucleus. All in one large fragment of cytoplasm.
ᵉ Two large nuclei with early signs of chromatin condensation (3·2 C and 3·9 C), two intermediate with advanced chromatin clumping, and the rest definitely degenerated.
ᶠ All nuclei with strands of condensed chromatin, no nucleoli.
ᵍ All nuclei with strands of condensed chromatin, no nucleoli.
ʰ Degenerating nucleus or degenerated anaphase.
ⁱ Degenerated metaphase.

than that of freshly isolated oocytes. Eight oocytes fragmented at different times of culture (from 13 to 106 h).

The pronuclei or nuclei found in parthenogenones were weakly visible, even under phase-contrast. A pronucleus was a round or oval, sharply bordered structure containing several nucleoli; a single, large nucleolus was never seen. Refractiveness of the cytoplasm in the vicinity of the pronucleus was the same as elsewhere in the egg.

Feulgen staining of monkey parthenogenones

To get more information about the pattern of parthenogenetic development, eight non-activated oocytes and 23 activated oocytes or parthenogenones were re-examined after Feulgen staining. One non-activated oocyte that remained arrested in culture for 48 h appeared to be a primary oocyte in metaphase of the first meiotic division; surprisingly, the estimated F-DNA content of metaphase was 5·0 C. Seven other non-activated oocytes, which had been arrested or fragmented in culture, possessed a small amount of Feulgen-positive material contained in remnants of structures that were difficult to identify.

Of the eight stained oocytes that extruded a polar body and remained arrested in a single cell stage (PB), all appeared to be secondary oocytes. Metaphases of secondary oocytes contained 1·5–2·1 C F–DNA. However, another oocyte (Table 2, No. 1) that had extruded a polar body during the first 8 h of culture but later was presumed to have fragmented, was an 8-cell parthenogenone with first and second polar bodies present (Fig. 6). F-DNA content of its six measurable nuclei was 1·8–2·1 C. Around some nuclei, regular ghosts corresponding to the cytoplasm of blastomeres were visible. The polar body showing the distribution of chromatin characteristic for the first polar bodies (PB I) contained 1·8 C F-DNA; the one with the distribution of chromatin characteristic for the second polar bodies (PB II) contained 1·0 C F-DNA. On the basis of the appearance and F-DNA content of its polar bodies this parthenogenone was classified as a possible haploid, originating from activation of a normal secondary oocyte.

Fourteen oocytes that extruded a single polar body and formed a single pronucleus (PB + PR) were stained. Four of them, which subsequently were arrested or fragmented in culture, showed the presence of metaphase II or disseminated chromosomes after staining.

One oocyte that had extruded a single polar body and formed what appeared to be a single pronucleus was found to contain two extended, normal-looking pronuclei, each with several nucleoli, when fixed after 106 h of culture. The F-DNA contents of the pronuclei were 2·4 C and 2·8 C. The polar body contained 1·8 C F-DNA and had chromatin distribution characteristic for the PB I. The presumably single pronucleus of this parthenogenone was barely discernible in culture, and the presence of an overlapping pronucleus, therefore, may have been overlooked.

Nine remaining stained parthenogenones of this group cleaved in culture

at least once, and results of staining essentially confirmed this observation (Table 2). Two degenerated parthenogenones (Nos. 2 and 4) showed obvious abnormalities in the distribution of nuclear material between blastomeres. Degenerative changes, namely the formation of strands of chromatin, chromatin clumping, and disintegration of nuclei (Figs. 7 and 8) were also evident in the parthenogenone No. 5 (Table 2). On the basis of its appearance and the analysis of its F-DNA content, this parthenogenone was considered to contain ten cells, with one nucleus in, or nearly in, the G_2 phase disintegrated into three fragments. Another nine nuclei contained 1·9–3·9 C F-DNA. The only polar body present was PB I.

Three other cleaved parthenogenones of PB + PR pattern (Nos. 6, 7, and 8) fixed within zonae pellucidae at 101 h of culture also showed the presence of the single polar body with chromatin distributed characteristically as for PB I. Two of them contained from approximately 3·0 C to 4·0 C F-DNA per nucleus. The third one, in the 6-cell stage, possessed nuclei with 3·0–6·7 C F-DNA per nucleus.

Three cleaved PB + PR parthenogenones fixed after 44 h of culture were essentially in the 8-cell stage. In one of them (No. 10) one nucleus was probably disintegrated into two parts or was in an unusual anaphase stage. Another seven nuclei of this parthenogenone contained 2·7–3·9 C F-DNA. In two others (Nos. 3 and 9) nuclei contained 1·2–1·4 to 2·5–2·7 C F-DNA per nucleus. Unfortunately polar bodies of these parthenogenones were lost during fixation after zona removal.

Five oocytes that were at the germinal vesicle stage at the beginning of culture were also stained after 44 h culture. One that developed a single structure resembling a pronucleus with many nucleoli contained 4·6 C F-DNA. Another one that underwent a germinal vesicle breakdown and extruded a single polar body in culture contained a metaphase II with 2·0 C F-DNA.

