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Changes in patterns of protein synthesis in axolotl oocytes during progesterone-induced maturation

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SUMMARY

Patterns of protein phosphorylation and synthesis during axolotl (*Ambystoma mexicanum*) oocyte maturation were studied by incorporation of [^{32}P]orthophosphate and [^{35}S]methionine into polypeptides, followed by two-dimensional gel electrophoresis. Various alterations were observed after progesterone treatment: *de novo* appearance of [^{35}S]methionine-labelled polypeptides, a quantitative increase in previously synthesized proteins and a quantitative decrease in or disappearance of other previously synthesized proteins. Changes in ^{32}P- and ^{35}S-labelling were observed very early during maturation. Neither prior oocyte enucleation nor α-amanitin treatment had a significant effect on these changes. Stimulation with MPF provided the same final protein pattern as PG treatment. However, cholera toxin inhibited all the changes seen during maturation. Comparisons between the patterns of [^{35}S]methionine- and [^{32}P]phosphate-labelling provide further information on the biochemical events that take place during oocyte maturation.

INTRODUCTION

Amphibian oocytes arrested in the first meiotic prophase progress to the second metaphase after exposure to progesterone. All the physiological, cytological and biochemical changes taking place during that period are referred to as oocyte maturation (for a review see Masui & Clarke, 1979). Studies on *Xenopus* showed that oocytes undergo biochemical changes prior to germinal vesicle breakdown (GVBD) and the first meiotic division. There is initially an increase in free calcium concentration (Wasserman, Pinto, O'Connor & Smith, 1980) and a fall in cAMP levels (Maller, Butcher & Krebs, 1979), followed by a general increase in protein synthesis (Brachet *et al.* 1974; Shih, O'Connor, Keem & Smith, 1978; Wasserman, Richter & Smith, 1982) and phosphorylation (Maller, Wu & Gerhart, 1977). A cytoplasmic factor called maturation promoting factor (MPF) appears at this time. Studies on protein phosphorylation have been mainly directed on ribosomal protein S6 (Hanocq-Quertier & Baltus, 1981). Although most studies agree that there is a general increase in protein synthesis, there are divergences about the

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qualitative changes in protein synthesis observed during oocyte maturation. Two-dimensional polyacrylamide gel electrophoresis studies showed either disappearance of polypeptides (Ballantine, Woodland & Sturgess, 1979), little change (Wasserman *et al.* 1982), or some alterations including synthesis of new proteins (Younglai, Godeau & Baulieu, 1981; Younglai, Godeau, Mulvihill & Baulieu, 1982). One study on axolotl oocytes (Jäckle & Eagleson, 1980) found three extra proteins after maturation. The authors suggested that this might be due to post-translational effects such as phosphorylation or deamination rather than *de novo* synthesis.

We used axolotl oocytes since meiotic maturation lasts longer than in *Xenopus*. GVBD takes place 9 to 10 h after progesterone treatment, and the first polar body forms around 15 h at 18 °C. It is quite possible to follow these morphological events under a dissecting microscope.

In this study we investigated the changes in protein phosphorylation in parallel with patterns of protein synthesis occurring during meiotic maturation induced either by progesterone or MPF. To extend these observations we carried out studies on enucleated oocytes as well as on the effects of treatment with α-amanitin and cholera toxin (which was described to inhibit progesterone-induced maturation, Godeau *et al.* 1980). Similar experiments were carried out using non-equilibrium pH gel electrophoresis (NEpHGE).

MATERIAL AND METHODS

Adult axolotl females were bred in the laboratory, *Xenopus* females (for preparation of MPF) came from Snake Farm (Fish Hoek, Cape Town, South Africa). Full-grown oocytes were manually isolated with watchmaker's forceps and Pascheff's scissors. All experiments were carried out in modified Barth's solution buffered with Hepes (10 mM) (MBS-H) (88 mM-NaCl, 1 mM-KCl, 2·4 mM-$NaHCO_3$, 0·82 mM-$MgSO_4$, 0·33 mM-$Ca(NO_3)_2$, 0·41 mM-$CaCl_2$). Meiotic maturation was induced by 10 μM-progesterone in MBS-H, pH 8·4 for axolotl oocytes, or by progesterone 10 μg ml^{-1} in MBS-H, pH 7·4 for *Xenopus* oocytes. Timing of axolotl oocyte maturation was carried out at 18 °C.

