



145
592
THS

THE EFFECT OF ADENOSINE TRIPHOSPHATE
ON THE STIMULATION OF CLEAVAGE
IN THE EGGS OF RANA PIPIENS

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY

Ronald J. Pfohl

1962



ABSTRACT

THE EFFECT OF ADENOSINE TRIPHOSPHATE ON THE STIMULATION OF CLEAVAGE IN THE EGGS OF RANA PIPIENS

by Ronald J. Pfohl

A series of experiments were conducted to determine what effect the injection of adenosine triphosphate (ATP) into parthenogenetically stimulated eggs of Rana pipiens would have on the number which successfully cleaved to the blastula stage.

In general, the experimental procedure involved the injection into eggs of approximately 0.2-0.3 lambda quantities of 1.61×10^{-3} M ATP solutions in Niu-Twitty or Steinberg buffer. In some cases the design of the experiments was such that the injection process effected the activation of eggs smeared with blood. In other cases the injection was performed two hours after the eggs had been activated either by fertilization or by smearing the eggs with blood and pricking them with a fine glass needle.

The percentages of eggs which cleaved when injected with ATP were compared with the percentages which cleaved when injected with only the buffer solution. The results obtained, though not conclusive, indicate that the ATP injected into the eggs caused an increase in the numbers which cleaved as compared with those simply injected with the buffer medium.

Ronald J. Pfohl

The predominant effect of the ATP seems to stem from an ability to aid the egg in overcoming the injury it has sustained in the injection procedure. Utilization of the high energy moiety of the ATP molecule and its possible sites of action are discussed with reference to the mode of action of ATP in the enhancement of cleavage.

THE EFFECT OF ADENOSINE TRIPHOSPHATE ON THE
STIMULATION OF CLEAVAGE
IN THE EGGS OF RANA PIPPIENS

By

Ronald J. Pfohl

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Zoology

1962

8/16/22
5/3/64

ACKNOWLEDGEMENTS

The author wishes to express his sincere gratitude to Dr. John R. Shaver for initiating this investigation and for his continued confidence, interest, and encouragement during the course of this study.

Thanks are also due Dr. Philip Clark and Dr. Lester Wolterink for their constructive criticism in preparing the final manuscript.

Finally, the author wishes to express his appreciation to "Mac" Henderson for her understanding and frequent assistance on numerous occasions.

TABLE OF CONTENTS

	Page
LIST OF TABLES	iv
LIST OF APPENDICES	v
INTRODUCTION	1
A. Parthenogenesis, With Special Reference to the Eggs of Frogs	1
B. A Review of Some Aspects of Cell Division	5
1. Sol-gel Transformations; Cell Cortex in Cleavage	5
2. The Spindle in Mitosis	8
3. Energetics of Cell Division	11
MATERIALS AND METHODS	21
RESULTS	26
DISCUSSION	37
SUMMARY	45
APPENDICES	47
LITERATURE CITED	51

LIST OF TABLES

Table		Page
1A	Pooled data from frogs III-IX; Activation simultaneous with injection of Niu-Twitty buffer (pH 7.0-7.5) or 0.1% ATP solution (pH 6.0-6.5)	28
1B	Paired observations; Eggs smeared with blood and injected with Niu-Twitty buffer or with ATP; Values given are percentages of eggs which developed to the blastula stage	28
2A	Pooled data from frogs X and XI; Niu-Twitty buffer (pH 6.6) or 0.1% ATP solution (pH 6.6) injected about 2 hours after activation . .	30
2B	Paired observations; Pricked blood-smeared eggs and fertilized eggs injected with Niu-Twitty buffer or with ATP two hours after being activated; Values given are percentages of eggs which developed to the blastula stage	30
3A	Pooled data from frogs XIII and XIV; Activation simultaneous with injection of Steinberg's medium (pH 6.85) and 0.1% ATP solution (pH 6.65)	33
3B	Paired observations; Eggs smeared with blood and injected with Steinberg's medium or with ATP; Values given are percentages of eggs which developed to the blastula stage .	33
4A	Pooled data from frogs XII and XIII; Steinberg's medium (pH 6.85) or 0.1% ATP solution (pH 6.65) injected about 2 hours after activation	35
4B	Paired observations; Pricked blood-smeared eggs and fertilized eggs injected with Steinberg's medium or with ATP two hours after being activated; Values given are percentages of eggs which developed to the blastula stage	35

LIST OF APPENDICES

Appendix	Page
I Data from frogs I-IX; Activation simultaneous with the injection of Niu-Twitty buffer (pH 7.0-7.5) or 0.01, 0.1, or 0.5% ATP solution (pH 6.0-6.5); Values in the table indicate the numbers of eggs which were treated and which cleaved to the blastula stage in each category	47
II Data from frogs X and XI; Niu-Twitty buffer (pH 6.6) or 0.1% ATP solution (pH 6.6) injected about 2 hours after activation; Values in the table indicate the numbers of eggs which were treated and which cleaved to the blastula stage in each category	48
III Data from frogs XIII and XIV; Activation simultaneous with the injection of Steinberg's medium (pH 6.35) or 0.1% ATP solution (pH 6.65); Values in the table indicate the numbers of eggs which were treated and which cleaved to the blastula stage in each category	49
IV Data from frogs XII and XIII; Steinberg's medium (pH 6.35) or 0.1% ATP solution (pH 6.65) injected about 2 hours after activation; Values in the table indicate the numbers of eggs which were treated and which cleaved to the blastula stage in each category	50

INTRODUCTION

The aim of this investigation was to determine what affect the injection of adenosine triphosphate (ATP) into parthenogenetically stimulated frog eggs had on the number which successfully cleaved to the blastula stage.

To familiarize the reader with the experimental material, a brief discussion of parthenogenesis in the eggs of frogs will be given. Following this, evidence concerning certain sol-gel transformations in relation to the cell cortex and to the spindle in cleavage and mitosis and their associations with ATP will be discussed. The evidence supporting a role for ATP in the process of cell division points, with some reservations, to the high energy moiety of ATP as the probable factor of functional significance. This review of the literature serves as the basis for the study undertaken and reported on in this paper.

A. Parthenogenesis, With Special Reference to the Eggs of Frogs

Fertilization, as defined by Lord Rothschild (1956, p. 1), is "the incitement of an egg to development by a spermatozoon, together with the transmission of male hereditary material to the egg." That the male nucleus is not always necessary for normal development is shown by the occurrence of natural or spontaneous parthenogenesis (i.e. development of the egg without fertilization) in such animals as the aphids, butterflies, bees, silkworms, and certain of the

crustacea (Loeb, 1913, chapter V).

Observations on the occurrence of natural parthenogenesis were the starting point for investigations upon artificial parthenogenesis. It soon became apparent that a great number and diversity of treatments, including puncture, heat, cold, ultra-violet radiation, acids, bases, isotonic salt solutions, hyper- and hypotonic solutions, fat solvents, and some alkaloids, were capable of inciting the eggs of some species of animals to develop. This array of agents contrasts strikingly with the high degree of specificity in fertilization and does not lend support to the idea that the parthenogenetic agent represents the effective component(s) of the spermatozoon. A number of theories, reviewed by Tyler (1941), have been advanced to account for the same response of the egg to a variety of agents.

The frog egg appears to be exceptional in that it has been possible to incite it to develop completely only by the use of one type of procedure, to be described below.

One may regard the initiation of parthenogenetic development in the frog egg as involving two phases. The first is an "activation" phase consisting of the separation of the vitelline membrane with subsequent formation of the perivitelline space, rotation of orientation, formation of the grey crescent region, and completion of the second maturation division. Many of the parthenogenetic agents which successfully incite other eggs to develop are capable only of stimulating the frog egg to undergo this first phase.

The events observed in this first phase may occasionally be followed by so-called "abortive" cleavages which in effect may involve one or two irregular cleavages or superficial indications of attempts at cleavage. Such events are not generally considered to involve bona fide initiation of cleavage, although migration of chromosomes and certain sol-gel transformations may result.

Successfully inciting a frog egg to undergo further parthenogenetic development therefore involves a second phase. Bataillon (1911) used the terms "first factor" and "second factor" to designate the components necessary to initiate the two phases. The necessity of a "second factor" for the initiation of genuine cleavage in the frog egg was established by Bataillon in 1912 (cited by Bataillon, 1929). He showed that complete cleavage in the frog egg could be accomplished by introducing a cellular element into the cytoplasm at the time of activation. This may be achieved simply by pricking the egg ("first factor") with a fine glass needle in the presence of blood, lymph, or the brei of some tissues ("second factor").

