ACTION OF PROGESTERONE AS A CLEAVAGE INHIBITOR OF RABBIT OVA IN VITRO

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Summary. The manner whereby progesterone acts as an inhibitor of cleavage of mammalian ova was studied using rabbit ova in vitro.

Progesterone blocked cleavage when it was present in the culture medium at concentrations of 10 µg/ml or more, and exerted this same effect on all stages up to the morula. The growth of blastocysts was not affected. The action was reversible: ova resumed cleavage within 2 to 3 hr after removal to progesterone-free media. The reversal was time-specific and was used to phase the subsequent cleavage of ova inhibited for varying periods of time. The inhibition was overcome by increasing the concentrations of amino acids or of the serum component in the medium. Supplementing the progesterone media with oestradiol did not reverse the inhibition.

Autoradiographs of sections of ova that were kept in 14C-labelled progesterone for 8 hr, showed silver grains concentrated on the surface of the ovum and the zona pellucida.

It is concluded that progesterone blocks cleavage by limiting the supply of protein or amino acids and thus inhibiting protein synthesis within the ovum. Presumably this is done by the progesterone aggregating on the surface of the cell or its protective coating.

The inhibitory action appears to be timed in relation to some event that precedes mitosis by an hour or two. It is therefore suggested that the protein synthesis involved is related to formation of the mitotic apparatus or to chromosome condensation.

INTRODUCTION

That progesterone had a 'toxic' effect on development of early mammalian embryos grown in vitro was observed by Whitten (1957). Working with mouse ova from the eight-celled to the blastula stage, he noted that development was inhibited by concentrations of 4 µg/ml and above, and that blastulae were more sensitive than earlier stages.

Because pregnancy serum is one component of the media used in attempts to culture rabbit embryos and because such serum contains progesterone, which according to some reports is in concentrations approaching the inhibitory level (Forbes, 1953), it became necessary to study the effect of progesterone on rabbit ova of different ages grown in vitro and clarify the nature of any inhibition observed.
METHODS AND MATERIALS

Ova were flushed with physiological saline from the oviducts or uteri of female New Zealand White rabbits at different times post coitum and collected in large sterile covered watch-glasses. They were transferred, via capillary pipettes, to smaller watch-glasses (25 mm diameter) containing culture medium F10 (Ham, 1963) with 10% normal rabbit serum. Progesterone (crystallized; Calbiochem Co.) dissolved in 95% ethyl alcohol was added to the medium in desired concentration. The ova were kept at 37°C in a water-jacketed incubator provided with a 5% CO₂ atmosphere and high humidity. For autoradiography, ova were sectioned at 5 μ and stained with nuclear fast red.

RESULTS

Rabbit ova were removed from the oviducts 15 hr post coitum as single cells, 7 to 10 hr before the first cleavage would occur. They were subjected to media containing different concentrations of progesterone from 0·001 μg/ml up to 1 mg/ml. As shown in Text-fig. 1, cleavage was inhibited with fair reliability only when the concentration was 10 μg/ml or better.† When progesterone is present as 7 μg/ml of the medium the rate of division is slowed so that ova in this medium have fallen one cleavage behind the controls in progesterone-free medium by about 36 hr post coitum.

When oestradiol in concentrations from 0·001 μg/ml to 1 μg/ml, was included in media containing 10 μg/ml of progesterone, rabbit ova grown therein failed to cleave. Oestradiol, therefore, in these concentrations, does not overcome the effect of progesterone.