Cholera toxin and α-amanitin were purchased from Sigma (St Louis), [^{35}S]methionine and [^{32}P]orthophosphate from Amersham (Belgium). Oocyte enucleation was performed according to Ford & Gurdon (1977) with minor modifications. In order to obtain MPF, we used *Xenopus* oocyte cytoplasm, which is easier to inject than axolotl cytoplasm. The feasibility of this transfer has been tested elsewhere (Reynhout & Smith, 1974).

In a typical experiment, twenty oocytes were each microinjected with 100 nl of the radioactive solution. Radioactive solution was 90 % of radiochemical adjusted to MBS-H molarity by appropriate salts. ^{35}S was 1200 Ci mmol^{-1} (411 mCi ml^{-1}) γ-[^{32}P]ATP 3000 Ci mmol^{-1} (10 mCi ml^{-1}). Oocytes were incubated for 1 h in modified Barth's solution, Hepes buffered. The oocytes were manually defolliculated and homogenized in lysis buffer (100 μl oocyte^{-1}: 50 mM-Tris, 150 mM-NaCl, 2 % NP40, 2 mM-PMSF and 0·15 i.u. ml^{-1} aprotinin) for 15 min before centrifugation (5 min at 10 000 *g*).

In the case of ^{32}P experiments, NaF (5 mM) and glycerophosphate (50 mM) were added to lysis buffer; and an additional control was performed adding γ-[^{32}P]ATP to a non-labelled oocyte extract in order to estimate *in vitro* phosphorylation of proteins.

10 μl of lysate supernatant were used for the measurement of TCA-precipitable radioactivity, 50 μl of lysate were mixed with 50 μl of O'Farrell lysis buffer for isoelectric focusing, followed by 10 % polyacrylamide SDS slab gel electrophoresis (O'Farrell, 1975). Fixed amounts of protein,

equivalent to half of an oocyte, were loaded on each gel. NEpHGE (pH 3·5–10) was carried out according to the method described by O'Farrell, Goodman & O'Farrell (1977).

Gels containing ^{35}S were prepared for fluorography (Chamberlain, 1979), and the dried gels containing ^{32}P were exposed under an intensifying screen to X-ray film at −80 °C. Comparison of ^{35}S-labelled and ^{32}P-labelled patterns was done using silver staining prior to fluorography (method described by Oakley, Kirsch & Morris (1980)) with methanol-acetic acid fixation prior to glutaraldehyde impregnation.

RESULTS

Four separate experiments on [^{32}P]orthophosphate incorporation were carried out on four different females; likewise four females were used for the [^{35}S]methionine experiments. In all cases it was checked that GVBD occurred around 10 h of maturation and that the first polar body was completely formed by 15 h at 18 °C, in at least 90 % of the oocytes. Identical amounts of total protein were subjected to focusing, in order to highlight changes in the synthesis of individual proteins during maturation. Proteins referred as p1 to p36 in the text are numbered 1 to 36 on the figure for more clarity.