Bataillon believed that the "second factor" induces the formation of a normal mitotic figure and must include material from a nucleated cell. Einsele (1930), however, demonstrated the cleavage-initiating capacity of supernatant fluids which were obtained after centrifugation of homogenized frog tissues and injected into virgin frog eggs. Shaver (1953) substantiated the work of Einsele and carried the

analysis beyond that of previous workers by determining which fractions of the cell were the most active. He accomplished this by homogenizing and differentially centrifuging adult and embryonic frog tissues and injecting the fractions thus obtained into the eggs. The large granule fraction, possibly mitochondrial in nature, proved to be the most effective in initiation of cleavage. When frog eggs were injected with the large granule fraction obtained from frog blood cell homogenates, 20.8% of the eggs cleaved to the blastula stage. A maximum of 44.6% cleavage to blastulae was obtained when the eggs were injected with the large granule fraction obtained from the homogenate of early frog gastrulae. In contrast to the findings of Bataillon (1919), Shaver (1953) reported that injection of whole frog serum into the eggs of frogs resulted in 11.7% cleaving to the blastula stage. Since the "second factor" appears to be principally concerned with the stimulation of bona fide cleavage, Shaver (1953) preferred to call the active agent (or agents) the "cleavage initiating substance" (CIS). The chemical nature of the CIS in the cytoplasmic granules is not yet known.

In this investigation, the eggs of frogs were incited to develop by the usual technique, namely smearing the eggs lightly with frog blood and pricking them with a fine glass needle or micropipette. The percentage of eggs smeared with blood that one can normally expect to cleave to blastomeres (of approximately the size attained by embryos in Shumway

stages 8 or 9) following artificial activation is generally within the range of 5-20%. Why only 15% of the eggs parthenogenetically stimulated actually cleave is not well understood. Apparently, formation of a "proper" astral system is necessary for segmentation of the egg to occur normally (Herlant, 1913). Parthenogenesis in the egg of the frog does not occur spontaneously in nature. It is likely that, with the present procedures, the proper combination of a number of factors, such as the condition of the egg and the precise stimulation applied to it, occurs only in a small percentage of attempts. Also, injury may be sustained by certain eggs when punctured. It is remarkable, therefore, that even 5-20% of the eggs should cleave. Obviously, then, an increase in the proportion which undergo successful cleavage may be the consequence of any of a number of possible mechanisms.

On the basis of the evidence presented in the following review, consideration was given to the possibility that ATP added to the system might aid the egg in overcoming factors which often hinder its cleavage.

B. A Review of Some Aspects of Cell Division

1. Sol-gel Transformations; Cell Cortex in Cleavage

The gelation of many protoplasmic systems is an endothermic process involving volume increase. Gel systems of such a nature may be referred to as type II according to the Freundlich classification (Marsland and Brown, 1942; Marsland,

1948).

The interconversion of protoplasm from the sol to the gel state or vice versa is thought to play an important part in cell division. Although the structure of protoplasmic gels and the nature of the alterations thereof in sol-gel transformations have not been clearly determined, X-ray diffraction and electron microscopy studies and theoretical considerations based on the physical properties of certain gel systems suggest that the gelation process probably represents the formation of a 3-dimensional network from fibrillar units present in the system (Ferry, 1948; Kopac, 1950).

Gelation has been shown to be correlated with furrow formation in the eggs of certain marine animals and in the eggs of Rana pipiens (Marsland and Landau, 1954). With an increase in temperature, a greater pressure is required to block the first cleavage furrow (i.e. a temperature increase favors gelation while a pressure increase favors solation). Thus, when the "structural strength" (based on the relative resistance to displacement of visible granules through the gel in eggs of marine species) of the cortical gel systems investigated by Marsland and coworkers falls to a certain critical level, the furrowing ceases.

A number of theories have been advanced to explain cleavage. They are critically analyzed by Wolpert (1960). In the egg of a frog the new cell surface is formed de novo, and the only theory directly applicable to this material is that of Selman and Waddington (1955).

Using ciné-film techniques, local vital staining and serial sections, Selman and Waddington (1955) conclude that the new unpigmented cortex, by which the daughter blastomeres remain in contact after cleavage, is first formed as a sheet of gel (in later stages they describe it as being a double layer) which grows downward through the cytoplasm from the animal toward the vegetal surface by a process involving gelation at its lower edge. They suggest that 1) cortical gel is being synthesized before cleavage as an additional layer beneath the pigmented cortex already existing, 2) a change from gel to sol occurs at the inner surface of the pigmented cortex with subsequent transfer of the material to the required region during cleavage, and 3) a change from sol to gel takes place along the lower edge of the new unpigmented cortex being formed ahead of the furrow. They also conclude that the gel layer contracts immediately after its formation, and in this way produces the "dipping in" of the new furrow and all the observed surface movements.

It must be realized that the above description is purely morphological. If the sol-gel transformations described do indeed occur, and if the gelation is an endothermic process involving volume increase, no explanation is available, to the knowledge of the present author, with regard to the nature of the energy transformations or exchanges occurring in the synthesis of the gel and subsequent transition to the sol state, followed by gelation in the furrow region. Does the temperature increase or pressure

applied to the eggs of R. pipiens in the experiments of Marsland and Landau (1954) affect the initial synthesis of the gel or the later sol to gel transformation in the formation of the new cell surface? Furthermore, no adequate description is given of the force vectors likely to be involved in the contraction postulated by Selman and Waddington (1955). Another process, possibly involving ATP, must be invoked on the basis that if the gelation process itself involves a volume increase as postulated, one would expect the gelation process to result in elongation rather than contraction of gel structures.

2. The Spindle in Mitosis

The preceding discussion has been concerned principally with gel-sol transformations in the cell cortex. This phenomenon is thought to be of importance also in the establishment of the mitotic apparatus. The initial development by Mazia and Dan (1952) of a method for isolating the mitotic apparatus of sea urchins has opened new fields of investigation concerning this structure. Recently, Mazia and coworkers (1961) have utilized dithiodiglycol (DTDG) to obtain what are possibly "native" mitotic apparatuses.

The mitotic apparatus has been defined (Mazia and Dan, 1952) as the "ensemble of structures constituting the 'chromatic' and 'achromatic' figures in the classical descriptions of mitosis. It includes spindles, asters, centrioles, nuclei (before breakdown) and chromosomal structures (after breakdown of the nuclear membrane)."

The mitotic apparatus has often been described as a gel whose microscopically visible fibrous elements are regions where the submicroscopic fibrils are condensed and oriented (Mazia, 1955). The growth of the spindle or aster may thus represent the transformation of some substance from a disordered to an ordered condition. Observations on the effects of antimitotic agents such as colchicine lead one to the conclusion that the formation of the mitotic apparatus is separable into two processes, a polymerization process giving rise to a shapeless gel and a second process of condensing and orienting the elements of the gel into the fiber system observed in the normal mitotic apparatus (Mazia, 1955). It was originally thought that the molecules in the mitotic apparatus were held together in polymers by disulfide bridges, chemical bonds between sulfur atoms on neighboring protein molecules (Mazia, 1955). More recent experience with the isolated mitotic apparatus emphasizes its instability and directs attention to weaker interactions that still involve sulfur-containing groups. Hydrogen bonding has played a prominent part in the theory of protein-to-protein interactions and must inevitably enter into our thinking about the intermolecular linkages functional in the lateral bonding which establishes the fibrous pattern of the mitotic apparatus observable with the light microscope (Gross and Spindel, 1960; Huggins, 1962). Inoué (1959) observed that the birefringence of the spindles in eggs of the annelid Chaetopterus increases with temperature in the range of 10°

to 40°C. The effect is reversible and leads to the conception of the spindle as a gel in a temperature-sensitive equilibrium. A thermodynamic analysis of this equilibrium gives results which are in agreement with the proposed lability of the spindle structure and which are consistent with the idea that the orientation depends on very weak bonding. That the material comprising the mitotic apparatus may be a Freundlich type II gel is also indicated by the observations of Pease (1941, 1946) and of Marsland and coworkers (1960) that high pressure causes the solation of the spindle-aster complex.

The function of the spindle in the movement of chromosomes is a question still open to much debate. Of the various concepts postulated to account for chromosome movement, the most credible ones are those which involve two mechanisms. According to Ris (1943), the chromosomal fibers attach the chromosomes to the proper spindle pole and contract, drawing the chromosomes toward the poles. The spindle fibers, extending the length of the spindle, apparently hold the poles of the spindle apart and elongate in the second half of anaphase. The two movements are complementary and very probably alternate as the "motor" of chromosomal movement.

In summary, the evidence presented suggests that there are at least two sol-gel systems in the cell which concern us: the cell surface (cortex) and the spindle. The cleavage of the frog egg is thought to involve the process of sol-gel

transformations in the formation of a new cell surface. The formation of the spindle presumably involves a sol-gel transformation with subsequent orientation of the amorphous gel into an ordered fibrous structure. The mechanics of mitosis, still a matter of much controversy, are thought to involve a contraction of chromosomal fibers and an elongation of spindle fibers.

3. Energetics of Cell Division

Having briefly discussed some aspects of the mechanics involved in cell division, our attention is now turned to the basic metabolic pattern by which the cell provides energy (protoplasmic gelations are endothermic) for the formation of its essential gel structures.

