EARLY EVENTS IN MATURATION OF THE OOCYTES OF STARFISH *MARTHASTERIA GLACIALIS*: PARTIAL CHARACTERIZATION OF A PHOSPHORYLATED FACTOR DURING OOCYTE MATURATION

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ABSTRACT

EARLY EVENTS IN MATURATION OF THE OOCYTES OF STARFISH MARTHASTERIA GLACIALIS: PARTIAL CHARACTERIZATION OF A PHOSPHORYLATED FACTOR DURING OOCYTE MATURATION

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In starfish Marthasteria glacialis, full-grown oocytes are arrested at the prophase of meiosis. Meiosis is re-initiated by a relay hormone, 1-methyladenine. A high level of protein phosphorylation stimulated as early as five minutes after the addition of the hormone to the oocytes appears to be a major prerequisite for meiosis induction. This phenomenon is also observed when cortices isolated from immature oocytes are treated with 1-methyladenine. The focus of the study described in this thesis was to identify and partially characterize a phosphorylated factor found in the cortices of oocytes that were treated with 1-methyladenine in the presence of ^{32}P ATP using an "in vitro" and an "in vivo" system. It is believed that this factor is the maturation promoting factor. The phosphorylated factor was found to be a cortical protein having an apparent molecular weight of 17,000 daltons.

Dedicated to a very good friend, Colette Jeannaut.

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CHAPTER 1

INTRODUCTION

Weismann (cited by Wilson, 1925), based on his embryological studies, concluded that a continuity of the germ cells from generation to generation was maintained during sexual reproduction. He also observed that the fertilized eggs divided to produce, on one hand, the cell material by which the characteristics of the species are maintained, and on the other hand, the material responsible for the propagation of the species. This type of differentiation shows the progressive developmental potential and the specialized functions of the embryonic totipotent cells.

The final development of the embryo produces the conception of a multicellular individual which appears to be a dual organism in which germ cells and somatic cells lead to a quasi-independent life.

Once the primordial germ cells have migrated to form the locus of the gonads, they multiply and produce oogonia cells in females and spermatogonia cells in males. These cells are larger and have different distribution of cytoplasmic organelles from the primordial germ cells (Giese and Pierse, 1974).

The population of oogonia increases greatly in a relatively short period of time, by a finite number of mitotic divisions. Shortly after the last division of the oogonium, the cell enters an interphase in which DNA is organized in preparation for meiosis. During

this preparation, the oogonium goes through the phases leading to the diplotene stage of the meiosis prophase. This equally applies to oogenesis as well as to spermatogenesis (Austin and Short, 1972). At this stage, the similarities end. The spermatogonium continues the meiosis, but the oogonium becomes transformed into an oocyte by entering a "resting period" which is terminated shortly ~~after or~~ before ovulation.

The emergence from this stationary stage is followed by the operation of external or internal stimuli that control the ripening or maturation of the oocyte (Sundararaj & Guswami, 1973; Papkoff et al, 1973; Kanatani, 1973; Dorée et al, 1976a). So far this mechanism of maturation is not well understood, but it can only be recognized at the terminal stage where the cellular, morphological and physiological characteristics and molecular components typical of this process become apparent (Rutter et al, 1973).

CHAPTER II

MATURATION OF OOCYTES IN ASTEROIDEA

II.1 Introduction

The control of gonadal functions has been recognized to depend directly or indirectly on the neuroendocrine systems in vertebrates (Papkoff et al, 1973; Licht and Papkoff, 1974a, 1974b; Licht et al, 1977) and also in invertebrates (Kanatani, 1973; Clark, 1965; Vicent, 1966; Wells, 1960; Adiyodi and Adiyodi, 1970; Chaet and McConnaughy, 1959). These neurosecretory hormones (gonadorophins) have been isolated and characterized in all the vertebrates (Papkoff et al, 1973; Licht et al, 1977) but in invertebrates, they have only been characterized in Asteroidea (Kanatani et al, 1971).

II.2 Neurosecretory Hormone

Chaet and McConnaughy (1959) reported the presence of an active substance in Asteria forbesii responsible of gamete-shedding. This was shown by injecting a water extract of the nerve into the coelomic cavity of the starfish.

After the findings of Chaet and McConnaughy (1959), other workers in the field reported the presence of the gamete-shedding substance (GSS) in 29 different species (Kanatani, 1973) and it was further shown that this factor lacked species-specificity (Hartman and Chaet, 1962; Chaet, 1966; Kanatani, 1973).

GSS was reported to be present in the radial nerve of both male and female (Chaet and McConnaughy, 1959; Kanatani and Ohguri, 1966; Chaet, 1966a) in equal physiological levels (Chaet, 1966a; Kanatani and Ohguri, 1966). However, Kanatani and Ohguri (1966) found this substance was also present in other regions of the starfish in which nervous tissues were present; later, these results were confirmed by Atwood and Simon (1971). GSS was found to be a polypeptide (Chaet, 1966a, 1967; Kanatani and Shirai, 1967; Shuetz, 1969).

Kanatani et al (1971) reported that GSS was an acidic polypeptide with a molecular weight of approximately 2,200 daltons. A series of functions were attributed to this neurosecretory hormone. At one point, it was postulated that the nerve extracts contained a "contraction factor" (Chaet et al, 1964; Chaet, 1966a) and a maturation factor (Chaet 1966a, 1966b). These factors were proposed based on the evidence that the shedding (Chaet et al, 1964; Kanatani and Ohguri, 1966; Kanatani, 1964; Chaet and McConnaughy, 1959) and maturation (Kanatani, 1964; Chaet, 1966a, 1966b) of the oocytes were induced by the injection of nerve extracts into the coelomic cavity of the starfish. However, Kanatani (1967) demonstrated that the nerve substance did not contain the "contraction factor". This was shown by testing previously gel filtrated nerve extracts on starfish ovarian walls attached to a kymograph. Not much contraction was produced although such extracts had a strong capacity to induce spawning in normal ovaries of starfish.

It was also shown independently by Kanatani and Shirai (1967)

and Shuetz and Biggers (1967) that both gamete release and oocyte maturation were not produced by the direct action of GSS, but that of a second substance which was newly biosynthesized in the gonads under the influence of GSS, Kanatani (1972) proposed a new name for the neurosécretory hormone and for the secondary factor, based on their physiological functions. The neurosecretory substance (Chaet and McConnaughy, 1959) called gamete-shedding substance was renamed Gohad-Stimulating Substance (GSS), since it acted on the ovary to stimulate the production of the secondary factor. This secondary factor was designated by Kanatani and Shirai (1967) as a meiosis-inducing substance and renamed "Maturation-inducing Substance" (MIS).

II.3 MATURATION-INDUCING SUBSTANCE

II.3.1 NATURE OF THE FACTOR

Maturation-inducing Substance was isolated by gel filtration from the supernatant of ovarian fragments that were treated with GSS (Kanatani and Shirai, 1967, 1970; Shuetz and Bigger, 1967; Shuetz, 1969a), and was shown to have spawning and maturation-inducing activity when used on other preparations of ovarian fragments.

Gel filtration of the mixture of extracts of ovaries without previous treatment of GSS did not show any MIS activity. It was also shown that MIS was not contained in the radial nerve extracts from where GSS was extracted (Hirai and Kanatani, 1971). However, MIS was only present when the ovarian fragments were incubated with GSS.

From the above, it was concluded that the action of GSS was only to produce MIS that will function as a relay hormone (Kanatani, 1972; 1973) in the ovary.

The production of MIS under the influence of GSS has been shown to be present in twelve (12) different starfish species so far studied (Kanatani, 1969, 1973; Shuetz and Bigger, 1967; Shuetz, 1969b). The chemcial structure of MIS was reported by Kanatani et al (1969) and it was shown to be a purine base molecule methylated at the first ring position, 1-methyladenine (1-MA).

The induction of spawning and maturation of oocytes by synthetic 1-MA has been reported in 19 starfish species (Kanatani, 1973) and it is believed to be a general meiosis-inducing agent in Asteroidea.

II.3.2. BIOLOGICAL ACTIVITY OF ADENINE DERIVATIVES

A series of adenine derivatives were tested for their biological activity (Kanatani and Shirai, 1971; Kanatani, 1972), but only analogous structures having an alkyl substituent at the position N-1 and an imino group attached to position C-6 induced maturation and spawning of oocytes in starfish.

Later, it was shown by Dorée et al (1976), that the degree of the biological activity present in the substituted adenines with N-1 substituents depends on their polar characterization; 1-benzyloxymethyladenine was shown to be 10^3 times less active than 1-benzyladenine, and it was observed that 1-carboxy-methyladenine did not show

any activity. It was also pointed out that at pH 7, 50% of the alkyladenines exist in the protonated form with a delocalized position charge in the purine ring. Therefore, it may be concluded that any substituent placed at position N-1 that will stabilize the positive charge donated to the molecule by the protonation of the imino group, will thus produce biological activity.

With respect to the binding abilities, it was shown that bulky groups as benzyladenine attached to N-1 and C-6 do not suppress biological activity or inhibit binding with the receptor. However, by adding a second substituent to the purine ring position N-7, or N-9, as in 1, 7-dibenzyladenine, 1, 9-dimethyladenine, and 1, 9-dibenzyladenine, it suppressed biological activity even when the substituent at position N-9 was a small methyl group (Dorée et al, 1976a). This portion of the purine ring was suggested to be the possible binding position of the hormone with the external receptor.

II.3.3 SITE OF ACTION ON THE OOCYTE OF THE MEIOSIS-INDUCING SUBSTANCE

Kanatani and Hiramoto (1970) suggested that 1-MA acts on the oocytes on the external site of the membrane during the reinitiation of meiosis. This was shown by microinjecting 1-MA into isolated oocytes from Asteria pectifera and comparing the results obtained with those from the oocytes treated with external application of the hormone.

The following were observed from these experiments: (1) the oocytes treated with 1-MA externally underwent maturation as seen by the germinal vesicle breakdown in oocytes. However, the oocytes that were injected with the hormone failed to maturate, but once transfered to sea water containing 1-MA underwent breakdown of germinal vesicles within 20 minutes followed with meiosis maturation. Dettlaf et al (1964) and Masui (1972) proposed that the mechanism of oocyte maturation in starfish as so far elucidated is similar to that of amphibians. Support for this statement was based on the work done by Masui and Markert (1971), Smith and Ecker (1969) and Dettlaf et al (1964). These authors showed that microinjection of MIS (progesterone) in oocytes of Rana pipiens did not promote oocyte maturation, whereas externally applied MIS lead to maturation.

Toole and Shuetz (1974) proposed that MIS (1-MA) may be required to enter the oocyte membrane of starfish to be converted into an intracellular active metabolite that induces maturation. This was ruled out by Dorée and Guerrier (1975). These authors, from competitive permeability studies with a specific inhibitor of the hormone uptake, demonstrated that threshold concentration of 10^{-7}M 1-MA was necessary to produce 100% maturation, lowering the concentration of the hormone, directly decreased the percentage of oocyte maturation, whereas an inactive hormone analogue, 1, 9-dimethyladenine, inhibited the permeability of 1-MA into the oocytes, but did not inhibit the biological response of the hormone, and concluded that 1-MA was not required to enter the oocytes of M-glacialis to induce maturation. Further evidence for the presence of a surface receptor was reported by Dorée

et al,(1976b), who incubated the oocytes with a non-ionic detergent, Triton X-100 (0.01 to 0.02%) for eight (8) minutes and abolished the biological response to the hormone. It is also possible that the detergent, inactivated or extracted recognition sites for the hormone. Furthermore, Kanatani (1978) reported that Triton X-100 wash of follicle-free oocytes contains a heat-stable, non-protein substance, and that oocytes incapacitated following Triton treatment had their responsiveness to 1-MA restored when they were incubated with this substance.

Godeau et al (1978) succeeded in inducing Xenopus oocytes to mature by exposing them to a polymer conjugated steroid. This showed that MIS in starfish as well as in Xenopus acted at an external site of the oocyte membrane. The nature of this site has not yet been characterized; however it has been shown by Kishimoto et al (1976) that in starfish the sulfhydryl content of oocyte cortical proteins was increased after the treatment with 1-MA. Non-structural analogues of 1-MA, i.e. disulfide-reducing agents such as dithiotheritol (DTT) and 2,3-dimercapto-1-propanol (BAL) (Kishimoto et al, 1976); Kishimoto and Kanatani, 1973) have been shown to mimick the action of 1-MA in the ability to induce oocyte maturation. Furthermore, it was shown that DTT, like 1-MA, acts on the oocyte from the outside (Kishimoto et al, 1976).