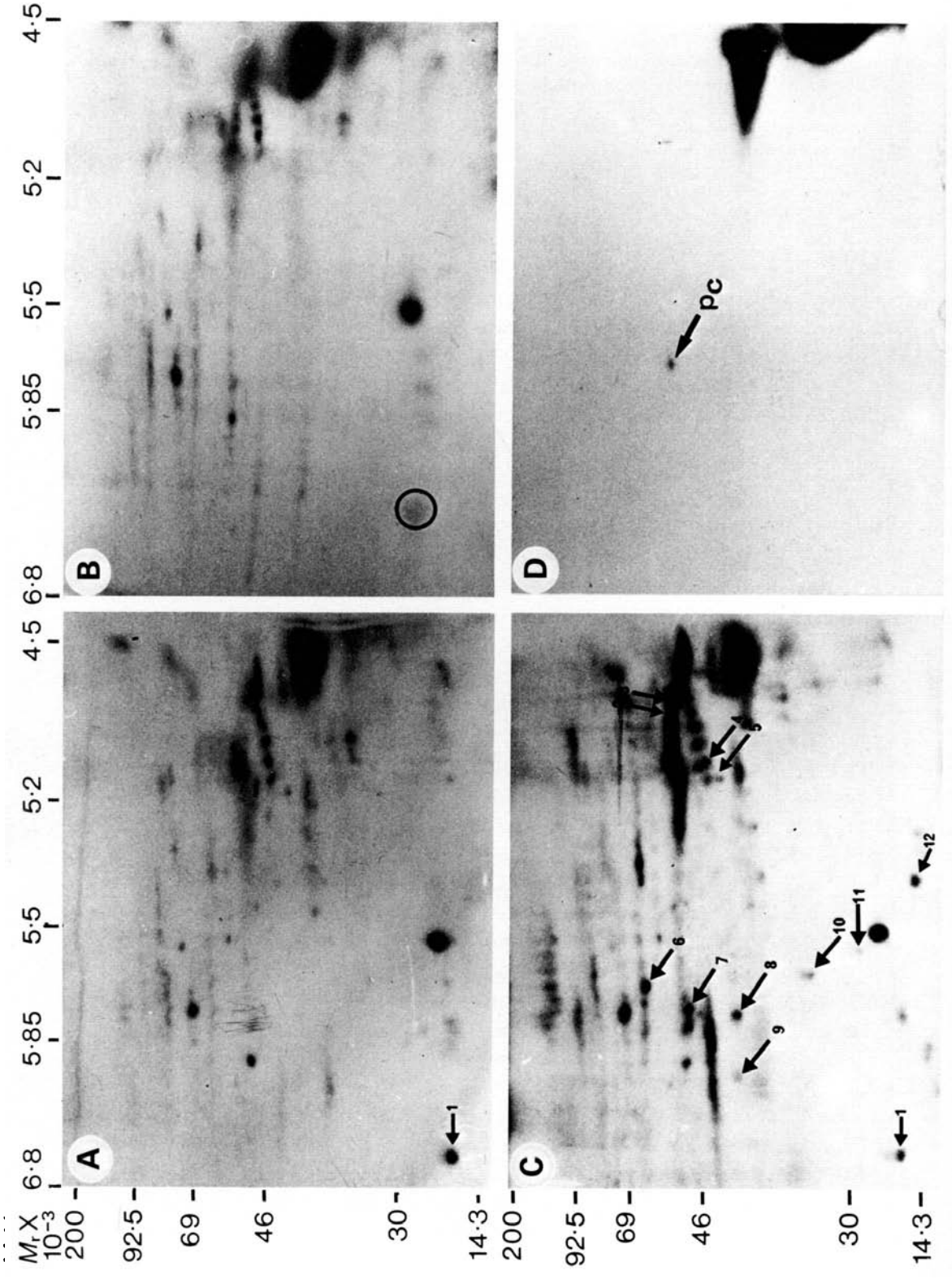
Fig. 1 shows the early dephosphorylation of the protein p1, within the third hour following progesterone treatment. At this time and subsequently, phosphorylation of 11 other proteins was observed (numbered p2 to p12). For all of these proteins, phosphorylation takes place around the time of GVBD. We also observed that the dephosphorylated protein p1 was rephosphorylated at GVBD.

Fig. 1D which is the *in vitro* phosphorylation control (using γ-[^{32}P]ATP) shows only one phosphorylated peptide, for 1 h incorporation.

Fig. 2 shows the two-dimensional fluorogram of ^{35}S-labelled polypeptides from control oocytes in the absence of progesterone. We were able to resolve around 500 separate polypeptides. Fig. 3A,B,C shows magnifications of areas *A*, *B* and *C* respectively on Fig. 2: the patterns of protein synthesis that were observed during maturation.

Synthesis of proteins p13–p16 was clearly seen in control oocytes, decreased during maturation and was undetectable at GVBD. Protein p13, which is synthesized between hours 4 and 5, was undetectable between hours 7 and 8, while p14, p15 and p16, which are still synthesized between hours 7 and 8, were undetectable between hours 9 and 10 (detail of timing for p15 is not shown).