The role of adenosine triphosphate in intermediary metabolism is the subject of a vast literature. The energy of ATP may be made available for different processes in the cell (see Lipmann, 1941, and Lehninger, 1959), and it is the natural choice as the important metabolite contributing energy to the sol-gel cycle in cells.

Considerable evidence has been accumulated indicating that ATP does play a prominent role in the process of gelation and cell movement. Runnstrom, on the basis of gross observations and centrifugation experiments (Runnstrom, 1949; Runnstrom and Kriszat, 1950a,b) on unfertilized marine eggs, concludes that the addition of ATP increases the rigidity of the submicroscopic fabric of the cytoplasm, and suggests

that this increase is due to a reinforcement of the bonds between the submicroscopic gel-forming molecular complexes. Membrane formation, cleavage and further development of fertilized eggs were improved upon addition of ATP.

As early as 1942, Marsland and Brown suggested that the greater velocity of gelation and solation reactions observed in protoplasmic systems as compared to methylcellulose and gelatin systems might be due to the intervention of an ATPase enzyme system which in itself may be modified under various conditions of temperature and pressure.

Marsland, Landau, and Zimmerman (Landau, et al, 1955; Marsland, et al, 1953) showed that whenever the gel state of cortical cytoplasm is "weakened" by lower temperature or by higher pressure there is a corresponding fall in furrowing strength (as determined by the amount of pressure or decrease in temperature required to inhibit furrowing) and that whenever the "structural strength" of the cell cortex is fortified by higher temperature, lower pressure, or by ATP added to the medium about 30 minutes prior to furrowing, the "furrowing strength" correspondingly increases. Furthermore, the minimum pressures required to block furrows are distinctly higher when ATP is added. At atmospheric pressure, the added ATP enabled the eggs to complete their furrowing at temperatures ordinarily too low to allow for successful cleavage. Their work indicates that the ATP system plays an important role in the metabolism of the cell which determines when and where gel structures are to be formed. Adenosine monophos-

phate, adenosine, or inorganic phosphate, used in place of ATP, were virtually ineffective. Thus the high energy moiety of ATP is evidently important in the action observed.

The importance of ATP in cell division is further emphasized by results obtained from studies of cell models. Hoffmann-Berling (1960) and Weber (1955) have reviewed evidence, most of it supplied by their own work, in support of the role played by ATP in the mechanisms involved in active movement. Similarities in the physical and chemical properties of extracted contractile proteins from sarcoma cells and actomyosin were noted. The contraction of actomyosin and the contraction of cell models prepared from fibroblasts were shown to be dependent upon similar ionic strengths and similar concentrations of Mg ions and ATP. Both were shown to have ATPase activities. Contraction was initiated by ATP (or ITP) only. AMP, creatine phosphate, other organic phosphates and inorganic polyphosphates were ineffective, thus emphasizing again the importance of the high energy labile phosphate group. If ATP was in supraoptimal concentrations or if its hydrolysis was inhibited, elongation of the models would occur. These workers observed, for example, that in fibroblast models prepared from cells in early anaphase and subjected to conditions which activate contractile mechanisms but exclude elongation mechanisms, the chromosomes would move apart, apparently due to contraction of chromosomal fibers. In the second portion of anaphase, however, the spindle bodies actively elongate. Active elongation of

the spindle bodies has been observed in grasshopper spermatocytes by Bêlâr (cited by Hoffmann-Berling, 1960). Spindle elongation, brought about by the inner portions of the spindle, is initiated by supraoptimal concentrations of ATP. Thus ATP is thought to have a dual action on the contractile protein structures. Elongation apparently involves the binding but not the splitting of ATP, whereas upon splitting (by contractile ATPases) the protein complexes contract. It is of interest here that ATPase has recently been found in association with the soluble fraction obtained from the mitotic apparatuses of sea urchin eggs isolated by the DTDG method (Mazia, Chaffee and Iverson, 1961).

Cytokinesis of the fibroblast models, as reviewed by Hoffmann-Berling (1960), has likewise been shown to involve an elongation and contraction under the influence of physiological concentrations of ATP. AMP and inorganic phosphate were ineffective. It is not clear how the contraction of cell cortical material of the egg of an amphibian, for example, might occur in such a way that it would aid in cytokinesis. As shown by Selman and Waddington (1955), a rounding up of the cells occurs prior to cleavage. Possibly a contractile mechanism involving cell cortical material is important in this phenomenon.

Further support for the importance of ATP in cell division may be obtained from studies involving various types of inhibitors.

Mersalyl acid (Salyrgan), a sulfhydryl-blocking

agent, is known to inhibit ATPase (see Weber, 1955). A fibroblast model behaves like actomyosin when treated with Salyrgan. Contraction, initially induced by ATP, is stopped reversibly by addition of Salyrgan to the medium. Although it has an inhibitory effect upon a wide variety of metabolic enzymes in which catalytic activity depends, at least partly, upon the pattern of -SH radicals in the protein moiety of their structure, Zimmerman, Landau, and Marsland (Zimmerman, et al, 1957; Marsland, 1956; Landau, et al, 1954) believe that its most specific effect is on the ATPase system. These workers observed that treating marine eggs with Salyrgan delayed and reduced the building up of the cortical gel structure associated with the first mitosis. The furrowing potency of the eggs was also lower.

Another inhibitor which has been widely used is 2,4-dinitrophenol (DNP). This compound has been known for some time to uncouple phosphorylation from oxidation (Loomis and Lipmann, 1949). Villee and coworkers (1949) reported that DNP reduces the uptake of P^{32} and its subsequent incorporation into nucleic acids and phosphoproteins in fertilized Arbacia eggs. These workers as well as a number of others (McElroy, 1947) have shown an increase in oxygen consumption and an inhibition of mitosis upon addition of DNP. The ability of an inhibitor such as DNP to increase respiration of an egg above the normal level and simultaneously inhibit cell division is believed to be due to the diversion of oxidative energy from phosphorylative synthesis to the combus-

tion of carbohydrate reserves (Hughes, 1952). Since biological endergonic processes draw the energy they require principally from ATP, and not from oxidation, it is expected that inhibition of ATP synthesis will arrest mitotic activity.

Brachet in 1954 (cited in Brachet, 1957, p. 177) showed that cleavage of amphibian morulae is inhibited by DNP. Clowes and coworkers (1950) demonstrated that the concentration of a substituted phenol that will block oxidative phosphorylation in cell-free particulate systems of Arbacia will also inhibit cleavage in the intact egg. Barnett (1953) was able to partially reverse inhibition of cleavage in sea urchin eggs by addition of ATP, whereas addition of adenylic acid, inorganic phosphate and pyrophosphate did not stimulate division of the inhibited eggs. ATP was capable of completely reversing cleavage inhibition by cyanide and anaerobiosis. Kriszat and Runnstrom (1951) also were able to remove a DNP block to cell division in marine eggs by adding ATP.

It thus seems apparent from studies on the effects of DNP on cell division that the generation of phosphate bonds forms a necessary link in the mechanism of cell division.

Sodium azide is known to inhibit transphosphorylation and ATPase activity (Meyerhof, 1945). Krah1 and coworkers (1941) noted that sodium azide stopped cell division in sea urchin eggs. Spiegelman and Moog (cited in Brachet, 1950, p. 169) found that a trace of azide will stop segmentation of Rana pipiens eggs. They suggest that this effect is due to the inhibition of an azide-sensitive apyrase. Barth and

Jaeger (1947) have shown that there exist at least three apyrase-protein complexes in the frog egg which are capable of hydrolyzing ATP, and which differ characteristically in pH optima for activity as well as in degree of thermolability. When the apyrase activity of the apyrase-protein fraction is tested at successive stages of development, the enzymes are found to differ in the periods of development at which they attain maximum activity. Barth and Jaeger suggest that apyrase-protein complexes are potential links between energy-producing and energy-utilizing systems of specific regions in the developing egg. Shaver, Subtelny, and Wania (1952) have shown that inhibition of the development of frog eggs with sodium azide at gastrulation also inhibits the cleavage initiating capacity of granules isolated from homogenates of such blocked embryos.

The action of heparin on the cleavage of eggs presents some indirect evidence for the role of ATP in cytokinesis. D. Harding (1949, 1951) and Shaver (1949) have noted that heparin or heparin-like polysaccharides inhibit to some extent the activating effect of blood in frog eggs. Cell division in tissue cultures is also inhibited by heparin (see D. Harding, 1949). A number of workers cited by C.V. Harding (1951) have shown the antagonistic effects of heparin and ATP in the processes involved in fertilization and cleavage of marine eggs as well as structural changes in other protoplasmic systems. The suggestion is made that heparin might interfere with phosphate metabolism at the cell surface.

Contrary to the evidence thus far presented, some workers question whether the ATP added to cell systems is actually the effective agent. Adenosine triphosphate is a large, highly polar molecule, and it is questionable whether it can penetrate into the cell in significant amounts. On the basis of experiments performed on sea urchin eggs, Litchfield and Whiteley (1959) conclude that ATP neither penetrates nor is adsorbed to the surface of fertilized eggs. Wolpert (1961) recently retracted his original report (1953) that ATP plays a role in the cleavage of the sea urchin egg. He attributes his original results to artifact.