CHAPTER III

PHYSIOLOGICAL AND BIOCHEMICAL CHANGES PRODUCED BY MIS DURING GERMINAL VESICLE BREAKDOWN

III.1 Introduction

The full grown oocytes of starfish are arrested at the prophase of meiosis until they are reinitiated by a relay hormone 1-MA. These changes occur as a normal process of sexual reproduction shortly before or after ovulation.

The term "maturation" is being used to describe completion of meiosis as defined by Wilson (1925), who stated that "maturation is accomplished in the oocyte by the breakdown of the germinal vesicle followed with successive meiotic divisions in the course of which the oocyte buds forth two polar bodies and it represents the final stage of the "ripening" of the egg. In the oocytes of the starfish Marthasterias glacialis, the response to the hormone primarily occurs by the germinal vesicle breakdown observed 18 minutes (Guerrier and Dorée, 1975; Guerrier and Dorée, 1975) after the external addition of 10^{-7}M 1-MA (threshold concentration). Changes were not observed by raising the hormone concentration (Dorée et al, 1976a). The germinal vesicle breakdown was followed by the release of the first and second polar bodies 80 minutes and 2 hours later, respectively (Dorée et al, 1976c).

It has also been reported (Guerrier and Dorée, 1975) that the germinal vesicle breakdown process in the oocyte of starfish has two

phases, one hormonal dependent, and the other hormonal independent. The first phase, which lasts about 4 minutes 30 seconds from the time of the hormone addition, requires the presence of the 1-MA, whereas the second phase, established between the 4.50th to the 18th minute, does not require the exogeneous supply of 1-MA.

If the hormone is applied in two periods during the hormonal-dependent phase of 2 minutes and 15 seconds each, it can give a biological response only if the lapse between the two applications is not more than 7.5 minutes. The above observations suggest that during maturation, MIS triggers reversible changes during the hormonal-dependent phase, followed by an irreversible change that provides the actual mode to reinitiate maturation.

III.2 MACROMOLECULAR BIOSYNTHESIS DURING GERMINAL VESICLE BREAKDOWN

It has been observed that neither protein synthesis nor nucleic acid synthesis seem to be required during the two steps that preceed germinal vesicle breakdown (Guerrier and Dorée, 1975).

III.2.1 RNA Biosynthesis

Guerrier and Dorée (1975) reproted that the presence of actynomycin D (100ug/ml) plus the hormone in the incubation medium did not inhibit germinal vesicle breakdown induced by the relay hormone, but it prevented the formation of the two polar bodies when the inhibitor of RNA synthesis was present during the hormonal-dependent phase.

III.2.2 Protein Synthesis

It was shown (Guerrier and Dorée, 1975; Guerrier et al, 1977) that the inhibition of protein synthesis by emetine (20 ug/ml), cycloheximide (100 ug/ml) or puromycin (100 ug/ml) did not block the germinal vesicle breakdown that is induced by the hormone. On the other hand, biosynthesis of RNA is necessary during the dependent hormonal phase. This RNA is probably required for changes that follow germinal vesicle breakdown.

III.3 ROLE OF Ca^{++} IONS DURING GERMINAL VESICLE BREAKDOWN

Kanatani (1964) and Shuetz and Bigger (1967) showed that Ca^{++} ions can trigger meiosis of oocytes of starfish when they were isolated in normal or Ca^{++} enriched artificial sea water. It was proposed that this ion acted by releasing 1-MA from the follicle cells.

This process of spontaneous maturation has been questioned by Guerrier et al (1978). They showed that an increase of calcium concentration in the incubation medium of oocytes of M-glacialis, isolated in Ca^{++} free sea water (Ca^{++} FASW) mimicked the response to 1-MA. The oocytes incubated in calcium-rich artificial sea water were shown to exhibit normal germinal vesicle breakdown in about 18 minutes. When maturation of these calcium-treated oocytes was completed and they were fertilized, the embryos developed normally to the bipinnaria larvae (Guerrier et al, 1978). Taking another approach, Moreau

and Guerrier (1978a) have shown that about 0.5 to 1.5 μM of Ca^{++} ions was released in less than one second after the addition of $2X10^{-7}$M 1-MA (threshold concentration).

Furthermore, it was shown by the microinjection of EGTA (a Ca^{++} chelating agent that lowers the light response produced by Ca^{++} -aequorin interaction) into oocytes lowers the internal level of Ca^{++} released by the external addition of the hormone that was necessary to allow meiosis reinitiation (Moreau et al, 1978b, 1978c). The inhibition of the meiosis response may be due to insufficient quantities of free Ca^{++} ions. However, the injection of EGTA after the production of the Ca^{++} response was no longer effective in inhibiting meiosis reinitiation. It was also shown (Moreau et al, 1978b) that a divalent action ionophore (A 23187) causes release of Ca^{++} ions 20 times higher than the levels observed during the external application of 1-MA; however, no maturation was observed in the oocytes. Instead, it was found that the fertilization membranes of these oocytes became elevated in a way which resembled what is seen during insemination of mature oocytes (Moreau et al, 1978b; Guerrier et al, 1978; Shuetz, 1975).

It is very possible that the levels of Ca^{++} released have to be within an appropriate range to induce meiosis reinitiation (Masui and Clark, 1979, unpublished). A series of inhibitors, e.g. D 600, isoptin, Mn^{++}, theophylline, procaine hydrochloride and emetine affect in an apparent competitive way, both the biological response and the release of Ca^{++}. They can thus prevent meiosis, unless the concentration of 1-MA is increased (Moreau et al, 1978b, 1978c) , which allows the Ca^{++} surge to attains the the threshold value.

There is no doubt that the intracellular release of calcium in the oocyte of starfish is a primary event that occurs during hormonal induction and it is a compulsory prerequisite for triggering meiosis reinitiation (Moreau et al, 1978b, 1978c). Dorée et al (1978) demonstrated also that 1-MA can induce the release of calcium ions from isolated plasma membranes of oocytes of M. glacialis. It was also shown that the same inhibitors that act "in vivo" by blocking the release of Ca^{++} ions competitively with the hormone (Moreau et al, 1978b) inhibited the release of Ca^{++} in the "in vitro" system (Dorée et al, 1978). This seems to suggest a way to isolate "in vitro" the whole system present in the cortices of oocytes responsible for the hormonal binding and calcium release.

From the above discussion it is reasonable to conclude that Ca^{++} release is a primary event induced by the hormone interaction which is absolutely required to trigger meiosis reinitiation in oocytes of the starfish.

Presently, the mechanisms by which calcium acts is not yet well understood, but at the present it is believed that calcium release is responsible for the stimulation of two irreversible events that are may be responsible for meiosis reinitiation. These two events are the activation of the Na^{+}-K^{+} pump (Guerrier et al, 1979) and the activation of a cortical phosphoprotein kinase (Guerrier et al, 1975, 1977). It must be emphasized however that only the latter event may have some significance in the process of maturation. The inactivation of Na^{+}-K^{+} pump with ouabain neither blocks the Ca^{++} release nor the

biological response.

In contrast, the endogeneous phosphorylation of a cortical substrate (Guerrier et al, 1975, 1977) was shown to be inhibited by chemicals that block the calcium release (Moreau et al, 1978) and the biological response to the hormone. This may mean that the release of calcium, followed by the activation of a cortical kinase play an important role in meiosis reinitiation in oocytes of starfish.

III.4 PROTEIN PHOSPHORYLATION

The stimulation of protein phosphorylation has been shown to be an irreversible step that occurs in oocytes of starfish. This phosphorylation appears to occur after the intracellular release of calcium ions from the plasma membrane and is caused by the external action of the hormone 1-MA (Guerrier et al, 1975, 1977).

The increase in the activity of the global protein kinase was observed (Guerrier et al, 1975) during the interaction of the hormone with oocytes from M. glacialis and A. rubens, by using endogenous and exogenous substrates. Kinetic studies were performed "in vivo" by Guerrier et al (1977) on the basis of the dependence of the external presence of the hormone. It was observed that the levels of phosphorylation were sharply decreased if the hormone was washed from the incubation medium at the 4th minute, inducing only 13% of the oocytes to undergo meiosis; however, elimination of the hormone at any time during the hormonal independent phase did not affect the levels of phosphorylation and all the oocytes underwent germinal vesicle breakdown at the 18th

minute.

This means that the hormone was required only during the dependent phase, inducing the production of sufficient levels of phosphorylation for G.V.B.D. The incorporation of ^{32}P on an endogenous phosphorylated factor was increased more rapidly in the cortex than in the endoplasm of oocyte (Guerrier et al, 1977). The differences of phosphorylation levels present between the control and the hormonal treated oocytes was shown to be due to protein kinase activity, and not to a decrease in activity of phosphatase (Guerrier et al, 1977).

The steady state level of phosphorylation in the cortex was reached between the 8th and 10th minute after the addition of the hormone, whereas the steady state is achieved during the 30th minute in the endoplasm, 12 minutes after G.V.B.D. Furthermore, the relationship between G.V.B.D. and phosphorylation levels was shown by a noticable decrease of the ^{32}P incorporation into the endogenous substrate, when inhibitors of G.V.B.D. were present in the incubation medium. All inhibitors such as emetine, caffeine, diamide and protease inhibitor Leupeptin were shown to compete with the hormone, but a slight increase in the hormone concentration produced G.V.B.D. and restored the phosphorylation levels to normal.

This kind of evidence suggests a close relationship between protein phosphorylation and G.V.B.D. Is this phosphorylated factor(s) responsible for the reinitiation of maturation?

Kishimoto and Kanatani (1976) showed that a maturation promoting factor (MPF) appears in the cytoplasm of the oocyte of starfish 13 minutes after the external addition of 1-MA. This MPF reached a steady state at the 20th minute, during germinal vesicle breakdown, and stayed at a plateau up to the 40th minute. The level of the maturation promoting factor decreased and almost disappeared during the release of the first polar body.

This correlates very well the findings of Guerrier et al (1977), who have shown that the cortical phosphorylated levels were increased very rapidly during the 5th minute after external addition of the hormone, reaching a plateau during the 9th minute. Germinal vesicle breakdown was found to occur at the 18th minute, whereas the phosphorylated levels were at a plateau in the cortex and more than 50% of the final levels of phosphorylation found during the 30th minute were present in the cytoplasm.

The differences in the ^{32}P incorporation between the control and the hormone-treated oocytes were maintained until the maturation reached the formation of the first polar body. The results obtained by Guerrier et al (1977) and Kishimoto and Kanatani (1976) link the maturation promoting factor with a phosphorylated factor that may be responsible for the induction of maturation in starfish oocytes. In other words, the MPF may be a cortical phosphorylated factor.

CHAPTER IV

MATURATION PROMOTING FACTOR

IV.1 Nature of the Factor

The "maturation promoting factor" (MPF) was the name given by Dettlaf et al (1964) to the factor responsible for inducing maturation in oocytes of Rana pipiens. The presence of this factor in the cytoplasm of maturing nucleated and enucleated progesterone treated oocytes was suggested by the result of experiments involving microinjection of cytoplasm from these maturing oocytes into immature oocytes. The oocytes receiving the matured cytoplasm were induced to undergo germinal vesicle breakdown (Dettlaf et al, 1964; Masui and Markert, 1971). The MPF has been found to be present in other animals as Xenopus laevis (Schorderet-Slatkine and Drury, 1973), Ambystoma mexicanum (Reynhout and Smith, 1974), sturgeon (Dettlaf et al, 1977) and starfish (Kishimoto and Kanatani, 1976).

This factor has been shown to be a non-species specific factor in amphibians (Reynhout and Smith, 1974) and starfish (Kishimoto and Kanatani, 1977). Furthermore, it has been shown that the production of the MPF is a result of a more complicated mechanism than the simple product of the reaction between the receptor molecules and the hormone. This means that the reaction between the receptor and the hormone is not necessary for the production of the MPF. The fact that other hormone non-analogues can induce maturation as well as the natural hormone has already been discussed.