It is probable that proteins p17 and p18 are subjected to an acidic shift under the action of progesterone. This shift in p17 was already visible within 1 h after hormone treatment. For p18 this shift was more progressive and became most noticeable between hours 9 and 10. An acidic shift is more likely than newly synthesized protein, because it is possible to follow the decrease in labelling of one polypeptide at the same time as an increase in the next polypeptide labelling (this is particularly clear in the case of p18). Only peptide mapping of these polypeptides will give a conclusive answer. Proteins p19–p35 are newly synthesized proteins which were not detectable in control preparations. Protein p33 was


M_r X 10^{-3}
200
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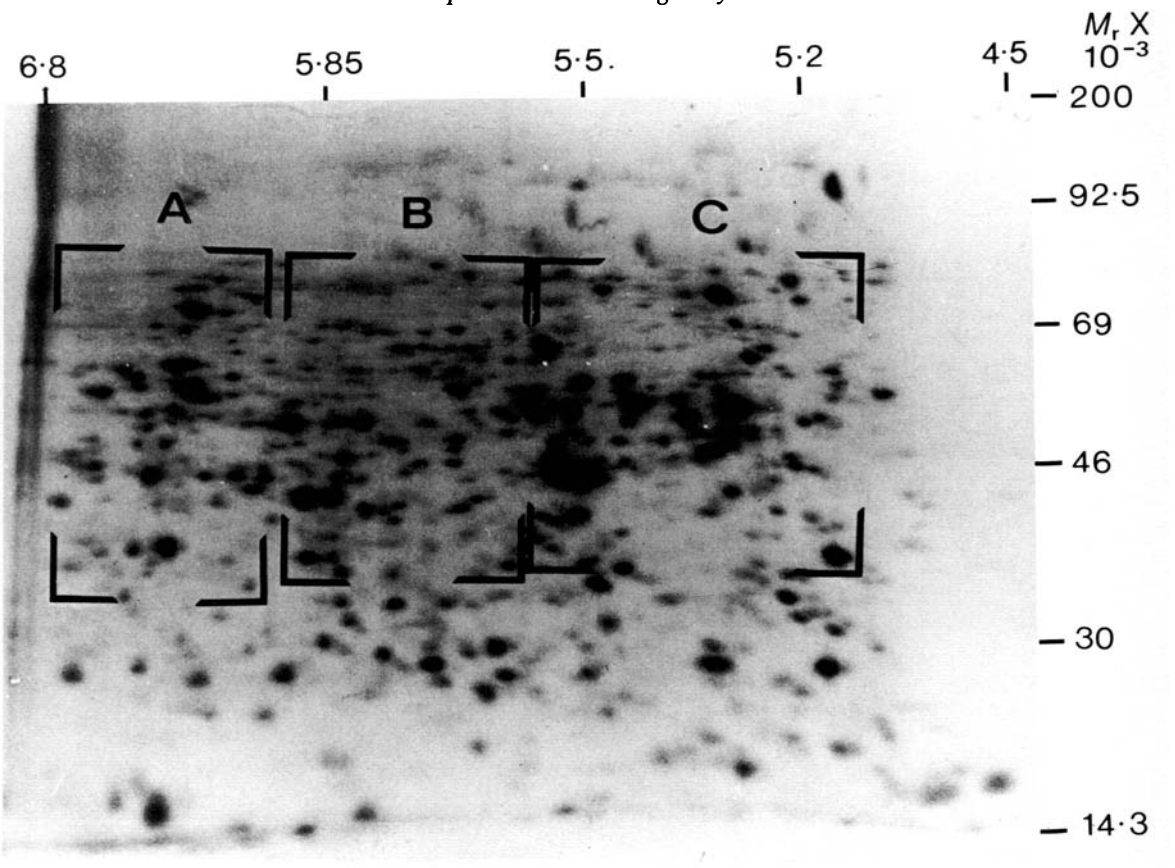
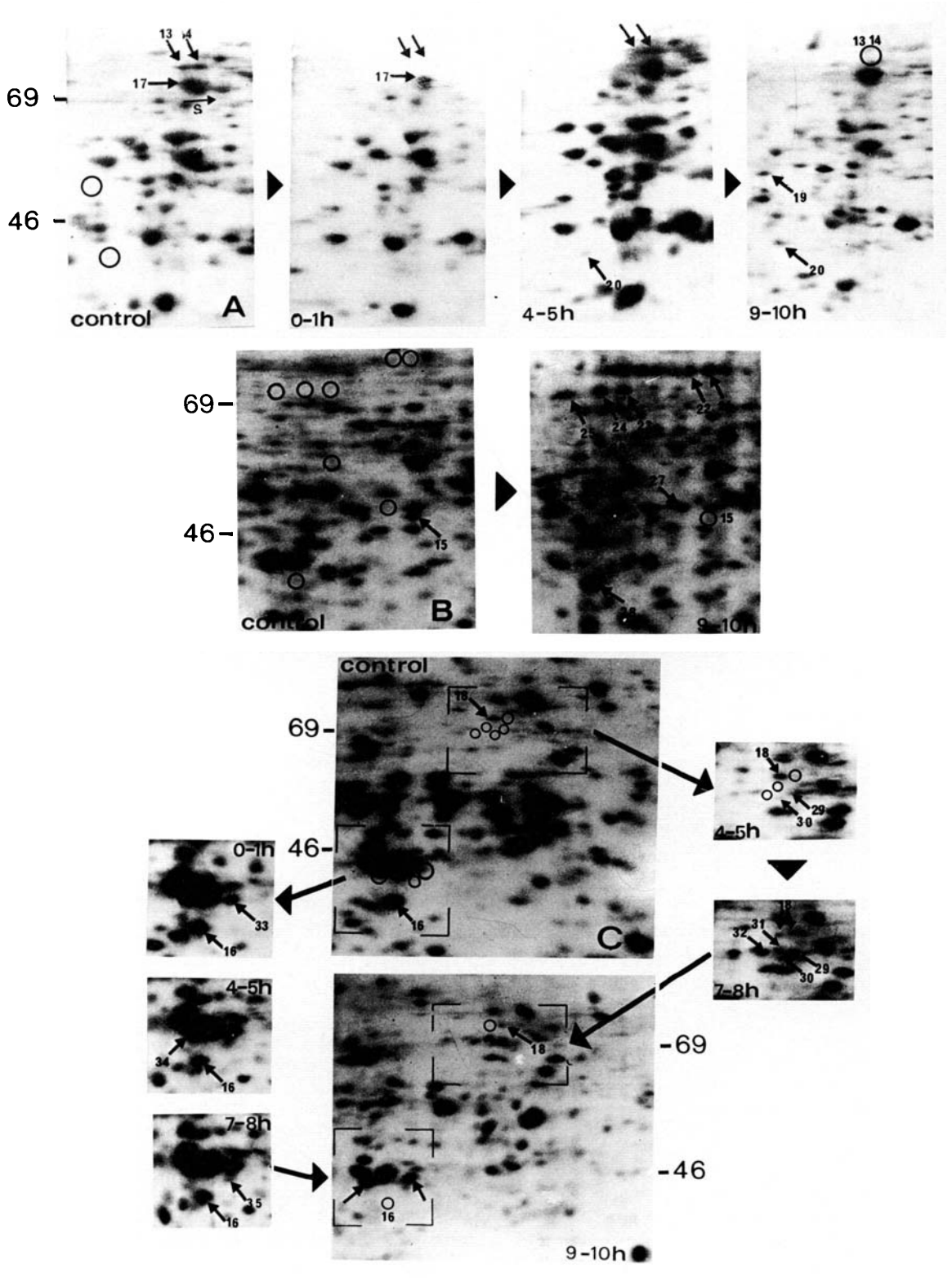