The situation may be such, as suggested by Lindberg (1950) and Runnstrom and Kriszat (1950a), that the energy from ATP in the surrounding medium is transferred across the cell surface to the superficial layers of the cytoplasm.

Some indication that ATP may be of importance in cell division is indirectly obtained from the behavior of mitochondria during cell division.

Mitochondria are cytoplasmic granules possessing a complex and characteristic structure. They contain an array of enzymes, including, in a specific way, the most important oxidative enzymes. They are capable, if properly supplied with soluble cofactors, ions, and substrates, of oxidative phosphorylations. The mitochondrion is, in essence, a cellular machine for converting energy liberated by oxidation of the citric acid cycle substrates into the bond energy of ATP. This appears to be a universal property common to

all mitochondria, regardless of their source (Green, 1960).

Monroy (1957) believes that activation of mitochondria is one of the events of the activation of the egg. He gives evidence for an increased activity of mitochondrial ATPase as a result of fertilization.

Mitochondria have been observed to aggregate about the spindle during cellular division (see De Robertis, et al, 1960, pp. 172, 326-327).

Finally, Shaver (1953) has shown that the large granule fraction obtained upon differential centrifugation of homogenates of frog gastrulae is the most active in initiation of cleavage. This fraction is thought to consist predominantly of mitochondria.

The preceding does not in itself constitute evidence in support of the role of ATP in cell division or egg activation, but merely suggests that such a role is in accord with observations on mitochondria.

This review has been concerned with some of the evidence supporting a functional role for ATP in the energetics of cell division and structural changes in the cell in general. Admittedly, the bulk of the evidence has been concerned with animal material other than that of amphibians, but the evidence which has been obtained directly from the eggs of amphibians seems to this author to warrant the assumption that the protoplasmic gel of the eggs of amphibians is of a type similar to that of the cell models and marine eggs so commonly studied. Under this assumption, the results obtained

in the present study on the effect of ATP on the initiation of cleavage in parthenogenetically stimulated frog eggs will be presented.

MATERIALS AND METHODS

Female frogs of the species Rana pipiens, obtained from dealers in Vermont and Wisconsin, were induced to ovulate prior to the onset of breeding season by intracoelomic injections of two or more anterior pituitary bodies from frogs of the same species (Rugh, 1934). Eggs were extruded onto clean glass slides. Following the treatment, experimental or control, the slides with eggs were placed in aerated tap water in finger bowls which were appropriately labelled.

Since the purpose of this investigation was to determine the effect of ATP on the cleavage of parthenogenetically stimulated frog eggs, it was necessary to employ a number of controls in order to eliminate the possibility of other factors being responsible for the results obtained. The following controls were utilized for the eggs from every frog used: fertilized eggs; unsmeared eggs, pricked; eggs smeared with blood and pricked; injection of Niu-Twitty or Steinberg buffer into unsmeared eggs; and injection of buffer into eggs smeared with blood.

Eggs were inseminated at the start of an experiment with a sperm suspension prepared by macerating two testes in approximately 10 cc. of 1/10 full strength Holtfreter's solution. One or two slides of eggs thus treated served as a control to give an indication of the viability of the eggs. Another fertilized control was generally prepared at the

termination of a particular experiment in order to determine whether the viability of the eggs remained at a desirably high level throughout the experiment. If a fertilized control showed less than 70% cleavage the results in that experiment were usually discarded.

Throughout all other portions of the experiment particular care was taken to maintain sperm sterility. In the unsmeared pricked control, eggs were punctured in the animal hemisphere with micropipettes similar to those used in the injections. Care was taken to avoid disturbing the area of the maturation spindle. If any sperm, blood cells or other tissue cells or debris were present which might initiate cleavage, their presence should have been detected by this control.

The control in which eggs smeared with blood were punctured gave an indication of the responsiveness of the eggs to artificial activation. In the instances where high percentages (15% and 33%) of successful cleavages resulted from this artificial activation, inspite of the low percentages (50% and 53%, respectively) obtained in the fertilized controls (see data from frogs VI and XIV in Appendices I and III), the results were still retained.

The results of injecting ATP into eggs which had been smeared with blood were compared with those obtained by injecting ATP into unsmeared eggs as well as those obtained by injecting smeared and unsmeared eggs with the buffer solution. The injection of smeared eggs with buffer was

necessary since the control in which smeared eggs were simply pricked is inadequate for the purpose of comparison with the injected experimentals, due to the fact that injection of a substance into the egg may in itself have some deleterious effect.

The disodium salt of adenosine triphosphate, obtained from Nutritional Biochemical Corporation (Cleveland, Ohio), was dissolved in Niu-Twitty solution or Steinberg's medium, the pH of which was adjusted to about 7.0 prior to making the ATP solution (Niu and Twitty, 1953; Steinberg, 1957). In alkaline solutions, ATP is quickly converted to 5-adenylic acid and sodium pyrophosphate even in the presence of ice; therefore solutions should not be allowed to become alkaline for more than a few seconds. If the pH of the ATP solution is maintained below 7.4, the refrigerated solution should remain stable for about one week (vide Merck Index of Chemicals and Drugs, 1960, p. 21). The pH of the prepared solutions was generally maintained between 6.0-7.0.

The injection apparatus consisted of a Luer Lock 10 cc. syringe fitted with a female Luer Slip adapter for the attachment of polyethylene tubing (I.D. 0.030" x O.D. 0.048"). The micropipettes, attached to the other end of the polyethylene tubing, were hand-drawn from capillary tubing over a micro-burner. The syringe and polyethylene tubing system was filled with distilled water, constituting a simple hydraulic system.

Before the micropipettes were attached to this system they were filled with the solution to be injected. It was

found advisable to fill a number of pipettes with the solutions and store them in vials before beginning the experiment since during the course of making the injections the pipettes may become clogged or the tips may be broken off. Undue delay in filling another pipette while in the process of injecting a slide of eggs caused the eggs to dry excessively, with deleterious results.

Prior to attaching the filled micropipette to the hydraulic system the plunger was withdrawn slightly so as to provide an air space between the distilled water in the system and the solution in the pipette. This air space was maintained at all times to avoid dilution and contamination of the solution with the distilled water.

The amount of solution injected into the egg was generally of the order of 0.2-0.3 λ and was always kept below a volume causing visible swelling of the eggs. Furthermore there was no assurance that all the injected solution remained in the egg after the pipette was withdrawn.

Following activation many eggs will exhibit a superficial wrinkling effect and some may undergo one or two irregular cleavages and then cease dividing. To avoid confusing true cell division with these aberrant forms the eggs were observed at the blastula stage (Shumway stages 8-9) at which time the blastomeres will give the embryo a characteristic appearance (more than 64 cells in a total blastula). Partial blastulae were counted as having cleaved, since the blastomeres in similar cases have been shown by Frazier (1951)

to be almost entirely nucleated.

Since the cleavage of the eggs was the only phenomenon under investigation, the development of the embryos beyond the blastula stage was not recorded.

RESULTS

The following three concentrations of ATP in Niu-Twitty solution were initially tested: 0.01% (1.61×10^{-4} M); 0.1% (1.61×10^{-3} M); and 0.5% (8.05×10^{-3} M). Different batches of eggs from each of the frogs III-V were injected with the three concentrations of ATP. Combining the results from the eggs of the three frogs, the injection of 0.01% ATP solution into blood-smeared eggs resulted in 6.8% cleavage; 0.1% ATP, 9.6% cleavage; and 0.5% ATP, 7.6% cleavage. The percentage of cleavages resulting from the control eggs which were smeared with blood and injected with Niu-Twitty buffer was 2.1%. The means of the differences between the percentages of blood-smeared control eggs which cleaved when injected simply with buffer and the percentages of blood-smeared eggs which cleaved when injected with ATP at concentrations of 0.1% and 0.5% were significantly different from 0 at the 5% level. The 0.1% solution of ATP was selected for the remaining injections.

In the first set of experiments the activation of the eggs was effected at the time of the injection. The results of injecting a 0.1% solution of ATP into the eggs of frogs III-IX are shown in Appendix I, and the percentages of cleavages calculated from the pooled data are shown in Table 1A. For cleavage to be initiated it was necessary to supply another factor, in this case whole blood, prior to the injection. ATP by itself was incapable of initiating cleavage. The 0.6% cleavage obtained in unsmeared eggs injected with

buffer, the 0.6% cleavage in unsmeared eggs injected with ATP and the 0.2% cleavage in unsmeared eggs simply pricked were probably due to contamination of the eggs with tissue debris.