The F-DNA content of three arrested primary oocytes was also determined; the oocytes were in the germinal vesicle stage after 44 h in culture. The values obtained (3·3, 3·6 and 3·7 C) suggest that the distributional error of F-DNA measurements in monkey parthenogenones is not significant.

DISCUSSION

The presence of blastomeres with nuclei containing relatively balanced DNA contents, the increase of the total DNA content in an egg, and ultrastructural observations (Vorbrodt *et al.* 1977), all show that some rhesus ovarian oocytes can undergo early parthenogenetic development *in vitro*.

The most common pattern of activation of oocytes we observed was the formation of a single polar body and single pronucleus. The analysis of four such cleaved parthenogenones stained with Feulgen method revealed that they developed from the activated primary oocytes. It is difficult to establish from

our material whether the pronucleus was formed directly from the retained chromosomes of metaphase I or after the extrusion of PB I maturation continued until metaphase II, and pronucleus was formed by the suppression of PB II. The former possibility is more likely because the appearance of pronucleus occurred within the first 8 h of the culture. Also, in some parthenogenones such pronucleus subsequently disappeared and typical metaphase II was observed after Feulgen staining. Present results thus suggest that, when isolated after germinal vesicle breakdown, monkey primary oocytes can undergo the first meiotic division and then pronucleus formation, DNA synthesis and cleavage *in vitro*. This pattern of activation could lead to the formation of diploid chimaeras or diploid heterozygotes. A similar mechanism might be involved in the formation of benign ovarian teratomas in humans (Linder *et al.* 1975). Our data, in addition, indicate that primary oocytes obtained from adult animals can develop parthenogenetically and that the breakdown of the germinal vesicle is necessary for *in vitro* activation of such oocytes. Early mouse parthenogenones arising from primary oocytes have also been observed (Abramczuk & Sawicki, 1975); however, in the case of the mouse oocytes, the only polar body had a nuclear morphology characteristic of PB II and contained 2 C F-DNA.

The F-DNA content of 2·1 C in PB I and the F-DNA contents of much more than 4·0 C in two nuclei of the parthenogenone No. 7 suggested that it could have developed from a primary, polyploid oocyte. The drop of F-DNA contents below 4·0 C in some nuclei of this parthenogenone can be attributed to degenerative changes. The finding in our material of one primary oocyte in metaphase I containing 5·0 C F-DNA, as well as the description of binuclear, and presumably polyploid, giant oocytes in rhesus monkey (Thompson & Zamboni, 1975), also support such an explanation. Another possible explanation, that the F-DNA content in two nuclei of the same parthenogenone was elevated above 4·0 C as a result of endoreduplication of DNA, seems not to be likely, since PB I contained 2·1 C F-DNA. It is generally believed that PB I cleaves or degenerates soon after extrusion but never synthesizes DNA.

Endoreduplication, however, probably occurred in two other 8-cell parthenogenones of the PB + PR pattern (Nos. 3 and 9) that possessed nuclei with F-DNA contents in the range of almost 1·0 C to considerably more than 2·0 C. Definite recognition is in this case impeded by the fact that polar bodies of these parthenogenones were lost after removal of zona pellucida.

Almost all oocytes that extruded a single polar body but did not form a pronucleus appeared to be secondary oocytes in metaphase II. Only one oocyte (No. 1) of this pattern was really activated and developed into a haploid parthenogenone of the 8-cell stage. These observations strengthen our feeling that at the start of culture the vast majority of oocytes were primary oocytes that had undergone germinal vesicle breakdown. In culture some of them completed maturation, whereas others began parthenogenesis without completion of meiosis.

Other unusual forms of the parthenogenetic activation of the rhesus oocyte included the formation of four pronuclei in one egg, and of a single pronucleus in two other eggs, without polar body extrusion in either. To our knowledge, such patterns of oocyte activation were only hypothesized by Austin (1961). Unfortunately, only one parthenogenone with a single pronucleus could be examined more closely. A value of 4·6 C F-DNA in its pronucleus suggests that it was the result of some DNA synthesis. That it resulted from the degeneration of a polyploid oocyte seems unlikely because the pronucleus looked normal.

Activation or fertilization of rhesus ovarian oocytes *in vitro* is very difficult (Suzuki & Mastroianni, 1968; Zamboni, 1972), possibly due to atretic changes in the majority of follicular oocytes in normally cycling monkeys. Several factors contributed to our successful activation of rhesus ovarian oocytes and the promotion of their development *in vitro*. The three most important, in our opinion, were the quality of the oocytes recovered after hormone stimulation, the correct culture conditions, and the maintaining of proper pH during isolation procedures. The importance of culture conditions is emphasized by the fact that the addition of EDTA to WM improved cleavage. EDTA has been recently shown to facilitate successful growth of mouse 1-cell embryos *in vitro*, probably by chelating some heavy metal ions (Abramczuk, Solter & Koprowski, 1977).

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