In starfish, it has been shown that substances other than 1-MA, (e.g. DTT, BAL, Ca^{++}) can induce meiosis reinitiation. Similar results were obtained for amphibians, where mercurials (Brachet et al, 1975), Ca^{++} ions (Wasserman and Masui, 1975), valinomycin (Baltus et al, 1977) and La^{3+} ions (Schordered-Slatkine et al, 1976), as well as the natural hormone progesterone can induce maturation.

It seems that the mechanism that produces the MPF is not involved in "de novo" synthesis of macromolecules such as RNA and protein, because their inhibition does not affect the G.V.B.D. (Guerrier and Dorée, 1975). The nature of the MPF has not yet been demonstrated, however there is a high correlation between phosphorylation and the production of MPF. Morril and Murphy (1972) showed that ^{32}P incorporation increases during maturation of oocytes of Rana pipiens. Maller et al (1977) also found that protein phosphorylation on Xenopus laevis was sharply increased a few hours after treatment with progesterone, reaching its highest level at G.V.B.D.

A similar phenomenon was observed by Guerrier et al (1977). Furthermore, Maller et al (1977) report that the microinjection of the cytoplasm of Xenopus hormone-treated oocytes into non-hormone-treated oocytes induces a burst of protein phosphorylation. The above suggests a close relationship between MPF and the increase in levels of phosphorylation. Therefore, it is very possible that a protein kinase may be activated during the process of oocyte maturation, for the purpose of phosphorylating a specific substrate.

More evidence for this can be obtained by injecting commercially prepared kinase into Ambystoma mexicanum (Wiblet et al, 1975) and Xenopus laevis (Moreau et al, 1976). Some attempts to isolate and characterize the MPF in amphibians have been performed by Wasserman and Masui (1976). They isolated a factor that was Mg-dependent, Ca^{++} sensitive and heat labile. The activity was found to exist in three different molecular weight fractions with an optimum pH between 6.5 and 6.8. Drury (1978) modified the Wasserman and Masui method by adding sodium fluoride (NaF) to the extraction medium; this appeared to be a better means of isolating MPF. Drury (1978) proposed that NaF stabilizes the MPF. The stabilization of MPF may be due to the inhibition of phosphoprotein phosphatases. This may open the possibility that the MPF in the oocytes of Xenopus laevis could be a phosphoprotein.

IV.2 MECHANISM OF ACTION OF THE HORMONE 1-MA

The mechanism in which the hormone acts to induce maturation in oocytes of starfish is not yet well understood. However, some indications show that the pattern in which the hormone acts can be explained by the scheme proposed by Masui and Clark (1979).

The scheme is presented in the following way: the hormone (MIS) acts in the external site of the oocyte, producing almost immediately a release of Ca^{++} ions. These calcium ions that are freed from a binding protein in turn activate the regulator site of a phosphodiesterase responsible for the lowering of the CAMP levels. The CAMP

dependent protein phosphorylation is arrested, increasing or activating a CAMP independent kinase. This phosphorylated factor is believed to be the active MPF and its non-phosphorylated form is the inactive precursor of MPF.

The only evidence that supports this theory of maturation of oocytes of starfish has been given by Guerrier and his associates, who demonstrated that the external addition of 1-MA in the incubation medium produces almost immediate release of Ca^{++} ions which trigger an irreversible event which results in the activation of the cortical kinase. This kinase was shown to phosphorylate a cortical substrate that possibly plays the role of a maturation promoting factor. With respect to the activation of a phosphodiesterase (PDE), only indirect evidence obtained by the use of PDE-specific inhibitors, i.e. theophylline, (1,3 dimethylxanthine) (Haring et al, 1976; Anders and Nielson, 1978; Bravo et al, 1978) that inhibits PDE and enhances the level of CAMP blocking the action of 1-MA in the oocytes of starfish (Dorée et al, 1976a; Moreau et al, 1976b) is available. Further studies that may explain the role of cyclic AMP and the phosphorylated factor in the maturation of oocytes of starfish may also prove the validity of the above mechanism.

CHAPTER V

PURPOSE OF THE PROJECT

If a specific maturation promoting factor produced as a result of the action of 1-MA on the oocyte surface, is released as a phosphorylated molecule into the cytoplasm and remains stable enough there so that it is able to promote maturation of other immature eggs when cytoplasm from these maturing oocytes containing this factor is injected into cytoplasm of immature oocytes, then it should be possible as a starting point in the study of the molecular aspects of the maturation process in starfish oocytes to isolate and identify this factor from oocytes that were treated with 1-MA in the presence of ^{32}P ATP. The results of this effort to identify and partially characterize the maturation factor from starfish oocytes are described in this thesis.

CHAPTER VI

MATERIALS AND METHODS.

VI.1 CHEMICALS

Adenosine 5'-(γ ^{32}P) triphosphate triethylammonium salt in 50% ethanol, Code PB.168 (3000 Ci/mM radioact. conc. 1 mCi/ml) and PB 108 (3.55 Ci/mM radioact. conc. 1 mCi/ml) were purchased from Amersham, Buckinghamshire, England.

Two batches of ^{32}P NaH_2PO_4 were obtained, one (PBS-40, 70 Ci/mg radioact. conc. 40 mCi/ml) from Amersham, Buckingamshire, England, and the second (P-32-S, 2 20 mCi/mg, radioact. conc. 40 mCi/ml) from C.E.A., France.

Unlabelled adenosine triphosphate, ethylene glycol bis (B-amino ethyl ether)-N, N, N', N',-tetra acetic acid (EGTA), ethylene diamine tetracetic acid (EDTA), 1-methyladenine (1-MA) and the protein markers were obtained from Sigma Chemical Company. All reagents used in the gel electrophoresis: bromophenol, sodium lauryl sulphate (SDS), acrylamide, N, N'-methylene-bis (Acrylamide), ammonium persulphate, β-mercaptoethanol were purchased from Eastman, Detergent Triton X-100 was obtained from Intertechnique, France.

Acetic acid, tris (hydroxymethyl amino methane, trichloro-acetic acid, folin ciocalteu phenol reagent were obtained from E. Merck, Darmastadt. NaH_2PO_4 and Na_2HPO_4 were obtained from Hinweis Fur Brd, Enschliealich, West Berlin. Coomasie brilliant blue R-250 was purchased from Schuchardt, Munchen. Sudan black B from R.A.L., France.

VI.2 BIOLOGICAL MATERIAL

Starfish Marthasteria glacialis were collected by specimen collectors of the Biological Station of Roscoff, France, off the French coast of Brittany. They were maintained in large aquariums with continous running sea water.

VI.3 COMPOSITION OF THE CALCIUM-FREE ARTIFICIAL SEA WATER

This medium was used to isolate follicle-free oocytes and it is called Shapiro's solution (Shapiro, 1941). The solution contained: 452.2 mM NaCl: 10.08 mM KCl; 29.8 mM $MgCl_2$; 17 mM $MgSO_4$ in 2mM Tris-HCl, pH 7.8

VI.4 PHOSPHATE BUFFER USED IN ENZYME ASSAY

This buffer was used in all the enzyme assays performed "in vitro";for simplicity it was called PK/2. The buffer contained: 6 mM $MgCl_2$, 30 mM Phosphate buffer (pH7.5) and just before using, 7.5 mg of Dithiothreitol (DTT) was added per 25 ml of the buffer.

VI.5 METHOD TO COLLECT IMMATURE OOCYTES

Ovaries were isolated in Shapiro's solution from the arms of the starfish Marthasterias glacialis. Ovaries were quickly dissected, torn with forceps in ice cold Shapiro's solution, and then they were filtered through cheese cloth to collect follicle-free oocytes. The oocytes were washed three times with Shapiro's solution and collected

each time by centrifuging them for 10 seconds in a bench clinical centrifuge.

"Immature follicle-free oocytes" were equilibrated with continuous stirring in Shapiro's solution for at least one hour. This procedure prevents more than 90% of the oocytes from undergoing spontaneous maturation (Dorée and Guerrier, 1975).

VI.5.1 Biological Test

After the equilibration, the oocytes were divided into two equal aliquots containing about $5X10^4$ oocytes/ml in suspension. One aliquot was used as a control, the other was incubated with the hormone, 1-MA 10^{-7}M. Both batches were incubated with continuous stirring at 23°C.

In all cases, the aliquots treated with the hormone showed germinal vesicle breakdown at around the 18th minute after the addition of the hormone (Guerrier and Dorée, 1975).

VI.5.2 Isolation of Oocytes, Cortices and Endoplasm

The cortices were isolated during the hormonal-independent period in the following way:

The oocytes were collected and washed twice in cold Shapiro's solution at low speed in a clinical centrifuge. Then the oocytes were divided in test tubes (a volume of 1 ml of packed oocytes each), washed

with 0.1M $MgCl_2$ in 10mM tris-HCl (pH 8.2) and homogenized in ice cold 0.1M $MgCl_2$ with 10 strokes of a hand homogenizer fitted with a Teflon pestle (Guerrier et al, 1977; Sakai, 1960; Kishimoto et al, 1976). A pellet was obtained by centrifuging at 10^3g in a clinical centrifuge for one minute. The first supernatant was collected (Endoplasmic fraction) and the pellet was washed twice with cold $MgCl_2$ to obtain pure cortices.

VI.5.3. Fractionation of Cortices

VI.5.3.1 KCl Soluble Fraction

A KCl-soluble fraction was obtained from the isolated cortices (Murofushi, 1974) by washing once with 1 mM EDTA containing 10 mM tris-HCl (pH 7.2) and 1 mM GSH to remove water soluble materials which contaminated the cortex preparation. Then the "EDTA-treated cortex fraction" was further extracted with 0.6M KCl in mM tris-HCl (pH 8.4). The supernatant was collected after being centrifuged at 10^3g for five minutes. This supernatant was called "KCl soluble fraction". The pellet was discarded.

VI.5.3.2. Triton Extractions

Cortices were resuspended in 5 mM phosphate buffer (pH 7.2) containing 0.2 Triton X 100 and incubated at 4°C. for thirty (30) minutes (Dorée et al, 1979). The supernatant was collected by centrifuging the mixture at 10^3g for five minutes and the pellet was discarded. The supernatant collected was called "plasma membrane

rich fraction". Further fractionation of the "plasma membrane rich fraction" (PMRF) was done by centrifuging it at 10^5g.

A 10^5g pellet and supernatant were prepared by centrifuging the PMRF at 10^5g for 45 minutes with a S.W. 50.1 Rotor in a Beckman L5-75 Ultracentrifuge.

VI.6 PRELIMINARY ASSAYS PERFORMED "IN VITRO" CONDITIONS

VI.6.1 Determination of ^{32}P-label Incorporated in the TCA-soluble Sub-fraction, "In vitro", Using Total Cortices, Plasma Membrane-rich Fraction and KCl-soluble Fraction from Treated and Non-treated with 1-MA.

The "in vitro" incorporation of the ^{32}P label in the TCA-insoluble sub-fraction was determined in duplicate as follows: Aliquots of 200 ul of each fraction were incubated with 100 ul of PK/2. To study the effect of calcium, to one of them 50 ul of 10 mM Ca^{++} was added, and to the other 50 ul of 10 mM EGTA.

The reaction was started by adding $6X10^{-2}$ mCi in 100 ul of a stock solution of ATP that contained 5 ml of $9.07X10^{-4}$M of unlabelled ATP and 200 ul of $3.8x10^{-4}$M (γ ^{32}P) ATP (20 mCi/mg, radioact. conc. 1 mCi/ml, purchased from C.E.A.) to a $8.86X10^{-4}$M total ATP concentration. The final assay volume was 450 ul.

The reaction mixture was incubated for 20 minutes at 23°C. Incubation was stopped by the addition of 2 ml of ice-cold 25% TCA(v/v), and it was kept overnight at 4°C. The first supernatant was used to

measure the ^{32}P free in solution. The TCA-insoluble sub-fraction was collected by centrifuging at 10^3g for one minute, and then it was washed three times with cold 10% TCA(v/v) and finally washed with 100% ethanol (Guerrier et al, 1977). The final TCA-insoluble sub-fraction was hydrolyzed with 3 ml of 0.5M NaOH for 10 minutes at 100°C.