The design of this experiment was such as to show any difference in the number of cleavages resulting from the two different treatments, namely, the injection of 1) Niu-Twitty buffer and 2) ATP into eggs smeared with blood. Since the eggs of different frogs may show some variation, genetic or otherwise, and since the eggs from each frog were subjected to both treatments, it was possible to apply a statistical test to the mean of the differences between the percentages of cleavages induced by the injection of buffer and the percentages induced by the injection of ATP into the blood-smeared eggs of each frog. This approach provides a more sensitive test than one based on group comparisons in which case the test is applied to the difference of the means of the two groups (Snedecor, 1953; Fisher, 1950).

The percentage of cleavages for each group of eggs from each frog and the differences between the two groups were calculated from the data in Appendix I and are shown in Table 1B.

The mean difference, $\bar{d}$, is 6.88 and the standard error of the mean difference, $s_{\bar{d}}$, is 2.23. Using these figures we may calculate "Student's" t value and find $t = 3.085$. With 6 degrees of freedom, the table value at the 5% level is 2.447. Thus at the 5% level, on the basis of the figures

Table 1A: Pooled data from frogs III-IX; Activation simultaneous with injection of Niu-Twitty buffer (pH 7.0-7.5) or 0.1% ATP solution (pH 6.0-6.5).

Treatment						Number of eggs treated	Blastulae	
Fertilized	Pricked	Eggs unsmeared	Eggs blood-smeared	Injection of NT	Injection of ATP		Number	%
x						1654	1417	85.7
	x	x				508	1	0.2
	x		x			545	76	13.9
		x		x		734	4	0.6
			x	x		986	50	5.1
		x			x	713	4	0.6
			x		x	1011	119	11.8

Table 1B: Paired observations; Eggs smeared with blood and injected with Niu-Twitty buffer or with ATP; Values given are percentages of eggs which developed to the blastula stage.

Frog	Treatment		Difference
	Eggs smeared with blood		
	Inj. with NT	Inj. with ATP	
III	4.55	12.36	7.81
IV	0.59	6.15	5.56
V	0.67	10.62	9.95
VI	13.55	15.11	1.56
VII	4.35	12.90	8.55
VIII	12.94	11.20	-1.74
IX	2.19	18.63	16.44

given, we may assert that the difference between the percentage of cleavages resulting from injection of buffer and the percentage resulting from the injection of ATP into eggs smeared with blood is significantly different from zero. One cannot, however, conclude from this significant difference that the injection of ATP does enhance the initiation of cleavage, because the pH of the Niu-Twitty buffer used in the control injections was not the same as the pH of the injected ATP solutions. In preparing the 0.1% solution, the addition of ATP to the Niu-Twitty buffer lowered the pH of the latter as much as 1.5 pH units. Whereas the pH of the injected buffer was generally 7.0-7.5, the pH of the ATP solution was 6.0-6.5. Maintaining the two solutions at the same pH would have eliminated or established the possibility that the acidity of the ATP solution was a factor contributing to the results obtained.

In the second series of experiments, the Niu-Twitty buffer and the 0.1% ATP solution were both maintained at pH 6.6. In this series of experiments, observations were made on the effect of injecting ATP into the eggs two hours after they had been activated; in other words, after the emission of the second polar body and before the first cleavage division. During the first hour after activation the egg is apparently in such a sensitive state that any disturbance, such as puncturing, will stop cleavage. Activation was accomplished by one of three methods: pricking

Table 2A: Pooled data from frogs X-XI; Niu-Twitty buffer (pH 6.6) or 0.1% ATP solution (pH 6.6) injected about 2 hours after activation.

Treatment						Number of eggs treated	Blastulae	
Fertilized	Pricked	Eggs unsmeared	Eggs blood-smeared	Injection of NT	Injection of ATP		Number	%
x						364	355	97.5
	x	x				197	0	0
	x		x			210	17	8.1
x				x		298	214	71.8
	x	x		x		181	2	1.1
	x		x	x		260	18	6.9
x					x	356	267	75.0
	x	x			x	191	0	0
	x		x		x	299	41	13.7

Table 2B: Paired observations; Pricked blood-smeared eggs and fertilized eggs injected with Niu-Twitty buffer or with ATP two hours after being activated; Values given are percentages of eggs which developed to the blastula stage.

Frog	Treatment		Difference
	Eggs smeared with blood and pricked		
	Inj. with NT	Inj. with ATP	
X	7.78	7.50	-0.28
XI	6.47	17.88	11.41
	Eggs fertilized		
	Inj. with NT	Inj. with ATP	
X	91.80	91.38	-0.42
XI	57.95	67.08	9.13

unsmeared eggs; pricking eggs smeared with blood; and fertilization.

The eggs of frogs X and XI were used in this series, and the percentages shown in Table 2A were calculated from a summation of the results shown in Appendix II.

Upon examining the percentages of blastulae shown in Table 2A, it is apparent that the injections of buffer and ATP into unsmeared eggs two hours after being activated by pricking had no appreciable effect. The 1.1% cleavage obtained upon injection of buffer into unsmeared eggs was probably due to contamination of the eggs with tissue debris. The lower percentages of blastulae obtained where fertilized eggs were injected with buffer and with ATP as compared to the untreated fertilized control were very likely due to the injury sustained by the eggs resulting from the injection process. The percentages of cleavages of eggs from each frog after each treatment are shown in Table 2B.

Testing the mean of the differences between the percentages of cleavages of fertilized eggs injected with buffer and of fertilized eggs injected with ATP shows that it is not significantly different from zero at the 5% level. Similarly, when testing the mean of the differences between the percentages of cleaving eggs obtained by injecting buffer and ATP into eggs smeared with blood and activated by pricking, it was shown to be not significantly different from zero at the 5% level.

An insufficient number of animals in this experiment

does not allow one to make any conclusive statements concerning the effectiveness of ATP in enhancing the cleavage initiation process. In the eggs of frog XI there was an appreciable increase in the percentages of cleavages when ATP was injected, particularly in the group activated by pricking. It is quite possible that if the number of animals had been larger the effect of ATP would have been statistically significant since the standard error of the mean would very probably have been considerably reduced.

In the next series of experiments, Steinberg's medium replaced the Niu-Twitty solution. The Niu-Twitty solution was not entirely satisfactory as a buffer because upon standing its pH had a tendency to rise due to the breakdown of the NaHCO_3 component of the buffer into Na and OH ions and CO_2 gas. Steinberg's medium is a solution having the same isotonic salt combination as the Niu-Twitty solution but containing a Tris-HCl buffer in substitution for the phosphate and bicarbonate buffering system of the Niu-Twitty solution.

Groups of eggs from frogs XIII and XIV were treated in a manner similar to those of frogs III-IX in the first series of experiments except that the buffer solution used was Steinberg's. The solutions of ATP prepared were of a 0.1% concentration. The pH of the Steinberg's medium to be injected as the control was 6.85 while that of the ATP solution (ATP dissolved in Steinberg's medium) was 6.65. The results obtained are shown in Tables 3A and 3B. The values given were compiled and calculated from the data in

Table 3A: Pooled data from frogs XIII and XIV; Activation simultaneous with injection of Steinberg's medium (pH 6.85) and 0.1% ATP solution (pH 6.65).

Treatment					Number of eggs treated	Blastulae		
Fertilized	Pricked	Eggs unsmearcd	Eggs blood-smearcd	Injection of SM		Injection of ATP	Number	%
x						434	335	77.2
	x	x				116	0	0
	x		x			143	32	22.4
		x		x		108	1	0.9
			x	x		169	31	18.3
		x			x	102	0	0
			x		x	199	29	13.1

Table 3B: Paired observations; Eggs smeared with blood and injected with Steinberg's medium or with ATP; Values given are percentages of eggs which developed to the blastula stage.

Frog	Treatment		Difference
	Eggs smeared with blood		
	Inj. with SM	Inj. with ATP	
XIII	17.92	17.80	- 0.12
XIV	19.05	6.17	-12.88

Appendix III.

From Table 3B we may calculate a mean difference of -6.50 and a standard error of the mean difference of 6.33. From these figures we obtain a "Student's" t value of -1.019, which with 1 degree of freedom is not significantly different from zero at the 5% level.

Finally, a series of eggs, smeared or unsmeared with blood, were injected with Steinberg's medium or ATP solution (0.1%) about two hours after activation by pricking or fertilization. The pHs of the injection media were the same as in the preceding series of experiments. The results, compiled from the data in Appendix IV, are summarized in Tables 4A and 4B.

When comparing the results shown in Table 4B obtained by injecting the control buffer and ATP into fertilized eggs, a mean difference of 3.36 and a standard error of the mean difference of 1.228 are calculated. These figures yield a "Student's" t value of 2.736. A mean difference of 0.595 and a standard error of the mean difference of 2.135 are calculated from the results obtained by injecting the control buffer and ATP into the eggs two hours after they had been smeared with blood and pricked. These figures yield a "Student's" t value of 0.272. With 1 degree of freedom neither of the mean differences obtained are significantly different from zero at the 5% level.

The inadequate sample size again does not allow one to make definite assertions concerning the effectiveness of

Table 4A: Pooled data from frogs XII and XIII; Steinberg's medium (pH 6.85) or 0.1% ATP solution (pH 6.65) injected about 2 hours after activation.