In addition to the single cell stage, rabbit ova of two, four, eight and sixteen cells, morula stages, and 5-, 6- and 7-day-old blastocysts were subjected to a medium containing 10 μg/ml of progesterone. All the early cleavage stages, including the sixteen-cell stage, were immediately blocked when the ova were put into the medium during the early phase of the cleavage. If subjected to progesterone when the next cleavage is imminent, the cells continue through that cleavage and then stop. Twelve blastocysts in progesterone-containing

* The 1 mg/ml concentration is not reliable because some precipitation of the hormone occurs.
† As a solvent for progesterone, ethyl alcohol was present in the medium at a maximum concentration of 0·025% by volume. An experiment designed to determine if the alcohol was exerting any toxic effect showed that cleavage still proceeded normally in vitro when the alcohol concentration exceeded that amount by twenty times. It was therefore ignored in the subsequent experiments.
media grew as well as the controls in normal media. Apparently rabbit blasto-
cysts are not sensitive to this concentration of progesterone, an observation
contrary to that of Whitten (1957) on mouse blasto-cysts. Morula stages vary
in their response to this hormone. Of ten morulae tested, six continued to grow
in the presence of progesterone while the other four did not.

Single-celled ova, removed from the mothers at various times post coitum and
immediately put into progesterone medium (10 μg/ml), were used to determine
the relationship of the inhibition to the time of the first cleavage. It may be
seen from Text-fig. 2, that cleavage is regularly inhibited only when the ova
are put into a medium containing progesterone within 22 hr after mating. A
few ova cleaved after transfer at this time, or as early as 17 hr p.c., but none
cleaved after transfer less than 17 hr p.c. Lewis & Gregory (1929) reported
that rabbit ova undergo the first cleavage between 22 and 25½ hr p.c. Inhibi-
tion is achieved therefore when the ovum is subjected to progesterone an hour
or two before it has initiated the programme sequence that leads to mitosis.

That ova will continue through cleavage if they are near that stage when
put into progesterone medium shows that this hormone, in the concentration
used here, does not kill them directly. The inhibition is removed by simply
removing the ova from progesterone into normal medium. Twelve single-celled
ova were kept in progesterone medium for 8 hr after the controls in normal
media had cleaved. Of these, ten underwent regular cleavage up to the morula
stage after return to normal medium. Using this method it has been possible to
keep ova in the single-cell stage until 48 hr post coitum (therefore, about 24 hr
past the expected time of the first division), and to initiate cleavage thereafter.
At least two changes of progesterone are necessary to achieve this prolonged
blockage of cleavage. Ova left in the same progesterone will usually start to
cleave after about 24 hr, presumably because of some denaturation of the
hormone.

It is possible to block and re-start cleavage repeatedly by transferring the
ovum to media with and without progesterone. In one experiment, two single-
celled ova were inhibited for 12 hr, then returned to progesterone-free media
for three cleavages (to reach the eight-cell stage), and again inhibited with
progesterone for 12 hr, after which cleavage was resumed to the morula stage
in normal media. In another case the same process permitted cleavage of three
ova to be halted at both the one- and four-cell stages with subsequent normal
development.
The observation that inhibition of cleavage of rabbit ova, related to the presence of progesterone, is time-dependent and can be reversed, led to the speculation that phasing of cleavage might be achieved by the use of this system. Ova, inhibited for varying periods of time, were removed from progesterone and the time of first cleavage carefully noted. In one experiment ova inhibited at 15, 18 and 21 hr respectively, all cleaved within a 1 hr period, approximately 2\(\frac{1}{2}\) hr after being returned to normal medium. Again, in two groups of three ova each, cleavage occurred 3 hr after removal from progesterone within a 1-hr period, one group having been inhibited for 14 hr, and the other for 10 hr, beyond the normal cleavage time of controls. In both cases cleavage appears to have been phased and to have occurred about 2\(\frac{1}{2}\) to 3 hr after release from inhibition. In mammalian somatic cells the period just preceding mitosis, the G2 period, is a time of protein synthesis. If mammalian ova follow the same pattern, the inhibition of cleavage by progesterone may be due to some effect of the hormone on protein synthesis. Increasing the protein-contributing factors of the media might therefore be expected to overcome the inhibition.