Aliquots of the NaOH digest were taken for protein determination according to Lowry et al (1951). Finally the label incorporated was measured using Cerenkow counting (Parker and Elrick, 1970) in an Intertechnique SL-30 Liquid Scintillation Counter.

VI.6.2 Influence of Isotopic Dilution

These studies were performed to observe the effect of the incorporation of the label using different concentrations of ATP solutions. Three different stock solutions were prepared as follows:

Solution "A": contained 20 ul of $3.8X10^{-4}M$ ($\gamma\ ^{32}P$) ATP solution (20 mCi/mg, radioact. conc. 1 mCi/ml) and 200 ul of $4X10^{-3}M$ unlabelled ATP; the final concentration was $3.67X10^{-3}M$ ATP.

Solution "B": contained 20 ul od $3.8X10^{-4}M$ ($\gamma\ ^{32}P$) ATP solution (20 mCi/mg, radioact. conc. 1 mCi/ml) and 200 ul of $4X10^{-4}M$ unlabelled ATP to a final concentration of $3.67X10^{-4}M$ ATP.

Solution "C": contained 20 ul of $3.8X10^{-4}M$ ($\gamma\ ^{32}P$) ATP solution (20 mCI/mg, radioact. conc, 1 mCi/ml) and 200 ul of PK/2 to a final concentration of $3.67X10^{-5}M$ ATP.

VI.6.2.1 Total Dephosphorylation Activity in the Enzymatic Assay

In these assays a "plasma membrane rich fraction" was used. The assay was performed in duplicate as follows: to each volume of 200 ul of PMR fraction, 150 ul of PK/2 was added. To one 50 ul aliquot of 10 mM EGTA solution, and to the other 50 ul of PK/2 was added. To reaction was started by adding 20 ul of either A,B, or C to a final volume of 370 ul at 23°C. Two incubation time periods were measured, one at 0 minute and the other at 20 minutes.

The enzyme reaction was stopped with 2 ml of ice-cold 25% TCA (v/v) solution, the proteins were allowed to precipitate at 4°C for at least three (3) hours. The free ^{32}P and ATP present in the supernatant was measured in the following way: 0.5 ml of the supernatant was added to 1 ml of a 20% active charcoal solution to absorb the ATP in solution and another 0.5 ml were added to 1.0 ml of distilled water to measure the total label present in the supernatant. After one minute incubation, the charcoal was centrifuged and 500 ul were taken from the two test tubes and the radioactivity was measured by Cerenkow counting (Parker and Elrick, 1970).

VI.6.2.2 Incorporation of the Label ^{32}P in the TCA Insoluble Sub-fraction

The volumes for the enzyme were similar to the one in Part VI.6.1. The enzyme reaction was stopped with 2 ml of ice-cold 25% TCA and then 50 ul of 2 mg/ml albumin solution were added to trap the insoluble TCA

sub-fraction in order to decrease any loss during the following washing: the mixture was incubated overnight.

The TCA-insoluble sub-fraction was washed three times with 10% TCA and once with 100% Ethanol. TCA-insoluble sub-fraction was hydrolyzed in 3 ml of 0.3M NaOH by incubating for 10 minutes at 100°C. and then radioactivity was measured by Cerenkow counting (Parker and Elrick, 1970).

VI.7 RELEASE OF CALCIUM IONS "IN VITRO" (AS MEMBRANE MARKER)

The release of Ca^{++} ions from the "plasma membrane rich fraction" in the presence of 1-MA was measured in the presence of the photoprotein Aequorin using the method described by Dorée et al (1978).

VI.8 PARTIAL CHARACTERIZATION OF THE PHOSPHORYLATED FACTOR

VI.8.1 "In vivo" System

About 4.4 mCi of ^{32}P NaH_2PO_4 (200 ul of P-32-S-2 and 100 ul of PBS 40. For spec. act. and radioact. conc., see section on materials) were injected into 60 ml of $5X10^4$ oocytes/ml in Shapiro's solution. The oocytes were incubated with gentle stirring for two hours at 23°C.

After the incubation, the eggs were divided into six portions of 10 ml and washed three times with Shapiro's solution and recuperated by centrifuging at 10^3g for five minutes in a clinical centrifuge.

The six fractions of packed eggs were pooled and diluted to 60 ml with Shapiro's solution. Then the pool was divided into two equal parts of 30 ml in two 100 ml beakers; one of them was used as a control and to the other 50 ul of 10^{-3}M 1-MA was added. Both fractions were incubated at 23°C. with continuous stirring.

Fractions of 10 ml were taken at 10 minutes, 30 minutes and 53 minutes. Each 10 ml fraction was treated in the following way: the oocytes were packed and washed once with 10 ml of cold Shapiro and then washed immediately with 10 ml of 0.1M $MgCl_2$ in 10 mM tris-HCl (pH 7.8). The packed oocytes were resuspended in 10 ml of 0.1M $MgCl_2$, homogenized with 10 strokes in a hand homogenizer. One ml of the total homogenate was kept. The remaining 9 ml were centrifuged for 2 minutes at 10^3g. 1 ml of the first supernatant was collected. The pellet was washed once with $MgCl_2$, obtaining clean cortices (Guerrier et al, 1977).

The cortices were solubilized in 200 ul of 2% SDS in 5 mM tris-HCl (pH 7.5) and incubated at 70°C. for 10 minutes for completed solubilization, then β-mercaptoethanol was added to a final concentration of 1% (v/v). To the total homogenate and endoplasm fraction a solution of 20% SDS (w/v) was added to a final concentration of 2% SDS and then incubated in 70°C water bath for 10 minutes. Each fraction was electrophoresed on 7% SDS acrylamide gels in duplicate.

The radioactivity present on the gel was scanned with a Berthold scanning device in an atmosphere of Methane. The densitogram of the gels

was obtained by using Vernon gel scanner apparatus.

VI.8.2 "In Vitro"

Cortices were freshly prepared from oocytes treated and non-treated with 1-MA. The plasma membrane rich fraction was extracted as in VI.5. The enzyme assay was performed as follows: 200 ul of (γ ^{32}P) ATP (PB 168. For spec. act. and conc., see section on materials) were injected in two different flasks and then taken to dryness. Then 50 ul of PK/2 were added to each. To one of these, 100 ul of plasma membrane rich fraction prepared from oocytes that were not treated with 1-MA was added, and to the other was added 100 ul of plasma membrane rich fraction prepared from oocytes treated with 1-MA, simultaneously. The enzyme mixture was incubated at 23°C. for 15 minutes. The reaction was stopped by adding a volume of 20% SDS (v/v) to a final concentration of 2% SDS. The mixture was incubated in a water bath at 100°C. for 2 minutes, after which the two fractions were electrophoresed on a 7% SDS acrylamide gel. The radioactivity pattern and densitogram of the gel were obtained as in Part VI.8.1.

VI.8.3 Control for the Presence of Non-specific Label Present in the SDS Polyacrylamide Gels

Freshly prepared "plasma membrane rich fraction" was obtained from oocytes non-treated with the hormone. The PMR fraction was divided into two volumes of 1.5 ml each; one was denatured by adding 150 ul of 20% SDS (v/v) and boiled for 2 minutes at 100°C., and to the other,

150 ul of distilled water was added and it was kept at 4°C.

The enzyme assay was performed as follows: two volumes of 200 ul of (γ ^{32}P) ATP (100 ul of PB 108 and 100 ul of PB 168. For spec. act. and conc., see section on materials) were reduced to dryness. To the two flasks, 50 ul of PK/2 were added, and the reaction was started by adding simultaneously, to one, 100 ul of PMR fraction denatured, and to the other, 100 ul of the active PMR fraction, incubated for 30 minutes at 23°C. The reaction was stopped by adding a volume of 20% SDS (v/v) to a final concentration of 2% SDS to eqch flask and then incubating them in a water bath at 100°C. for 2 minutes .

The fractions were electrophoresed as usual and the radioactivity present in the lower reservoir of the electrophoresis apparatus was determined by using activated charcoal as in VI.6.2.1.

VI.9 DISTRIBUTION OF THE ^{32}P LABEL IN THE ORGANIC AND AQUEOUS LAYER AFTER EXTRACTION DURING "IN VITRO" ASSAY

The phosphorous components of the cortices of starfish were extracted using the method of Palmer and Verpoorte (1971), with some modifications. The cortices were obtained in the usual way, and then they were washed with PK/2, the pellet was recuperated by cenrifuging at 10^3g for 5 minutes. The total pellet was diluted up to a volume of 4 ml with PK/2 and the reaction was started by adding 1 ml of 10^{-3}M ATP containing 200 mCi of (γ ^{32}P) ATP of PB 168 (For spec. act. and conc., see section on materials). The reaction mixture was incubated with gentle stirring for 30 minutes at 23°C.

After incubation, the mixture was cooled to $0^{o}C$. to slow down the reaction, and centrifuged at $10^{5}g$ for 15 minutes. The pellet was recuperated and washed twice with 10 mM EDTA to chelate any cationic ions. The pellet was recuperated every time by centrifuging at $10^{5}g$ for 15 minutes in a Beckman L5-75 ultracentrifuge.

Finally the pellet was diluted with 3.5 ml of 0.2% Triton (v/v) in 1 mM phosphate buffer (pH 7.2) to a total volume of 4.5 ml. The same volume of Butanol was added and the pellet was extracted for six hours at $4^{o}C$. under continuous stirring. A milky solution was observed. To obtain a clear Butanol phase, the solution was centrifuged overnight at $10^{3}g$ ($4^{o}C.$). Three phases were observed: a Butanol phase at the top, a water phase at the bottom, and a milky interphase. The Butanol phase of 2.8 ml volume was recuperated. To both the remaining water phase and interphase, a volume of 100% acetone was added. The mixture was brought almost to dryness under pressure using a Roto Vapor, and to the pellet, 3 ml of a chloroform-methanol-HCl (200:100:1) was added and incubated under continuous stirring for three hours at $4^{o}C$. The chloroform mixture was separated from the pellet.

The following steps were performed to determine the label incorporated in the Butanol, chloroform and protein fractions:

The organic phases (Butanol and chloroform phases) were washed with water several times until no more label was obtained in the aqueous phase. The protein pellet was washed in the usual way and

hydrolyzed in NaOH. Finally, the label in the three fractions was determined by using Cerenkow counting.

VI.9.1 Localization of the Lipidic Zone

The chloroform phase was taken almost to dryness under pressure using a Roto Vapor, and then prepared for electrophoresis in the same way as the other fractions. Phospholipid mixtures obtained from Boehringer Nannhein were treated in the same way to be used as standard to determine their positions on the gels. The gels were run in duplicate in the usual way. After electrophoresis, one gel was stained with Coomasie brilliant blue and the other with Sudan black B, as described in VI.11.1 and VI.11.3.

VI.10 ANALYTICAL METHODS

VI.10.1 Protein Concentration

Protein concentration was determined by the method of Lowry et al (1951), using crystalline bovine serum albumin as a standard.

VI.10.2 Cerenkow Counting

Radioactivity was counted in the Intertechnique Liquid Scintillation Counter. Samples in plastic vials were counted using Cerenkow settings. The Cerenkow setting is based on the reading of the S-1 of a faint bluish-white light produced by a high concentration of β-emitters of ^{32}P with a Emax of 1.71 Mev in a homogenous solution.

This type of radiation is generated when a charged particle travels through a medium faster than the speed of light through that medium, and it is known as Cerenkow radiation (Parker and Elrick, 1970).

An efficiency of 35% was obtained using this method.

VI.10.3 SDS Polyacrylamide Gel Electrophoresis

The gel electrophoresis was performed according to Weber and Osborn (1969), with some modifications. The apparatus used was a standard disc-electrophoresis apparatus connected to a power supply, VOKAM. The following stock solutions were used to prepare the gels:

Solution A: 0.1% SDS (w/v) in 1 M phosphate buffer (pH 7.5)

Solution A_1: 80 ml of A
20 ml of distilled water (D.W.)
0.23 ml of TEMED

Solution B: 28% Acrylamide (w/v)
0.74% Bis-acrylamide (w/v)
in 100 ml of D.W.