Treatment					Number of eggs treated	Blastulae		
Fertilized	Pricked	Eggs unsmeared	Eggs blood-smeared	Injection of SM		Injection of ATP	Number	%
x						375	350	93.3
	x	x				135	0	0
	x		x			171	51	29.8
x				x		290	170	58.6
	x	x		x		187	1	0.5
	x		x	x		237	16	6.8
x					x	253	150	59.3
	x	x			x	196	0	0
	x		x		x	248	19	7.7

Table 4B: Paired observations; Pricked blood-smeared eggs and fertilized eggs injected with Steinberg's medium or with ATP two hours after being activated; Values given are percentages of eggs which developed to the blastula stage.

Frog	Treatment		Difference
	Eggs smeared with blood and pricked		
	Inj. with SM	Inj. with ATP	
XII	8.33	11.11	2.78
XIII	5.13	3.54	-1.59
	Eggs fertilized		
	Inj. with SM	Inj. with ATP	
XII	36.17	38.41	2.24
XIII	79.87	84.35	4.48

ATP in enhancing cleavage.

DISCUSSION

A number of experiments, the results of which have been presented in the preceding section, have been performed in an attempt to ascertain what effect, if any, the introduction of ATP into unfertilized frog eggs would have on the percentage which successfully cleaved to the blastula stage. These experiments were suggested by evidence that ATP is functional in cell division and structural alterations of the cell (see Marsland, 1956; Runnstrom and Kriszat, 1950a,b; Hoffmann-Berling, 1960). The bulk of this evidence has been gathered from such materials as marine invertebrate eggs and fibroblast cell models. Some of the evidence mentioned was obtained from amphibian material, however, and was of such a nature as to suggest the presence of gel systems similar to those in marine invertebrate eggs and cell models. The lines of evidence indicating the presence of systems in the eggs of amphibians which may utilize ATP in the process of cell division are 1) the demonstration by Marsland and Landau (1954) that the gelation process in the eggs of R. pipiens is an endothermic process, 2) the work of Selman and Waddington (1955) which indicates that gelation is an important process in the de novo formation of the new cell surface in the cleavage of amphibian eggs, 3) the inhibition of cleavage in amphibian morulae by DNP, which is known to inhibit oxidative phosphorylation (see Brachet, 1957, p. 177), and 4) the demonstration by Spiegelman and Moog (cited by

Brachet, 1950, p. 169) that sodium azide, an inhibitor of transphosphorylation and ATPase activity, will stop segmentation of R. pipiens eggs. As might be expected, the presence of enzymes catalyzing ATP hydrolysis in the eggs of frogs has been shown by Barth and Jaeger (1947). Evidence of a more indirect nature is gained from sodium azide (Shaver, et al, 1952) and heparin (Shaver, 1949; Harding, D., 1949, 1951) inhibition studies in relation to the cleavage initiating substance in artificial parthenogenesis in amphibians. Finally, the large granule fraction, presumably mitochondrial in nature, obtained upon centrifuging homogenates of frog gastrulae, was found to have a high cleavage-initiating capacity in experimental parthenogenesis (Shaver, 1953). Although the results of the present investigation indicate that ATP per se has no cleavage initiating capacity (0.6, 0.0, 0.0, 0.0% cleavage when unsmeared eggs are injected with ATP; see Tables 1A, 2A, 3A, and 4A, respectively), it is possible that the ATP produced by mitochondria may have some function in increasing the number of successful cleavages.

Some experiments in this study were performed in such a way that the injection procedure also effected the activation process (Tables 1A,1B and 3A,3B), while in others the injection procedure followed activation by pricking or by fertilization by about two hours (Tables 2A,2B and 4A,4B). In 1958, Wolpert reported that treatment of sea urchin eggs with ATP ten minutes prior to cleavage resulted in the eggs showing considerable delay and abnormalities in cleavage, or

resulted in the eggs failing to cleave at all. Such results might be expected under the supposition that the eggs were unable to regulate the ATP supply within such a short time prior to cleavage and as a result a relaxation or elongation would be induced by the supraoptimal concentrations of ATP in a manner analogous to that described by Hoffmann-Berling (1960) in cell model systems. When Niu-Twitty buffer solution was used in the present study as a medium for the solution of ATP, however, no apparent difference in the response of the eggs to the injection of ATP at the time of activation and two hours after activation was noted (Tables 1A,1B and 2A,2B). Wolpert (1962) has since attributed his results to artifact due to pH changes.

It is apparent from the results presented that the injection of buffer into eggs smeared with blood had some detrimental effect on the eggs. The percentages of eggs cleaving to blastulae in this case (5.1, 6.9, 18.3, and 6.8 in series 1A-4A) were always less than the percentages obtained by simply puncturing smeared eggs (13.9, 8.1, 22.4, and 29.8, respectively). With the exception of series 2, the percentages of cleavages obtained upon injecting ATP into the eggs (11.8, 13.7, 13.1, 7.7) were also less than those obtained by the simple puncturing procedure, but, with the exception of series 3, the percentages were more than those obtained in the controls where buffer was injected into smeared eggs. The results of injecting ATP into the smeared eggs thus seemed to indicate that the ATP counter-

acted to some extent the detrimental effect of the injection process.

As was pointed out in the presentation of results, the sample sizes in the second, third, and fourth series were too small for tests of statistical significance to be meaningful. In the first series of experiments, the pH of the injected control solution was in the alkaline range, whereas the pH of the injected ATP solution was in the acid range. As a result, one wonders if the statistically significant data obtained in the first series of experiments are a result of the acidity of the ATP solution or of some other property inherent in the ATP molecule, such as its high energy transfer potential.

Acidity plays a role in R.S. Lillie's (1934) theory of activation, but this theory is based on observations on artificial parthenogenesis in marine invertebrate eggs. Sea urchin eggs have been successfully activated by acid treatment (Loeb, 1913), whereas successful cleavage has not been obtained through such treatment of frog eggs. Anderson (1956) has proposed a general theory in which he suggests that polyelectrolyte balance is important in sol-gel transformations. According to this concept, a shift of the polyelectrolytes toward polyanions promotes solation and a shift toward polycations promotes gelation. A shift toward polycations and hence toward gelation would be favored by a drop in pH. However, this author is not aware of any evidence indicating that acidity, per se, would have any stimulatory

effect in cell division in the eggs of amphibians.

If the acid pH of the ATP solution were of significance, one would expect the results obtained to be the same when injecting an ATP solution and a buffer control of similar pH. It was noted, however, that in the second series of experiments, with special reference to the eggs of frog XI, where injections were made two hours after activation, the ATP did show some positive effect in enhancing cleavage. The percentage of fertilized eggs injected with ATP two hours after fertilization which cleaved was about 16% greater than the percentage of control eggs which cleaved when injected with Niu-Twitty buffer two hours after fertilization. With regard to the eggs of frog XI, which were injected two hours after having been smeared with blood and pricked, it was found that the ratio of the percentage of eggs injected with ATP which cleaved to the percentage injected with Niu-Twitty buffer which cleaved was greater than 2.5. A slight increase in the percentage of cleavages of those eggs injected with ATP over the percentage of those controls injected with Steinberg's medium is also apparent in the results recorded in Tables 4A and 4B.

On the basis of the literature summarized in the first paragraph of this discussion, it is tempting to theorize that ATP mediates in the sol-gel transformations involved in the scheme of cell division in amphibian eggs as formulated by Selman and Waddington (1955) and in the formation and functioning of the mitotic apparatus. If one regards

the pH discrepancies of the first series of experiments in the present investigation as insignificant, then the results of this first series in addition to those of the second and fourth series, though inconclusive, are not in contradiction with the above conjecture, although the precise mode of action is undefined.

If ATPase is present in the mitotic apparatus of amphibian eggs as it is in the mitotic apparatus of the sea urchin egg (Mazia, Chaffee, and Iverson, 1961), it is possible that ATP injected into the interior of the egg may serve as an added energy source for the functioning of the mitotic spindle in chromosome movements. Rough calculations indicate that the amount of ATP injected into the egg introduced added energy of the order of 10^{-9} kcal/egg. Calculations based on labile phosphate determinations made by Barth and Jaeger (1947) on early cleavage stages (Shumway stages 4-7) of R. pipiens indicate that the energy available in the form of ATP (or ADP) is of the order of 10^{-8} to 10^{-9} kcal/egg. Thus the injection of ATP as performed in this study is likely to involve a 50-100% increase in the energy available to the egg. Caution is advisable, however, with regard to interpretations concerning the affect of ATP, since the present study did not investigate the possibility that AMP might be as effective as ATP. This possibility must be eliminated before the results obtained can be attributed to the high energy moiety of ATP.