Fig. 2. Two-dimensional fluorograms of [^{35}S]methionine-labelled polypeptides synthesized during progesterone-induced maturation. Control oocytes without progesterone. Areas A, B and C show the major occurring modifications. Magnifications of these three areas are represented on Fig. 3A,B,C respectively at different stages during progesterone-induced maturation.

present in very low amounts in controls but increased very markedly after progesterone treatment and was therefore included in this group (the exact timing of the appearance of all these new polypeptides is not shown for reasons of brevity).

Fig. 1. Two-dimensional autoradiograms of [^{32}P]phosphate-labelled polypeptides labelled during progesterone-induced maturation of axolotl oocytes. Each autoradiogram represents newly phosphorylated protein from half of an oocyte. (A) Control oocytes without progesterone; (B) incorporation 3–4 h after progesterone stimulation; (C) incorporation 11–12 h after progesterone stimulation; (D) *in vitro* phosphorylation of a non-labelled oocyte extract using γ-[P^{32}]ATP (see Materials and Methods). pc: *in vitro* phosphorylated polypeptide. Relative molecular mass marker proteins were myosin (200 K), phosphorylase b (92·5 K), bovine serum albumin (69 K), ovalbumin (46 K), carbonic anhydrase (30 K), lysozyme (14·3 K). The isoelectric pH range of the gels was 4·5–6·8. Major modifications are indicated by arrowheads and are numbered from 1 to 12.