Solution C: 8% SDS (w/v)

Solution D: 1% Ammonium persulfate (w/v)

The proportions of the above solutions to make a 7% Acrylamide gel were: 2 ml of A_1; 4 ml of B; 2 ml of C and after de-aeration, 8 ml of D were added.

The mixture was added gently to a set of tubes of 8 mm diameter per 12 cm long. Using a fine syringe, a few drops of water were layered on top of the gel solution to prevent a deep miniscus from forming once the gel was set. The gels were allowed to polymerize for about 30 minutes. The electrophoresis buffer was prepared by mixing 82.22 ml of 0.2 M sodium phosphate monobasic with 167.73 ml of 0.2 M sodium phosphate dibasic and diluted twice using D.W. and finally o.25% SDS (w/v) were added (pH 7.5). The gels were run at 5 MA/gel.

VI.10.3.1 Sample Preparation

The fractions were solubilized in 2% SDS (w/v) in 5 mM tris-HCl (pH 7.5) and 5% β-mercaptoethanol (v/v), followed by an incubation for 2 minutes at 100°C. Then 5 ul of a concentrated solution containing Bromophenol (tracking dye) and glycerol were added to each fraction before overlaying on top of the gel. About 100 ug of protein were placed on top of each gel. Electrophoresis was allowed to proceed until the tracking dye was 2 cm from the end of the gel.

VI.10.3.2 Standard Proteins

The following standard proteins were used to calibrate the gel:

. Bovine serum albumin	M.W. 68,000	Weber and Osborn, 1969
. β galactosidase (bovine)	M.W. 32,000	Chytil, F., 1965

. Trypsin M.W. 23,300 Weber & Osborn, 1969

. Lysozyme M.W. 14,300 Weber & Osborn, 1969

VI.11 STAINING AND DESTAINING

After electrophoresis, the gels were stained and destained in the following way:

VI.11.1 Total Proteins

Staining for proteins with Coomasie brilliant blue R-250: the gels were placed in test tubes containing a solution of 30% isopropyl alcohol (v/v), 12% acetic acid (v/v) and 0.025% Coomasie brilliant blue (w/v) for twelve (12) hours. Then they were destained with 7.5% acetic acid, in volumes of 200 ml/gel. Gels were preserved in acetic acid (7.5%).

VI.11.2 Glycoproteins

The gels were stained for glycoproteins using a Periodic Acid Schiff stain (PAS) as described by Zacharius et al (1969). The gels were destained and stored in 7.5% acetic acid (v/v).

VI.11.3 Lipid Staining

The gels were stained for the presence of lipids with Sudan black B. The staining of the gels was carried out in a saturated solution of Sudan black B in 60% v/v ethanol, to which 0.1 ml of a

25% NaOH (w/v) was added per 50 ml of the Sudan black B stock solution. The gels were stained for about 12 hours and destained in 50% ethanol (Sargent, 1965).

VI.11.4 Phosphoprotein Staining

Stain-All (Cationic carbocyanine dye) was used to visualize phosphoproteins (Green et al, 1973). After electrophoresis, the gels were incubated for 10 hours in 25% (v/v) isopropanol to remove SDS. The presence of SDS in the gels interferes with the dye.

A stock solution of the dye was prepared as follows: 0.1% stain-All was prepared in 100% formamide in a dark bottle covered with aluminium paper. The staining solution contained 10 ml of the stock stain, 10 ml formamide, 50 ml isopropanol, 1 ml 3 M tris-HCl pH 8.8 and distilled water to a volume of 200 ml. The gels were rinsed and incubated in the stain solution in the dark for twelve (12) hours; the gels were destained with distilled water until there was a clear background.

CHAPTER VII

RESULTS

VII.1 ISOLATION AND FRACTIONATION OF THE OOCYTES CORTICES

The modification of Sakai's (1960) method as described by Kishimoto et al (1976) was used to prepare cortices from starfish oocytes (i.e. normal oocytes and oocytes that were treated with 1-methyladenine). A typical preparation of cortices obtained by this method is shown in the micrograph in Figure 1. The preparation contains clean ghost membranes with very few cortical granules attached. Cortical preparations were subjected to two extractions: (1) the cortices were extracted with KCl according to the method of Murosfushi (1974) in order to isolate a cortical protein kinase, and (2) cortices were extracted with 0.2% Triton X 100 in 5 mM phosphate buffer pH 7.2, to isolate endogenous substrate. Triton X-100 is a non-ionic detergent that mobilizes membrane-bound protein forming small vesicles. Dorée et al (1978) reported that at least 40% of the total protein present in the cortices could be extracted by this method.

VII.2 PRELIMINARY STUDIES TO STANDARDIZE THE ENZYMATIC ASSAY "IN VITRO" CONDITIONS

VII.2.1 Determination of the Radioactivity (^{32}P) Incorporated into the TCA-insoluble Sub-fraction of the Three Cortical Fractions "In vitro" Conditions

Figure 1: Cortices isolated from starfish M. glacialis using the method of Sakai (1960), modified by Kishimoto et al (1976).

A standard enzyme assay "in vitro" was set up to determine the specific activity of ^{32}P label incorporated in the TCA-insoluble sub-fraction using the following cortical fractions: (1) Total cortices, (2) KCl fraction, and (3) plasma membrane rich fraction.

Using the same assay, the effects of millimolar concentrations of calcium and EGTA was also studied. The results in Figure 2, which are presented in the form of a histogram, show the levels of radioactivity per mg of protein incorporated into the three fractions. Further work was performed using "control EGTA-PMR fraction"; this fraction was shown to incorporate the highest levels of radioactivity in its TCA-insoluble sub-fractions.

VI.2.2 Isotopic Dilution

To establish the isotopic concentration necessary to maximize the incorporation of the label into the TCA insoluble sub-fraction of the "control EGTA-PMR fraction", three different ATP solutions with different isotopic dilutions were used in the enzyme assay. This assay was done in duplicate in order to study at the same time the effects of EGTA versus a control. The results summarized in Table 1 show a very small amount of the label is incorporated into the TCA-insoluble sub-fraction (0.14% to 1.67%), and it also shows that by decreasing the ATP concentration and increasing the specific activity of the isotopic solution, EGTA does not facilitate the incorporation of ^{32}P in the TCA-insoluble sub-fractions but rather shows an increase of the hydrolysis of ATP during the enzyme assay.

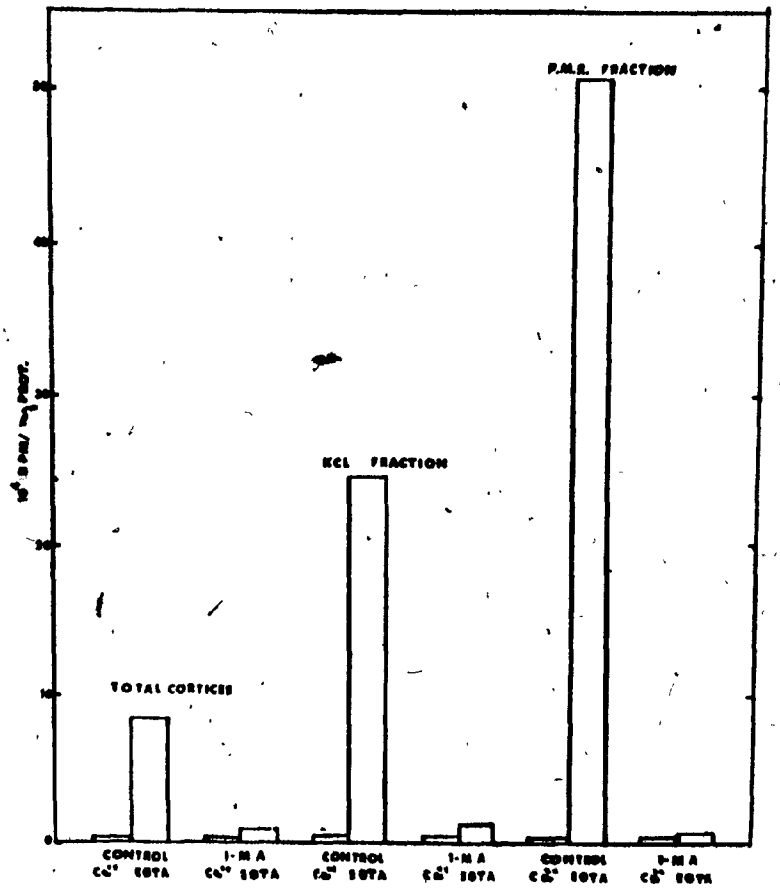

Figure 2: Incorporation of the ^{32}P "in vitro" from (γ ^{32}P)ATP into the total cortices, KCl fraction and P.M.R. fraction. The assay was performed as indicated in the Methods section.

TABLE 1

Distribution of radioactivity from three different isotopic solutions of (γ ^{32}P) ATP in the TCA soluble and insoluble fractions of the "in vitro" assay.

	Percentage of total ^{32}P radioactivity			
Assay[a]	TCA insoluble sub-fraction 20 min.	TCA soluble sub-fraction 20 min.	Spontaneous hydrolysis 0 minute	Remaining ATP 20 min.
A	0.14	5.84	9.17	84.85
A_{EGTA}	0.24	7.45		83.14
B	0.92	17.34	11.06	70.68
B_{EGTA}	0.95	26.65		61.34
C	1.67	35.70	11.11	51.52
C_{EGTA}	1.44	48.21		39.24

a. This assay was done in triplicate using three different isotopic dilutions, with or without EGTA, as explained in the Methods section. The protein concentration was 1 mg/ml. Counting efficiency was 35%.

VII.2.3 Release of Ca^{++} Ions "In vitro" as Membrane Marker

Dorée et al (1978) reported that the release of calcium is produced "in vitro" in the presence of the hormone 1-MA. It has been shown by Guerrier et al and Kanatani et al that the 1-MA receptor is present in the cortical region of the oocytes. Therefore, in this case, the release of calcium from the "control PMR fraction" in the presence of 1-MA is used as a membrane marker. Figure 3 shows the levels of calcium released from the "control PMR fraction" after the addition of the hormone; the levels of calcium were measured as described in the Methods section of the text.

VII.3 PARTIAL CHARACTERIZATION OF THE PHOSPHORYLATED FACTOR

The next step to identify and determine the nature of a phosphorylated factor observed on SDS acrylamide gels of extracts of cortices obtained after cortices were labelled with (γ ^{32}P) ATP while they were being treated with 1-MA under "in vivo" and "in vitro" conditions.

VII.3.1 "In vivo" Conditions

"In vivo" condition was established as follows: Immature starfish oocytes were isolated in Ca^{++} free artificial sea water (Shapiro's solution), then they were pre-charged with (^{32}P) NaH_2PO_4 washed and incubated with or without 1-MA. The protein and ^{32}P label pattern of cortices isolated from oocytes treated 10 minutes with 1-MA is

IN VITRO INDUCED RELEASE OF CALCIUM IONS FROM PLASMA MEMBRANE

Figure 3: Light response produced by the photoreaction of aequorin with the calcium ions. 500 ul of plasma membrane-rich fraction containing 50 ul of aequorin were tested. (A) 100 ul of 5mM tris-HCl (pH 7.2). (B) 50 ul of 10^{-5}M 1-MA. (C) 100 ul of 5 mM tris-HCl (pH 7.2). (D) 50 ul of 10^{-5}M 1-MA. (E) 250 ul of 3×10^{-7}M $CaCl_2$. Vertical and horizontal bars correspond respectively to 1.3 nA anode current and to 12 sec.

shown in Figure 4. The ^{32}P radioactivity incorporated in the band present between 0.8 and 1.0 relative mobility was approximately 300 cpm. The protein and ^{32}P label patterns of control cortices is shown in Figure 5. Figure 6 shows the protein and label patterns of the endoplasm obtained from oocytes treated 53 minutes with the hormone. In the control gel, 112 cpm were incorporated in the band present in the range of 0.8 and 1.0 relative mobility. The endoplasm obtained from oocytes treated with the hormone at 10, 30 and 53 minutes did not show any label in the gels.

VII.3.2 "In vitro" Conditions

For the "in vitro" system, the enzymatic assay was performed with the endogenous substrate and kinase present in the "plasma membrane rich fraction" obtained from cortices of oocytes treated with or without the hormone (1-MA) for 15 minutes.