Although the literature reviewed in this paper does

indicate a role involving ATP in the sol-gel transformations involved in cell cleavage and in the movements of the spindle, good experimental data elucidating the nature of the mechanisms involved are lacking. Likewise, information has not been obtained with regard to the mechanisms involving ATP in the present investigation. Perhaps a systematic biochemical and biophysical analysis of model gel systems might provide some interesting results with regard to the precise nature of the role played by ATP.

In view of the above considerations, how can one account for the results obtained in the third series of experiments (Tables 3A and 3B)? In this series the percentages of eggs, particularly those of frog XIV, which cleaved when injected with ATP were lower than the percentages which cleaved when injected with Steinberg's medium (control). The only explanation which seems feasible at this time involves the assumption that the amount of ATP injected into the eggs was in supraoptimal quantities. Supraoptimal concentrations of ATP are known to cause motile protein structures to elongate (Hoffmann-Berling, 1960). Under these conditions these same structures are unable to contract. Thus the postulated contraction of the spindle might be inhibited. Furthermore, if contraction is involved in the rounding up of a cell prior to cleavage (Selman and Waddington, 1955), supraoptimal concentrations of ATP may indirectly prevent cleavage by inhibiting the general cell surface contraction leading to the rounding up of the cell.

From this discussion it should be obvious that more extensive data are needed before any conclusions can be drawn with regard to the effectiveness of ATP in the enhancement of cleavage initiation in frog eggs. The speculations put forth in this discussion not only accentuate the limitations of this investigation, but also offer a number of interesting avenues of approach for the further investigation and clarification of this problem.

SUMMARY

There is a considerable literature concerned with the intracellular action of adenosine triphosphate. Much of it is particularly concerned with the role played by ATP in cell division. On the basis of the previous work reviewed in this paper, it was of interest to the present author to observe what effect, if any, ATP injected into parthenogenetically stimulated frog eggs would have on the number which successfully cleaved to the blastula stage.

The percentage of eggs in each experiment which cleaved following injection with ATP ($1.61 \times 10^{-3}M$) was compared with a control batch from the same frog in which the eggs were injected with Nig-Twitty or Steinberg buffer. The design of the experiments was such that in some cases (Tables 1 and 3) the injection of the buffer or ATP into eggs smeared with blood effected the parthenogenetic activation whereas in others (Tables 2 and 4) the injection was made approximately two hours after the eggs had been stimulated either by fertilization or by pricking blood-smeared eggs with a fine glass needle.

In all cases the percentage of control blood-smeared eggs injected with buffer which cleaved was less than the percentage of cleavages obtained by simply pricking blood-smeared eggs. Similarly treated eggs, when injected with ATP, also cleaved to a lesser extent than the pricked controls in three out of the four series of experiments (Tables 1A, 3A, and 4A). Cleavages occurred in these ATP-injected eggs

more frequently, however, than cleavages in the eggs injected with buffer in three of the four series of experiments (Tables 1A, 2A, and 4A).

These results seem to indicate that the ATP functioned in some way in aiding the egg in overcoming injury it had incurred as a result of the injection procedure.

The failure to maintain a proper pH in the control injections in one series of experiments and the small sample sizes in the subsequent three series of experiments prevent the author from drawing any definite conclusions with regard to the effectiveness of ATP in enhancing cleavage. There are indications, however, that ATP may be important and with this in mind, several possible modes of action for ATP are discussed.

Appendix I: Data from frogs I-IX; Activation simultaneous with the injection of Niu-Twitty buffer (pH 7.0-7.5) or 0.01, 0.1, or 0.5% ATP solution (pH 6.0-6.5); Values in the table indicate the numbers of eggs which were treated and which cleaved to the blastula stage in each category.

Frog		0.01%		0.1%		0.5%	
		Smeared, Inj. with buffer	Unsmearcd, Inj. with buffer	Smeared, Inj. with ATP	Unsmearcd, Inj. with ATP	Smeared, Inj. with ATP	Unsmearcd, Inj. with ATP
I	treated	77	44	50	113	110	115
	cleaved	27	0	9	0	8	4
II	treated	95	72	69	112	150	123
	cleaved	91	0	19	0	29	4
III	treated	331	112	66	104	198	66
	cleaved	302	0	9	1	9	0
IV	treated	331	96	119	84	170	72
	cleaved	300	0	17	0	1	0
V	treated	245	71	72	74	149	48
	cleaved	228	0	21	0	1	0
VI	treated	248	54	86	124	155	134
	cleaved	123	1	13	0	21	0
VII	treated	195	52	49	101	92	121
	cleaved	172	0	4	3	4	4
VIII	treated	93	60	76	104	85	108
	cleaved	91	0	5	0	11	0
IX	treated	211	63	77	143	137	109
	cleaved	201	0	7	0	3	19

Appendix II: Data from frogs X and XI; Niu-Twitty buffer (pH 6.6) or 0.1% ATP solution (pH 6.6) injected about 2 hours after activation; Values in the table indicate the numbers of eggs which were treated and which cleaved to the blastula stage in each category.

Frog		X				XI			
		treated	cleaved	treated	cleaved	treated	cleaved	treated	cleaved
	Fertilized	177	170	187	185				
	Unsmearcd, Pricked	88	0	109	0				
	Smeared, Pricked	54	11	156	6				
	Fertilized, Inj. with buffer	122	112	176	102				
	Unsmearcd, Pricked, Inj. with buffer	102	2	79	0				
	Smeared, Pricked, Inj. with buffer	90	7	170	11				
	Fertilized, Inj. with ATP	116	106	240	161				
	Unsmearcd, Pricked, Inj. with ATP	111	0	80	0				
	Smeared, Pricked, Inj. with ATP	120	9	179	32				

LITERATURE CITED

- Anderson, N. G. 1956. Cell division I: A theoretical approach to the primeval mechanism, the initiation of cell division, and chromosomal condensation. *Quart. Rev. Biol.*, 31: 169.
- Barnett, R. C. 1953. Cell division inhibition of Arbacia and Chaetopterus eggs and its reversal by Krebs cycle intermediates and certain phosphate compounds. *Biol. Bull.*, 104: 263.
- Barth, L. G. and Jaeger, L. 1947. The apyrase activity of various protein fractions of the frog's egg. *J. Cell. Comp. Physiol.*, 30: 111.
- Bataillon, E. 1911. Les deux facteurs de la parthenogénèse traumatique chez les amphibiens. *C. R. Acad. Sci.*, 152: 920. (Read in translation)
- Bataillon, E. 1919. Analyse de l'activation par la technique des oeufs nus et la polyspermie expérimentale chez les batraciens. *Ann. des Sci. Nat. et Zool.*, 10^{me} série, 3: 1. (Read in translation)
- Bataillon, E. 1929. Analyse de la fécondation par la parthenogénèse expérimentale. *Roux' Archiv f. Entwicklungs. d. Organ.*, 115: 707. (Read in translation)
- Brachet, J. 1950. Chemical Embryology, Second Edition. Interscience Publishers, Inc., New York.
- Brachet, J. 1957. Biochemical Cytology. Academic Press, New York.
- Clowes, G., Kelch, A. K., Strittmatter, C. F., and Walters, C. P. 1950. Action of nitro- and halophenols upon oxygen consumption and phosphorylation by a cell-free particulate system from Arbacia eggs. *J. Gen. Physiol.*, 33: 555.
- De Robertis, E., Nowinski, W., and Saez, F. 1960. General Cytology, Third Edition. W. B. Saunders Co., Philadelphia.
- Einsele, W. 1930. Entwicklungserregung von Froscheiern durch Injektion zellfreier Organextrakte. *Roux' Archiv f. Entwicklungs. d. Organ.*, 123: 279.
- Ferry, J. D. 1948. Protein gels. *Adv. in Protein Chem.*, 4: 1.

- Fisher, R. A. 1950. Statistical Methods for Research Workers, Eleventh Edition. Hafner Publishing Co., New York, pp. 117, 121-122.
- Frazier, R. L. 1951. A cytological and cytochemical study of parthenogenetic embryos of Rana pipiens. Master's Thesis, University of Missouri (Unpublished).
- Green, D. E. 1960. Structure and function in the mitochondrial electron-transport system. Radiation Research, Suppl. 2: 504.
- Gross, P. R. and Spindel, W. 1960. Heavy water inhibition of cell division: an approach to mechanism. Ann. N. Y. Acad. Sci., 90: 500.
- Harding, C. V. 1951. The action of certain polysaccharides on fertilization in the sea urchin egg. Exptl. Cell Research, 2: 403.
- Harding, D. 1949. Effect of heparin on artificial activation in the frog egg. Proc. Soc. Exptl. Biol. and Med., 71: 14.
- Harding, D. 1951. Effect of bacterial polysaccharide on artificial activation in the frog egg. Nature, 167: 355.
- Herlant, M. 1913. Etude sur les bases cytologiques du mécanisme de la parthénogénèse expérimentale chez les amphibiens. Arch. de Biol., 28: 505. (Read in translation)
- Hoffmann-Berling, H. 1960. Other mechanisms producing movements. In Comparative Biochemistry: A Comprehensive Treatise, Vol. II, M. Florkin and H. S. Mason, editors. Academic Press, New York, p. 341.
- Huggins, M. L. 1962. Physico-chemical aspects of hydrogen bonds and their application to biology. Am. Scientist, 50: 485.
- Hughes, A. 1952. Inhibitors and mitotic physiology. Symp. Soc. Exptl. Biol., 6: 256.
- Inoue, S. 1959. Motility of cilia and the mechanism of mitosis. In Biophysical Science- A Study Program, J. L. Oncley, editor. Wiley and Sons, Inc., New York, p. 402.
- Kopac, M. J. 1950. Physical properties of protoplasm. Ann. Rev. of Physiol., 12: 7.
- Krahl, M. E., Keltsch, A. K., Neubeck, C. E., and Clowes, G. 1941. Studies on cell metabolism and cell division V. Cytochrome oxidase activity in the eggs of Arbacia punctulata. J. Gen. Physiol., 24: 597.