69
46
control
A
0-1h
4-5h
9-10h
B
C
7-8h

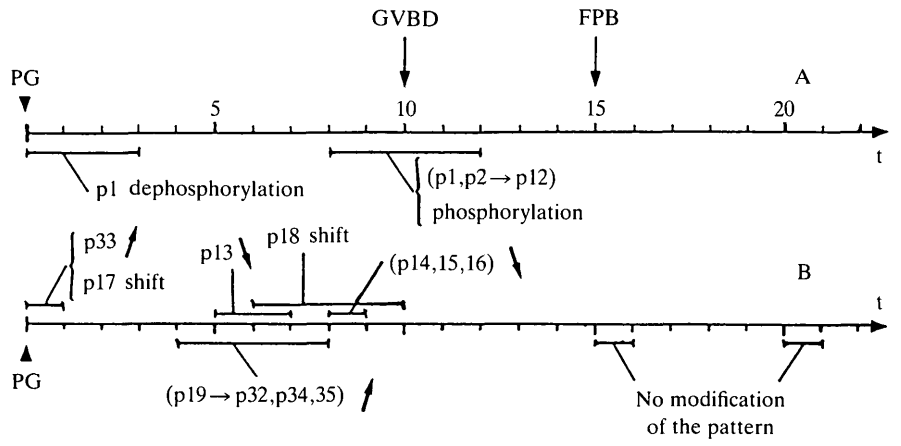


Fig. 4. (A) Changes in ^{32}P-labelled polypeptides during maturation. PG, time of hormone addition; GVBD, germinal vesicle breakdown; FPB, first polar body formation. (B) Changes in ^{35}S-labelled polypeptides. Short arrows symbolize the appearance of, or an increase in, synthesis (↗) or symbolize lack of, or reduced, synthesis (↘).

The synthesis of all these new proteins starts before GVBD: between 4–5 h for p20–p25, p29, p30, p34, p35 and between 7–8 h for p19, p26, p28, p31 and p32. Synthesis of p33 was clearly observed within 1 h of maturation.

Comparison between ^{32}P- and ^{35}S-labelled polypeptides on silver-stained gels shows possible identity of p6 (^{32}P) and p26 (^{35}S), and p8 (^{32}P) and p28 (^{35}S). The patterns of incorporation of [^{35}S]methionine in the later stages of maturation (15–16 h and 21–22 h) were very similar to those of the earlier incorporation of ^{35}S (9–10 h).

Cholera toxin at a final intraoocytic concentration of 10^{-8} M prior to progesterone treatment completely altered the observed pattern, whereas α-amanitin at a final concentration of 1 $\mu g\,ml^{-1}$ did not affect the action of progesterone on the patterns of protein synthesis. In the same way, enucleation had no effect.

Using NEpHGE we were unable to detect any significant alterations in basic polypeptide synthesis during maturation.

DISCUSSION

The present investigations represent the first detailed studies on protein phosphorylation and synthesis during axolotl oocyte maturation. The polypeptide

Fig. 3. Arrowheads 13 to 16 show the disappearance of four polypeptides. Arrowheads 17 and 18 show the acid shift of two polypeptides. Arrowheads 19 to 36 show the major new polypeptides synthesized. The circles symbolize the spaces where newly synthesized polypeptides appear, or the spaces where proteins were previously synthesized.

Table 1. *[^{35}S]methionine incorporation into polypeptides from axolotl oocytes at various stages of maturation*

	Incorporation (c.p.m. × 10^3/ovocytes)*
Control (no PG)	355
0–1 h after PG addition	371
4–5 h after PG addition	895
9–10 h after PG addition	957
9–10 h after PG addition + α-amanitin	788
4–5 h after MPF injection	1102

PG: progesterone, MPF: maturation-promoting factor.
* Means of the different experiments.

patterns were highly reproducible; marked differences in [^{35}S]methionine-labelled polypeptides were found between control oocytes, and oocytes at various stages of progesterone-induced maturation.

As in previous studies on *Xenopus* oocytes (Wasserman *et al.* 1982) we observed that the rate of protein synthesis in progesterone or MPF-induced maturation is about twice that of the rate in control or cholera toxin-treated oocytes (data not shown).