The protein and label pattern of the "1-MA-PMR fraction" is shown in Figure 8 ; most of the ^{32}P radioactivity was incorporated between 0.8 and 1.0 mobility. This was about 450 cpm, whereas the protein and label pattern of the "control PMR fraction" in Figure 7 shows that the label was incorporated as well in the 0.8 to 1.0 relative mobility and was about 3,150 cpm. The efficiency obtained in the Berthold scanning device in which the gels were scanned was 20% for the ^{32}P isotopes.

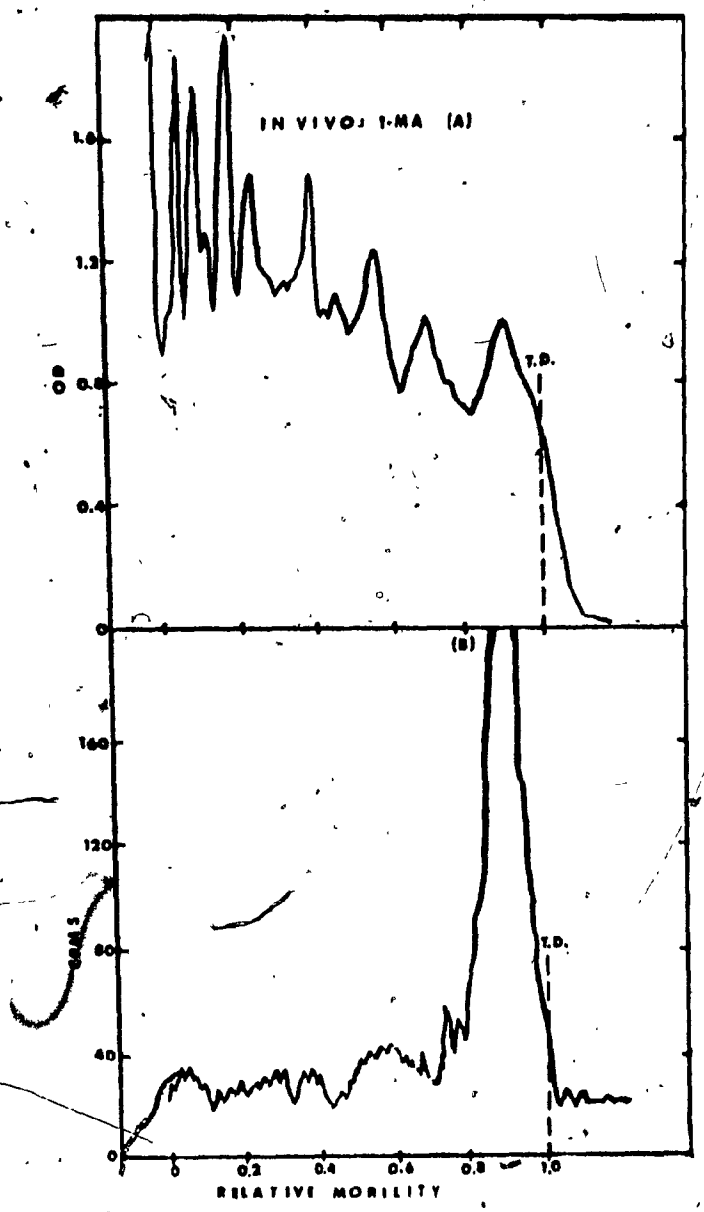

Figure 4: "In vivo" phosphorylation. The oocytes were pre-charged with (^{32}P) PO_4H_2Na and then incubated 10 minutes with the hormone 1-MA. The cortices were isolated as described in the Methods section. Protein (A) and label (B) pattern of the SDS polyacrylamide gel.

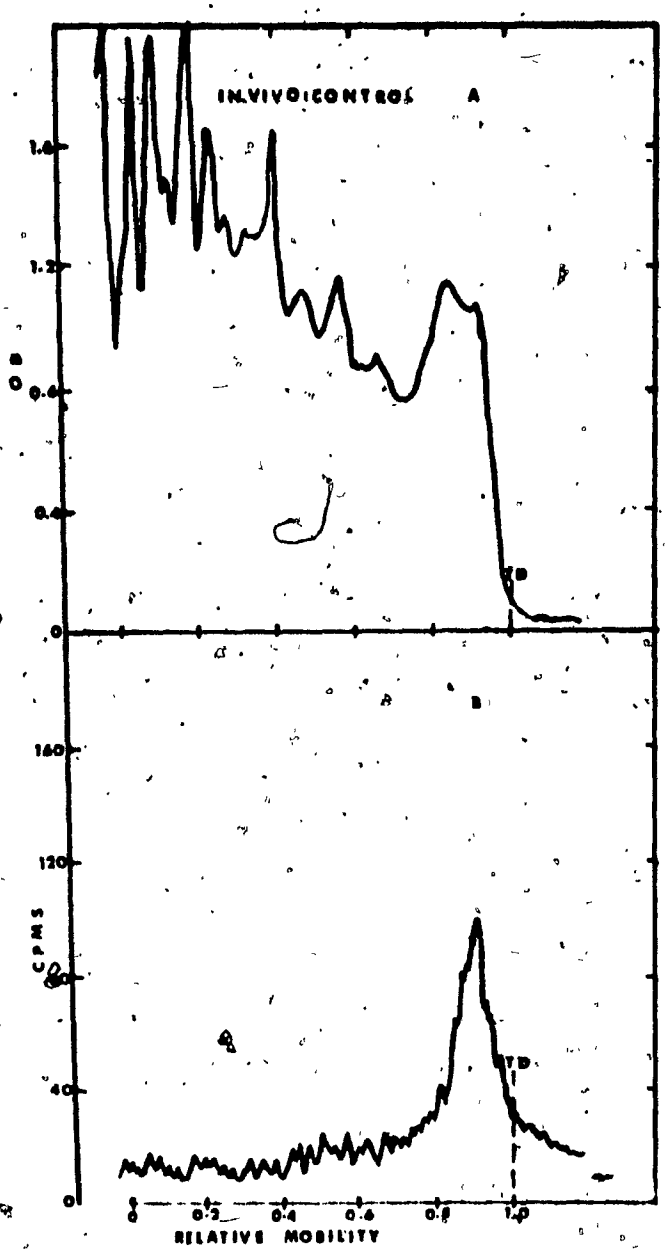

Figure 5: "In vivo" phosphorylation. The oocytes were pre-charged with $(^{32}P)PO_4H_2Na$ and then incubated 10 minutes without the hormone 1-MA. The cortices were isolated as described in the Methods section. Protein (A) and label (B) of SDS acrylamide gel.

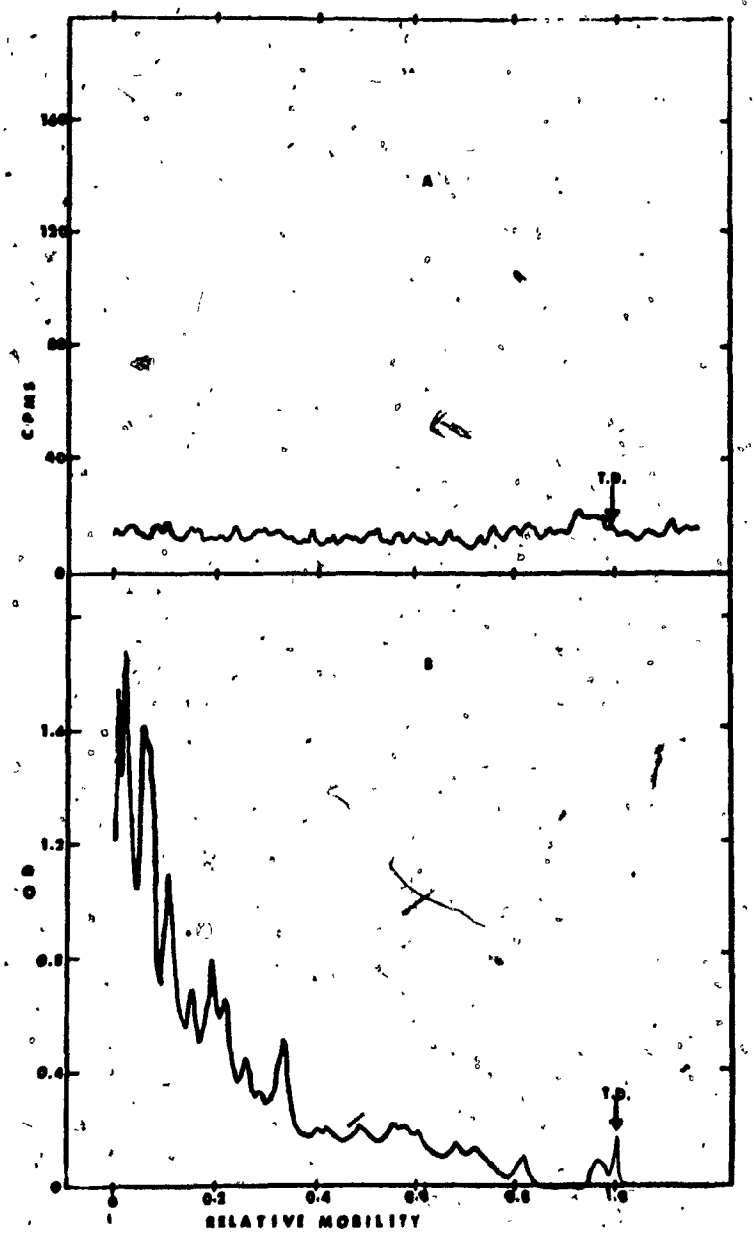

Figure 6: "In vivo" phosphorylation. The endoplasm from oocytes pre-charged with $(^{32}P)PO_4H_2Na$ and incubated 53 minutes with the hormone (1-MA) was isolated, dissolved and electrophoresed as described in the Methods section. Label (A) and protein (B) patterns of the SDS acrylamide gel.

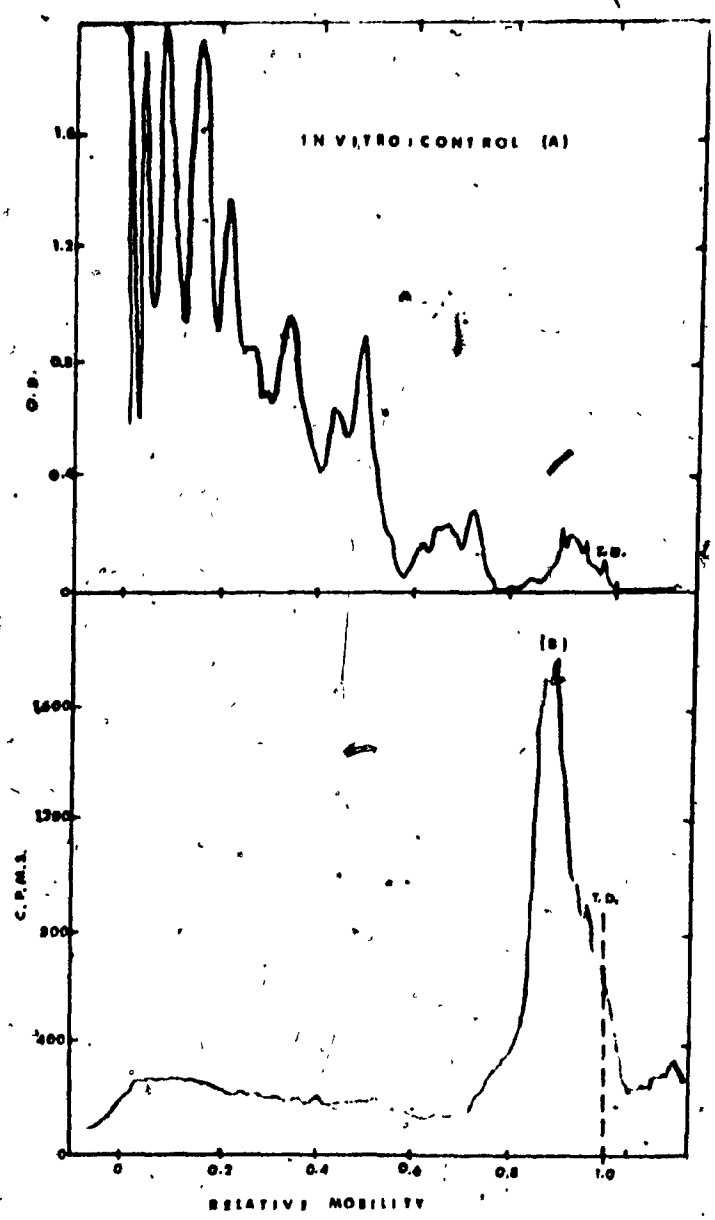

Figure 8 "In vitro" phosphorylation assay using endogenous kinase and substrate present in the plasma membrane-rich fraction obtained from control oocytes. The membranes were dissolved and electrophoresed as described in the Methods section. Total proteins (A) stained with Coomasie brilliant blue. Label (B) incorporated into the gel.