- Kriszat, G. and Runnstrom, J. 1951. Some aspects of adenosine triphosphate on the cytoplasmic state, division, and development of the sea urchin egg. Trans. N. Y. Acad. Sci., Ser. 2, 13: 162.
- Landau, J. V., Marsland, D. A., and Zimmerman, A. M. 1954. Kinetics of cell division: Action of mersalyl acid on pressure-temperature cleavage block in the eggs of Arbacia and Chaetopterus. Anat. Record, 120: 789.
- Landau, J. V., Zimmerman, A. M., and Marsland, D. A. 1955. The energetics of cell division; effects of adenosine triphosphate and related compounds on the furrowing capacity of marine eggs. J. Cell. Comp. Physiol., 45: 340.
- Lehninger, A. L. 1959. Respiratory-energy transformation. In Biophysical Science-A Study Program, J. L. Oncley, editor. Wiley and Sons, Inc., New York, p. 136.
- Lillie, R. S. 1934. The influence of hypertonic and hypotonic sea-water on the artificial activation of starfish eggs. Biol. Bull., 66: 361.
- Lindberg, O. 1950. On the surface reaction in the sea urchin egg. Exptl. Cell Research, 1: 105.
- Lipmann, F. 1941. Metabolic generation and utilization of phosphate bond energy. Adv. in Enzymology, 1: 99.
- Litchfield, J. B. and Whiteley, A. H. 1959. Studies on the mechanism of phosphate accumulation by sea urchin embryos. Biol. Bull. 117: 133.
- Loeb, J. 1913. Artificial Parthenogenesis and Fertilization. University of Chicago Press, Chicago.
- Loomis, W. F. and Lipmann, F. 1948. Reversible inhibition of the coupling between phosphorylation and oxidation. J. Biol. Chem., 173: 807.
- Marsland, D. A. 1943. Protoplasmic contractility. Pressure experiments on the motility of living cells. Sci. Monthly, 67: 193.
- Marsland, D. A. 1956. Protoplasmic contractility in relation to gel structure. Temperature-pressure experiments on cytokinesis and amoeboid movement. Int. Rev. Cytology, 5: 199.
- Marsland, D. A. and Brown, D. 1942. The effects of pressure on sol-gel equilibria, with special reference to myosin and other protoplasmic gels. J. Cell. Comp. Physiol., 20: 295.

- Marsland, D. A., Landau, J. V., and Zimmerman, A. M. 1953. Adenosine triphosphate as an energy source in cell division; pressure-temperature experiments on cleaving eggs of Arbacia and Chaetopterus. Biol. Bull., 105: 366.
- Marsland, D. A. and Landau, J. V. 1954. The mechanism of cell division; temperature-pressure experiments on the cortical gel system in various marine eggs. J. Exptl. Zool., 125: 507.
- Marsland, D. A., Zimmerman, A. M., and Auclair, W. 1960. Cell division: experimental induction of cleavage furrows in the eggs of Arbacia punctulata. Exptl. Cell Research, 21: 179.
- Mazia, D. 1955. The organization of the mitotic apparatus. Symp. Soc. Exptl. Biol., 9: 335.
- Mazia, D., Chaffee, R. R., and Iverson, R. M. 1961. Adenosine triphosphatase in the mitotic apparatus. Proc. Natl. Acad. Sci. U. S., 47: 788.
- Mazia, D. and Dan, K. 1952. The isolation and biochemical characterization of the mitotic apparatus of dividing cells. Proc. Natl. Acad. Sci. U. S., 39: 826.
- Mazia, D., Mitchison, J. M., Medina, H., and Harris, P. 1961. The direct isolation of the mitotic apparatus. J. Biophys. Biochem. Cytol., 10: 1.
- Mc Elroy, W. D. 1947. Mechanism of inhibition of cellular activity by narcotics. Quart. Rev. Biol., 22: 25.
- The Merck Index of Chemicals and Drugs, Seventh Edition, P. G. Stecher, editor. Merck and Co., Inc., Rahway, New Jersey, 1960, p. 21.
- Meyerhof, O. 1945. The origin of the reaction of Harden and Young in cell-free alcoholic fermentation. J. Biol. Chem., 157: 105.
- Monroy, A. 1957. An analysis of the process of fertilization and activation of the egg. Int. Rev. Cytology, 6: 107.
- Niu, M. C. and Twitty, V. C. 1953. The differentiation of gastrula ectoderm in medium conditioned by axial mesoderm. Proc. Natl. Acad. Sci. U. S., 39: 985.
- Pease, D. C. 1941. Hydrostatic pressure effects upon the spindle figure and chromosome movement. I. Experiments on the first meiotic division of Urechis eggs. J. Morph., 69: 405.

- Pease, D. C. 1946. Hydrostatic pressure effects upon the spindle figure and chromosome movement. II. Experiments on the meiotic divisions of Tradescantia pollen mother cells. Biol. Bull., 91: 145.
- Ris, H. 1943. A quantitative study of anaphase movement in the aphid Tamalia. Biol. Bull., 85: 164.
- Rothschild, Lord 1956. Fertilization. Wiley and Sons, Inc., New York.
- Rugh, R. 1934. Induced ovulation and artificial fertilization in the frog. Biol. Bull., 66: 22.
- Runnstrom, J. 1949. The mechanism of fertilization in Metazoa. Adv. in Enzymology, 9: 241.
- Runnstrom, J. and Kriszat, G. 1950a. On the effect of ATP and Ca on the cytoplasm of the egg of the sea urchin, Psammechinus miliaris. Exptl. Cell Research, 1: 284.
- Runnstrom, J. and Kriszat, G. 1950b. On the influence of ATP on the fertilization and segmentation of the sea urchin egg, Strongylocentrotus lividus. Exptl. Cell Research, 1: 497.
- Selman, G. G. and Waddington, C. H. 1955. The mechanism of cell division in the cleavage of the newt's egg. J. Exptl. Biol., 32: 700.
- Shaver, J. R. 1949. Experimental study of artificial parthenogenesis in the frog. Anat. Record, 105: 571.
- Shaver, J. R. 1953. Studies on the initiation of cleavage in the frog egg. J. Exptl. Zool., 122: 169.
- Shaver, J. R., Subtelny, S., and Wania, A. 1952. Studies on initiation of cleavage in the frog egg. Biol. Bull., 103: 282.
- Snedecor, G. W. 1953. Statistical Methods, Fourth Edition. Iowa State College Press, Ames, Iowa, pp. 43-45, 84-87.
- Steinberg, M. 1957. A non-nutrient culture medium for amphibian embryonic tissues. Carnegie Instit. of Wash. Year Book, 56: 347.
- Tyler, A. 1941. Artificial parthenogenesis. Camb. Phil. Soc. Biol. Rev., 16: 291.
- Villee, C. A., Lowens, M., Gordon, M., Leonard, E., and Rich, A. 1949. The incorporation of P³² into the nucleoproteins and phosphoproteins of the developing sea urchin embryo. J. Cell. Comp. Physiol., 33: 93.

- Weber, H. H. 1955. The link between metabolism and motility of cells and muscles. Symp. Soc. Exptl. Biol., 9: 271.
- Wolpert, L. 1953. Effect of adenosine triphosphate on cleavage of sea urchin eggs. Nature, 181: 716.
- Wolpert, L. 1960. The mechanics and mechanism of cleavage. Int. Rev. Cytology, 10: 163.
- Wolpert, L. 1961. A mechanism of cell cleavage. Pathologie-Biologie, 9: 517.
- Wolpert, L. 1962. Personal communication.
- Zimmerman, A. M., Landau, J. V., and Marsland, D. A. 1957. Cell division: A pressure-temperature analysis of the effects of sulfhydryl reagents on the cortical plasmogel structure and furrowing strength of dividing eggs, (Arbacia and Chaetopterus). J. Cell. Comp. Physiol., 49: 395.

ROOM USE ONLY

ROOM USE ONLY

~~JUL 20 '65~~

MICHIGAN STATE UNIVERSITY LIBRARIES



3 1293 03174 4893