Ova were put into progesterone-containing medium that was reinforced with amino acids up to the levels found in uterine milk by Gregoire, Gongsakdi & Rakoff (1961) namely: alanine (6-4 \(\times\) 10\(^{-2}\) g/l), glutamic acid (1-19 \(\times\) 10\(^{-1}\) g/l), glycine (2-68 \(\times\) 10\(^{-1}\) g/l), serine (1-04 \(\times\) 10\(^{-1}\) g/l) and threonine (4 \(\times\) 10\(^{-2}\) g/l). Of ten ova so tested, all initiated cleavage within the expected time range and continued uninterrupted into the morula stage. In the same way ten ova were kept in progesterone media where the serum component was raised to 30%. Cleavage was not inhibited in any of these ova. It appears that when the essentials for protein synthesis or supply are in high concentration, progesterone (at the concentration used) is unable to block cleavage of the mammalian ovum.

On the supposition that progesterone might interfere with the supply of essential amino acids or proteins to the ovum, it was decided to use autoradiography in an attempt to locate the region of progesterone action. Ova were removed from the female 16 hr post coitum and inhibited for 8 hr with \(^{14}\)C-labelled progesterone (-4-C-14). After sectioning, these ova were coated with emulsion and kept in a cold, dark room for 4 days before developing. In those sections where a pattern is apparent, the radioactive deposit is heaviest on the surface of the ovum or on the surface of the zona pellucida or both (Plate 1).

**DISCUSSION**

The results of the experiments reported here support the theory of steroid hormone action which says that the permeability of the cellular membranes is altered when the hormone reacts with protein of or on the membrane (Mueller, 1957). However, according to the evidence for this theory summarized by Villee (1961) the presence of the hormone *increases* the permeability of the membranes to various substances. The action of progesterone on mammalian ova *in vitro* appears to have the opposite effect, by inhibiting the transfer of amino acids or soluble proteins across the cell membrane. Presumably this is
Phase-contrast photographs of autoradiographed sections of rabbit ova after 8 hr in media containing $^{14}$C-labelled progesterone. Silver grains are concentrated around the surface of the zona pellucida (A) and the ovum (B).
achieved when progesterone aggregates on the surface of the ovum and/or zona pellucida. We have no evidence to show whether or not it ‘reacts’ with the protein of the cell membrane or zona pellucida or with some amino acid or protein component of the medium. Willmer (1961) has suggested a scheme whereby steroids and related compounds can be incorporated among the phospholipid and cholesterol molecules of the cell membrane. According to this scheme progesterone is presumed to penetrate so that the methyl ketone enters the cell membrane leaving the ketone as the terminal group on the outer surface. Proteins, polypeptides and amino acids can become attached to this exposed polar group. Whatever the mechanism of surface accumulation, the attachment must be quite weak because progesterone is easily washed off, as shown by the resumption of cleavage when the ovum is returned to normal medium.

The results reported in this paper show that mitosis in blastocyst and some morula stages is not blocked by progesterone. Other studies (Pincus & Werthesen, 1938) have shown that progesterone does not inhibit the growth in vitro of rabbit ova removed 68 to 77 hr post coitum and therefore in the late morula to early blastocyst stages. This may support the suggestion that the action of progesterone is a function of cell surface. As the number of cells increases, even though the total embryonic volume in the morula remains about the same as that in the single-celled stage, the cell surface area increases. By the blastocyst stage the total cell surface is quite large and the amount of progesterone used is not effective. Other hormones are also believed to exert an influence on protein synthesis in other ways. Manchester & Young (1961) conclude that “The mechanism of action of insulin in stimulating protein biosynthesis in muscle appears not to be in the translocation of amino acids across the cell membrane but in a process more intimately concerned with the intra-cellular synthesis of protein.”

In a recent review of some aspects of the problem of cell differentiation Flickinger (1963) describes the need for a sufficient supply of materials necessary for protein synthesis at times of specific gene action. When this supply is inadequate, normal differentiation does not proceed. Progesterone, in high concentration, apparently imposes such a severe limitation on the supply of these materials that the first step toward differentiation, namely the division of the ovum into two blastomeres, cannot proceed. Perhaps in more moderate concentrations at later stages, its action might contribute some control to the process of differentiation.