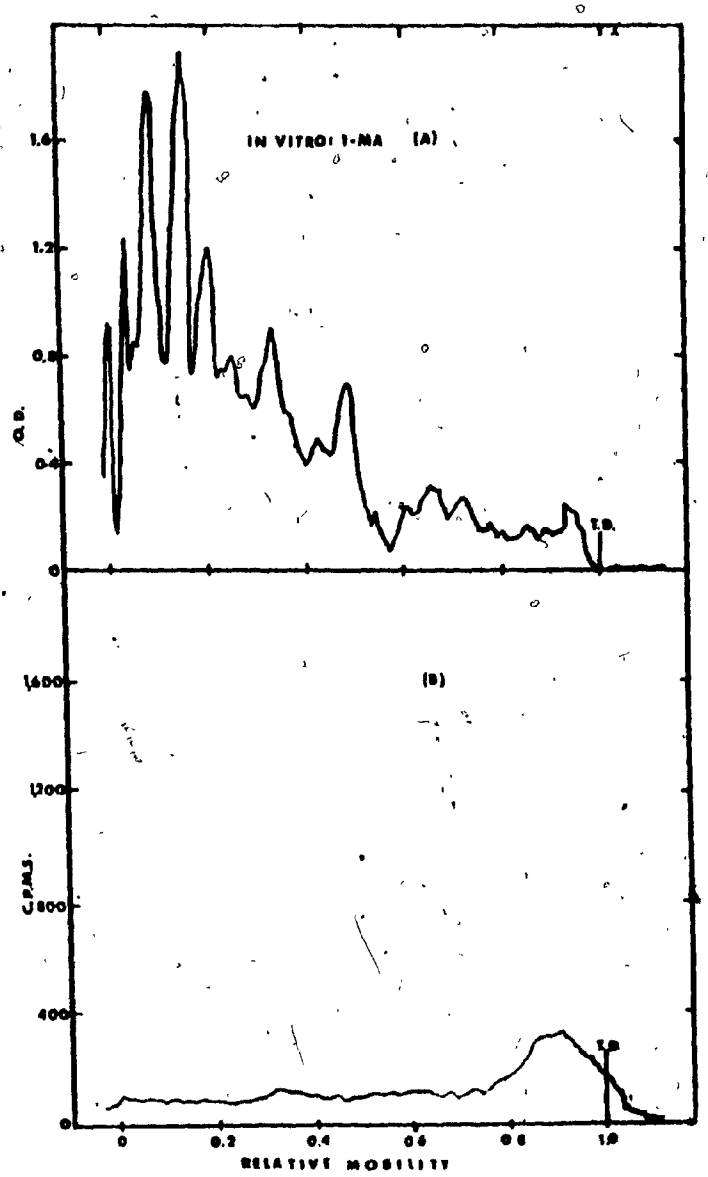

Figure 8: "In vitro" phosphorylation assay using endogenous kinase and substrate present in the plasma membrane-rich fraction obtained from oocytes treated with 1-MA. The membranes were dissolved and electrophoresed as described in the Methods section. Total proteins (A) stained with Coomasie brilliant blue. Label (B) incorporated into the gel.

VII.3.3 Control for Non-specific Label in the Gel

A control for non-specific label in the gel was established to show that none of the ^{32}P radioactivity incorporated in VII.3.1 and VII.3.2 of the SDS acrylamide gel was due to contamination, but only to incorporation of the ^{32}P into a substrate that had a specific relative mobility. The results in Figure 9 show that under the conditions established for this control (described in the Methods section), non-specific radioactivity was found present after the staining and destaining of the gel.

VII.3.4 Determination of the Apparent Molecular Weight of the Band that Incorporated the ^{32}P Radioactivity on SDSPAGE

Figure 10 shows the localization of the radioactive label in the gel with respect to the standard protein with known apparent molecular weight. The radioactivity was shown to be present in a band which migrates in a position between trypsin M.W. 23,300 and lysozyme M.W. 14,300.

VII.4 NATURE OF THE SUBSTRATE

After the determination of the relative mobility of the band that incorporates the radioactivity in the gel, the nature of the substrate was studied by using different staining procedures. Three different types of macromolecules were considered as possible substrates for the kinase enzyme, and they were:

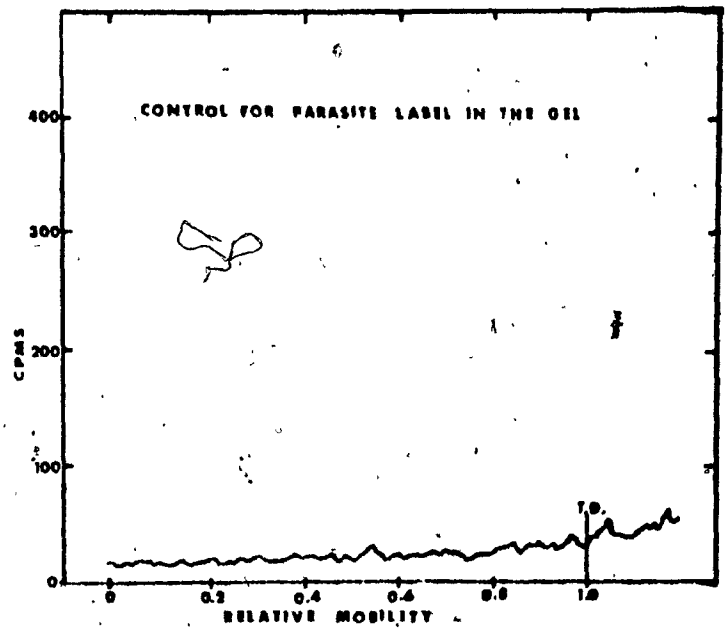

Figure 9: Control for the presence of non-specific parasite label present in the SDS acrylamide gel. The cortices were prepared and electrophoresed as described in the Methods section.

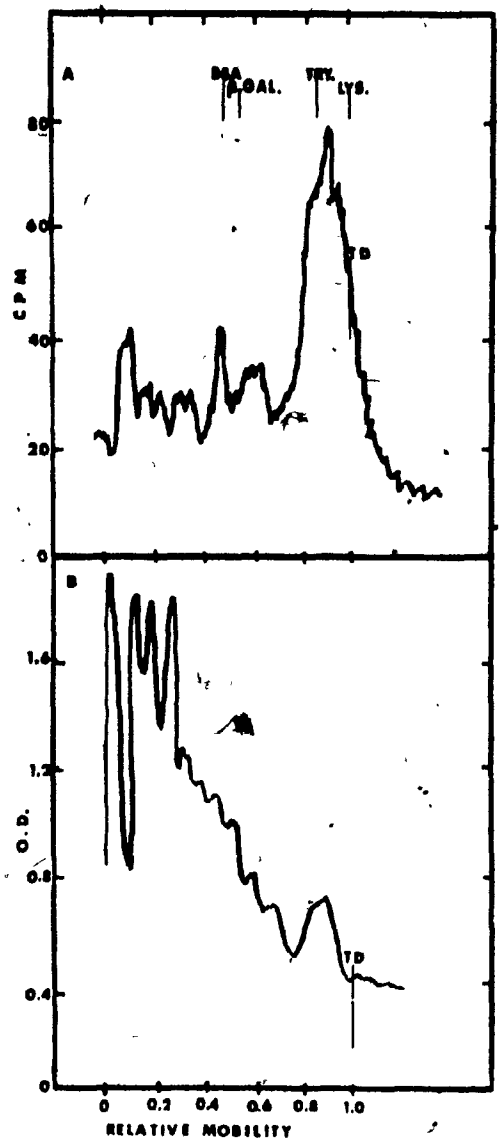

Figure 10: Localization of the radioactive label on the gel with standard proteins. The cortex fraction used in this assay was done with oocytes pre-charged with $(^{32}P)PO_4H_2Na$ and then incubated 30 minutes with the hormone. Label (A) and protein (B) pattern of the SDS acrylamide gel.

VII.4.1 Lipids

The extraction of the lipid fraction was performed by using the method of Palmer and Verpoorte (1971), with some modifications as described in the Methods section of the text. The results (Figure 11) showed that the chloroform phase contained 48% of the label. This agrees with results of Palmer and Verpoorte (1971), who reported that this phase contained the major part of the phosphorylated lipids. The chloroform phase was treated as described in the Methods section, electrophoresed and stained with Sudan black B, a specific stain for lipids. The pattern of the radioactivity and lipid zone were correlated after locating the radioactivity in the Berthold scanning device. Figure 12 shows that the position of the lipidic zone was located beyond the tracking dye.

VII.4.2 Glycoproteins

Another possible substrate was a glycoprotein. Electrophoresis was carried out in duplicate. One gel was stained with Coomasie brilliant blue to locate the proteins and the other with PAS as described in the Methods section of the text in order to locate carbohydrate-containing molecules. The data in Figure 13 shows that the major glycoproteins were correlated with proteins stained with Coomasie brilliant blue and they correspond to higher molecular weight species. However, positive Schiff reaction was observed in a broad band which migrated just below the tracking dye in the position that was shown to be occupied by lipid-containing molecules.

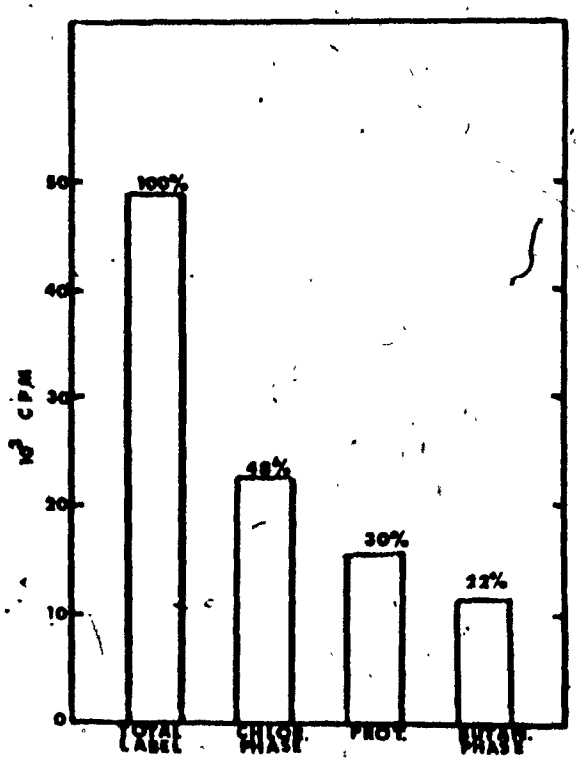

Figure 11: Distribution of the ^{32}P label in the organic and aqueous phase during "in vitro" enzyme assay.