At the time of GVBD, the observed wave of protein phosphorylation was similar to that reported by Maller *et al.* (1977) in *Xenopus* oocytes. More surprising is the early dephosphorylation observed in the first 3 h of maturation before GVBD. The identification of an early dephosphorylation is unique; investigations have commenced in order to substantiate its effect.

This observation supports the hypothesis that early protein dephosphorylation leading to the formation of MPF is the first major event following hormone treatment (Maller & Krebs, 1977; Huchon, Ozon & Demaille, 1981). However, it should be pointed out that in axolotl this protein was rephosphorylated during the later phosphorylation wave.

Considering the [^{35}S]methionine-labelled polypeptides, all the progesterone-induced changes took place prior to GVBD, and no changes were observed after this time. As found in previous studies on *Xenopus* oocytes (Younglai *et al.* 1982), all these changes were inhibited by cholera toxin, but were not significantly affected by either enucleation or α-amanitin treatment. It has been suggested that the changes in protein synthesis during maturation do not involve transcriptional processes, but rather reflect the effects of recruitment of pre-existing mRNAs (Richter, Wasserman & Smith, 1982).

We must also point out that these changes might not require components issued from germinal vesicle (enucleation experiments).

We were not able to observe differences in the patterns between progesterone and MPF-induced oocytes. We performed two-dimensional gels of [^{35}S]methionine and ^{32}P-labelled polypeptides of MPF-induced oocytes, between 4 and 5 h after MPF injection. At this time [^{35}S]methionine and [^{32}P]polypeptide

patterns were identical to those obtained with progesterone between 9 and 10 h of maturation. The precise timing of MPF appearance in axolotl oocyte after progesterone stimulation is not well known; MPF probably appears around 4 h of maturation.

Three main kinds of change were found in progesterone-induced oocytes.

(1) We observed a marked reduction in the synthesis of four polypeptides which became almost undetectable. The drop in synthesis of three of them occurred shortly before GVBD, between 8 and 9 h after progesterone treatment. We have previously shown (Gautier & Beetschen, 1985), that cytoplasmic changes occurring at this time are prerequisites for early induction of grey crescent formation. It could be suggested that the reduction in the synthesis of one of these proteins reflects the disappearance of an inhibitor of the symmetry reaction.

(2) Two possible post-translational acidic shifts of the polypeptides were observed. One takes place early on, during the first hour following exposure to progesterone, most probably before formation of MPF. We do not know the precise nature of these acidic shifts, but they are probably not phosphorylations, since there was no evidence of ^{32}P-labelling in these areas. Many other kinds of post-translational modification other than phosphorylation have been reported (Wold, 1981) and many of them can lower the pH_i values.

Again, only a partial enzymatic digestion of these polypeptides, followed by polyacrylamide gel electrophoresis (i.e. peptide mapping), will give a satisfactory answer.

(3) We obtained evidence for *de novo* synthesis of several proteins occurring between 4 and 8 h following exposure to progesterone, i.e. probably at the time of MPF formation in the axolotl oocyte. Other studies on *Xenopus* oocytes have described changes in protein synthesis during maturation prior to GVDB; MPF has been found to have almost instantaneous effects on translation (Wasserman *et al.* 1982). Studies on the distribution of total proteins in axolotl oocytes (Jäckle & Eagleson, 1980) demonstrated, using silver nitrate staining after two-dimensional electrophoresis, the appearance of three new proteins after maturation. These authors suggested that they represented post-translational modifications of pre-existing polypeptides rather than *de novo* synthesis. In the present study we produce evidence for *de novo* protein synthesis. Two-dimensional fluorography provides a more detailed picture of protein synthesis than total protein staining. In most cases, the newly synthesized polypeptides did not correspond to phosphorylated molecules or other post-translational modifications, as was shown by their relative positions on the fluorograms. Compared to the studies on *Xenopus* oocytes (Younglai *et al.* 1982; Wasserman *et al.* 1982) we observed more alterations in the axolotl oocyte during maturation both qualitatively and quantitatively. The appearance of early changes following exposure to progesterone, but prior to MPF formation, was particularly clear. The axolotl oocyte thus provides an improved model system for the investigation of the early biochemical events during maturation.

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