Although oestrogen and progesterone are known to have both counteracting and synergistic effects in different circumstances (Courrier, 1950; Hisaw & Hisaw, 1961), in this case oestrogen does not alter the capacity of progesterone to inhibit cleavage. These results support Whitten’s (1957) observation that oestradiol in concentrations of 0.05 to 0.2 µg/ml had no effect on reversing the inhibitory action of progesterone on mouse ova. Cochrane & Meyer (1957) observed that 4 µg of oestrone, given with progesterone to ovariectomized pregnant rats with delayed implantation, immediately initiated a resumption of embryonic development. Whatever influence oestrogens may have regarding developmental control of early mammalian embryos in a progesterone-rich
environment, it is not exerted directly upon the embryo in vitro. However, in view of the fact that the inhibition can be overcome by increasing the amino-acid concentration, and since oestrogen accelerates the production of amino acids in the fluids of the genital tract (Noall & Allen, 1961), it is conceivable that, if the same conditions exist in vivo, oestrogen may indirectly counter the inhibitory effect of progesterone. Cases of artificially-induced delayed implantation might follow this pattern, but the stage of arrested development has been shown to be late morula to early blastocyst (Cochrane & Meyer, 1957), which are the stages least reactive to progesterone in vitro.

Reports vary as to the serum progesterone levels reached during pregnancy or pseudopregnancy. Mikhail, Noall & Allen (1961) report a peak of only 2·36 µg/ml in the 16-day pregnant rabbit, while the highest level reported by Forbes (1953) was 7 µg/ml on Day 7. The highest of these concentrations would be sufficient to inhibit or reduce growth rate, but by this time any existing embryo is beyond the sensitive stage. Another young ovum in the genital tract at this time, however, would presumably be inhibited. Perhaps this system acts thereby as a second check to discourage superfoetation in the event of a mid-pregnancy ovulation and subsequent fertilization, a not uncommon circumstance in some species (Harrison & Neal, 1959; Shackelford, 1952) but not reported in the rabbit.

Whether progesterone exerts any direct effect in vivo on the embryo during early pregnancy remains debatable. In much of the work on delayed implantation, the presence of progesterone could be interpreted as having an inhibitory effect, but other studies (e.g. Adams, Hay & Lutwak-Mann, 1961; Smithberg & Runner, 1956) suggest that this is not the case. The present studies of the effects in vitro do not presume to present evidence for interpreting the in-vivo condition. The difference in effective concentration of progesterone between that needed to inhibit rabbit ova and that needed for mouse ova as reported by Witten (1957), may reflect differences in the species, in the stages studied, in the solvent or in the basic media used. The F10 medium used in these studies on rabbit eggs is rich in amino acids, and therefore a higher progesterone concentration would be needed to achieve an inhibition than would be required in a less nutritious medium.

The mammalian egg stores most of the materials it will need during early cleavage. This is demonstrated by its ability to begin development in far simpler media in vitro than that required by most other mammalian cells. However, as shown by Purshottam & Pincus (1961) and supported by the results described here, the ovum must be provided with some protein component or components, or with amino-acids from which the protein can be synthesized. Anything that interferes with this demand could therefore inhibit cleavage; progesterone appears to do so.

The relative success achieved in phasing the first cleavage of ova after removal from progesterone inhibition, points to a specific timed event that is especially sensitive to a limitation imposed on protein supply or synthesis. The first cleavage after removal of ova from the inhibiting progesterone occurs within 2½ to 3 hr, a time strongly reflective of the 1 to 2 hr limit previous to cleavage when progesterone is most effective. The slight difference can be
attributed to disturbances that undoubtedly result from the slight cooling and manipulation necessary to transfer the ova.

It appears that the sequence of events, originally disturbed by progesterone, continues after progesterone is removed. Apparently the event that is blocked occurs just before mitosis, by not more than a few hours at most, and one is led to suspect some temporary interference with the formation of the mitotic apparatus or chromosome condensation.

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REFERENCES