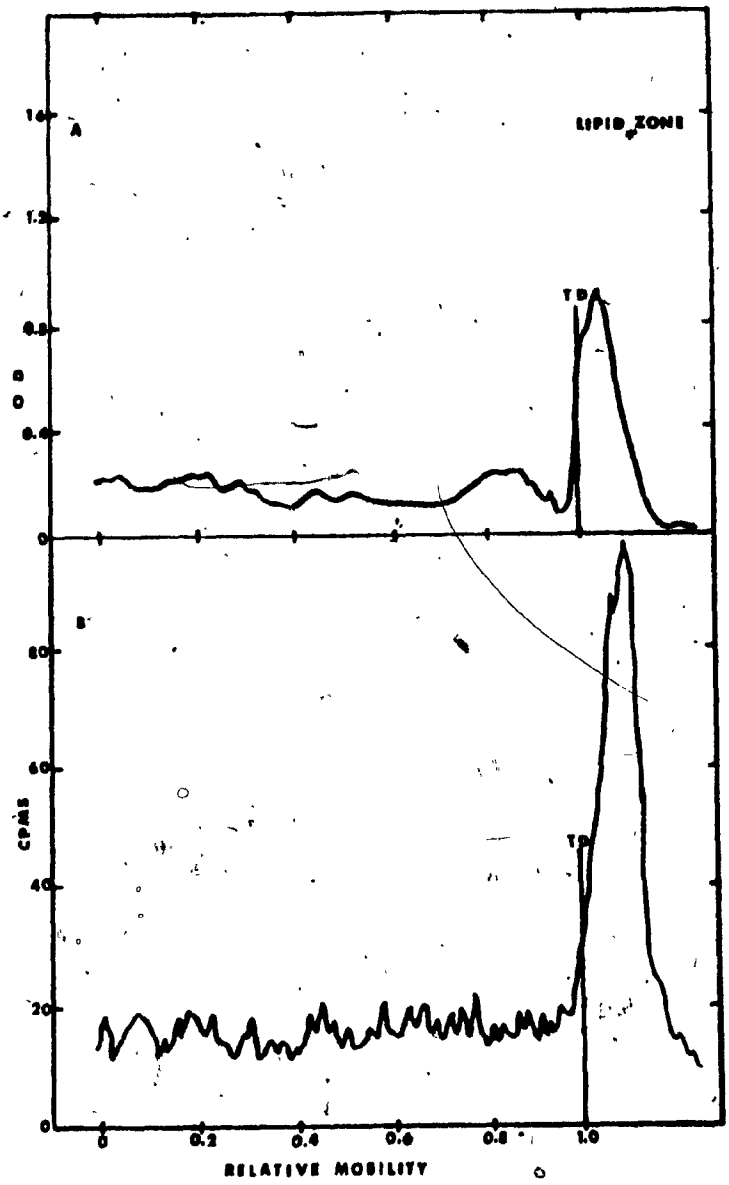

Figure 12: Incorporation of the ^{32}P label in the lipid zone. The extraction of the lipids from the oocytes cortices was done as described in the Methods section. The gel was stained for lipids (A) with Sudan black B. The label (B) incorporated in the gel corresponds to the lipid zone.

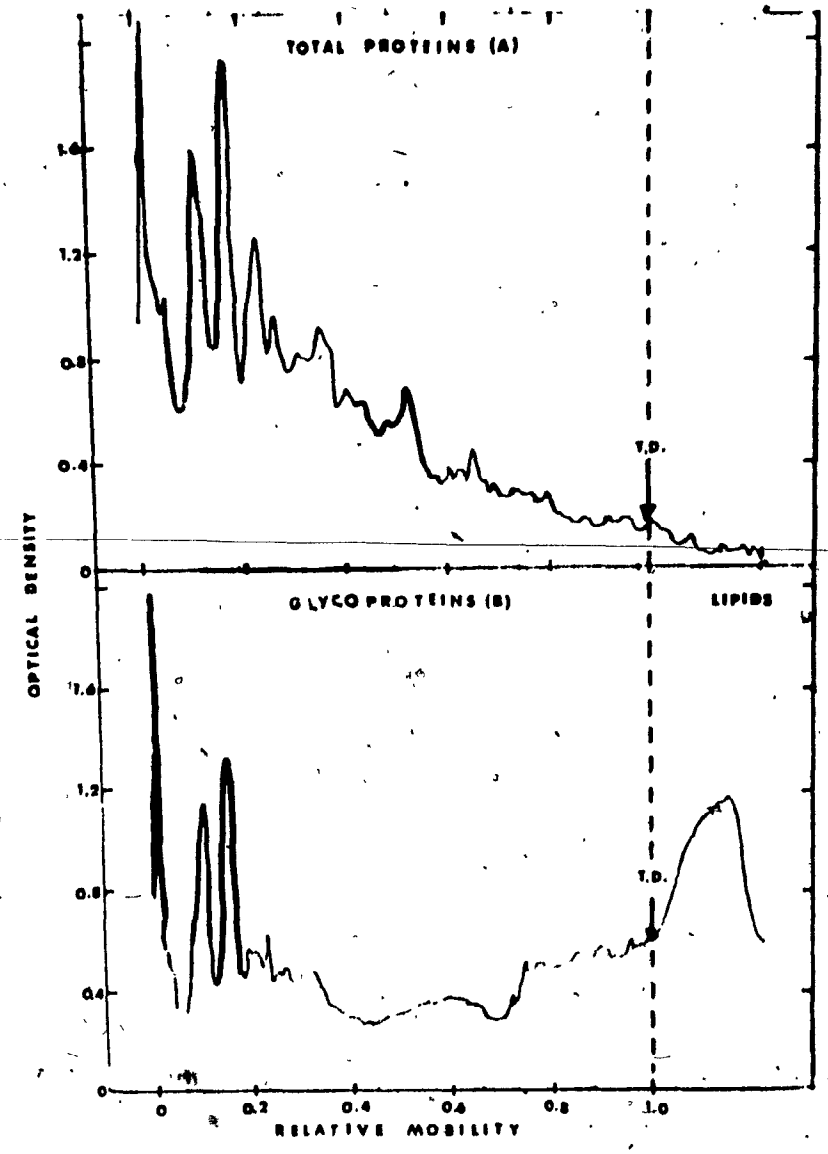

Figure 13: Densitograms of the plasma membrane-rich fraction obtained as described in the Methods section. Total proteins (A) stained with Coomasie brilliant blue. Glycoproteins (B) stained with PAS; the broad band just below the tracking dye reflects the Schiff-positive activity of the membrane lipids.

VII.4.3 Proteins

Stain-All was used to detect phosphoproteins on SDS gel of solubilized cortices. This stain is light sensitive. It showed the presence of a dark blue band characteristic of the phosphoproteins that had migrated in a position just above the tracking dye.

CHAPTER VIII

DISCUSSION

The preliminary studies were performed to determine which fraction, i.e. total cortices, KCl fraction and plasma membrane rich fraction, contained the maximum levels of endogenous substrate, and also to study the best conditions necessary to perform the enzymatic assay "in vitro".

However, before discussing the results obtained for the enzyme assay done in the preliminary studies, it is necessary to point out that the cortices used in the "in vitro" system are obtained from oocytes treated with or without the hormone 1-MA "in vivo". This means that the oocytes treated with the hormone have already depleted the substrate, whereas the ones that were not treated with the hormone contain the substrate and the kinase.

The results shown in Figure 2 can be explained in the following way:

1. the "control-EGTA fraction" incorporates the ^{32}P label in higher levels than the "1-MA-EGTA fraction" in the three cases this occurs due to the explanation given above.

2. a millimolar concentration of Ca^{++} ions inhibits almost 100% incorporation of ^{32}P label with respect to the one treated with EGTA.

3. the "control EGTA-PMR fraction" has 2.05 and 6.01 fold increase of radioactivity incorporated with respect to "control EGTA-KCl fraction" and "control EGTA-Total cortices" respectively.

The plasma membrane rich fraction was shown to contain the highest levels of the endogenous substrate. It was also necessary to measure all the parameters that might affect the availability of the ^{32}P from the (γ ^{32}P) ATP, as ATP spontaneous hydrolysis and dephosphorylating enzymes. Table 1 shows that increasing specific activity of the (γ ^{32}P) ATP solution increases the incorporation of the ^{32}P radioactive level in the TCA-insoluble sub-fraction from 0.14% to 1.67% and at the same time the free ^{32}P present in the assay medium is increased from 5.84% to 48.21%. In Table 1 it can also be observed that EGTA does not facilitate the incorporation of the label in the TCA-insoluble sub-fraction. This means that the assay requires a solution of (γ ^{32}P) ATP with a high specific activity to maximize the ^{32}P label incorporated into the phosphorylated factor.

Finally, the Standard Condition of the enzyme assay "in vitro" was established as the medium containing: "plasma membrane rich fraction", the buffer PK/2 (pH 7.5) and a high specific activity solution of (γ ^{32}P) ATP. Recently, Moreau et al (1978) studied a phenomenon that occurs when the hormone (1-MA) is added to starfish oocytes. This phenomenon is the intracellular release of calcium ions which correspond to the earliest response to the hormone recorded so far. The same phenomenon was shown to occur "in vitro", when the plasma membranes of the oocytes of starfish were incubated with the hormone 1-MA (Dorée et al, 1978). This characteristic of the plasma membrane was used as a membrane marker and it is shown in Figure 3 that the release of the Ca^{++} ions was produced by addition of the 1-MA to the "plasma membrane rich fraction".

Having already established the conditions for the "in vitro" enzyme assay, the next step was to separate the phosphorylated substrate by using SDS polyacrylamide gel electrophoresis. These assays were performed under "in vivo" and "in vitro" conditions as already defined. Under "in vivo" conditions, it was possible to observe that the label was incorporated in a molecule that migrated to a position on the gel very close to the tracking dye. It was also shown that the cortices treated with 1-MA (Figure 4) incorporates 2.7 fold more ^{32}P radioactivity than the control cortices (Figure 5). These results support the data obtained by Guerrier et al (1977), who showed an increase in the label incorporated in the cortices obtained from oocytes treated with 1-MA with respect to the control.

The endoplasm did not show incorporation of the label (Figure 6). This indicated that the phosphorylated factor was of cortical origin and not cytoplasmic. In the "in vitro" condition, it was found that ^{32}P radioactivity was incorporated into a molecule in the same molecular weight range as "in vivo". However, as explained before, it was expected to have the same range as that of the "control PMR fraction. This fraction will incorporate higher levels of radioactivity than the "1-MA-PMR fraction" and it was shown that "control PMR fraction" (Figure 7) has 7 fold increase in the label incorporated with respect to the "1-MA-PMR fraction" (Figure 8).

The ^{32}P radioactive substance seems to correlate with a band stained with Coomasie brilliant blue that migrates on the gel between 0.8 and 1.0 relative mobility and falls between trypsin M.W.

23,300 and lysozyme M.W. 14,300 used as molecular weight markers (Figure 10). However, the fact that the label correlates with a band stained with Coomasie brilliant blue does not necessarily mean it is a protein; therefore, in order to determine the nature of this molecule, the gels were subjected to differential staining in order to locate lipids, glycoproteins, and phosphoproteins.

During the extraction of lipids in the "in vitro" condition, it was observed that 48% of the label incorporated into the control cortices ocrresponded to the chloroform phase, whereas only 30% was incorporated into the proteins (Figure 11). This means that the condition for the "in vitro" system was optimal for other kinase as phosphatidyl-inositol kinase that incorporates ^{32}P from (γ ^{32}P) ATP into a lipid which co-chromatographs with diphosphoinositide (Buckley, 1976). The lipids were found to be present in a band stained with Sudan black B below the tracking dye. All the radioactive label deposited in the gel was found to be incorporated in this band (Figure 12). Furthermore, the lipid zone was also observed as a broad band below the tracking dye in the gels stained with PAS (Figure 13).

The position of the glycoproteins were found in the upper part of the gel. This may be due to their abnormal mobility produced by the changes in the net charge and differences in the stock radius in comparison with a polypeptide. The final stain used was Stain-All, which shows the presence of a dark blue band characteristic of a phosphoprotein. This band was found just above the tracking dye, having the same relative mobility as the band that incorporates the radioactive label "in vivo" and "in vitro".

It can be concluded from the results presented here that in the presence of 1-MA and(^{32}P) ATP, a protein in the cortices of starfish oocytes was phosphorylated perhaps by a protein kinase located in the cortices. It can be assumed that this kinase was activated by the action of the hormone 1-MA. The molecule that was phosphorylated appears to be a protein of a molecular weight of approximately 17,000 daltons. This finding agrees with a recent observation of Preddie et al (1979) who showed by using immunological methods and "cross-affinity" chromatography that a protein nolecule of 17,000 daltons becomes phosphorylated when cortices of starfish eggs were treated with (γ ^{32}P) ATP in the presence of 1-methyladenine.

The results of Guerrier et al (1977) and Kishimoto and Kanatani (1976) seem to suggest that the maturation promoting factor is similar to a phosphorylated factor that may be responsible for the induction of maturation in starfish eggs. The results presented here suggest that this 17,000 dalton species may very well be a maturation promoting factor in this species of starfish.

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