Effect of ascorbic acid sulfate on macromolecular synthesis and early morphogenesis of sea urchin embryos.

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EFFECT OF ASCORBIC ACID SULFATE ON MACROMOLECULAR SYNTHESIS
AND EARLY MORPHOGENESIS OF SEA UCHIN EMBRYOS

BY
Kandavanam Balasubramaniam

A Thesis
Submitted to the Faculty of Graduate Studies through the
Department of Biology in Partial Fulfillment
of the Requirements for the Degree of
Master of Science at the
University of Windsor

WINDSOR, ONTARIO, CANADA
1973
The role of ascorbic acid sulfate in embryonic development has been studied using embryos of the sea urchin, *Lytechinus pictus*, as the model system. Ascorbic acid sulfate was prepared from encysted brine shrimp embryos according to the method described by Mead and Finamore (1969). The eggs and the embryos of *Lytechinus pictus* were exposed continuously to various concentrations (1-5 mM) of ascorbic acid sulfate in the culture medium (sea water) and the effect of this compound on morphogenesis and on several biochemical parameters was investigated. It was observed that ascorbic acid sulfate at the concentrations tested retarded cleavage in 15-40% of the embryos compared to control embryos. In addition, protein and nucleic synthesis were inhibited by the treatment, whereas amino acid and nucleotide metabolism was relatively unaffected. The presence of multinucleate cells and numerous mononucleated in some treated embryos suggests that ascorbic acid sulfate may be interfering with both microtubular protein and cell membrane synthesis. Ascorbic acid sulfate also produces a significant number of "vegetalized", "animalized" and "radialized" embryos. The mechanism of induction of these embryos is not known but it may be related to interference in embryo protein synthesis in various regions of the embryo. The effect of ascorbic acid sulfate appears to be reversible in most cases and the embryos are able to recover from the effects of this compound with time of incubation even in the continued presence of ascorbic acid sulfate. The reasons for the latter observation are not clear but perhaps the embryo secretes an enzyme (s) which leads to the breakdown of ascorbic acid sulfate in the medium.
ACKNOWLEDGEMENTS

I would like to acknowledge the direction, advice and guidance as well as encouragement given to me by Dr. A.H. Warner during the preparation of this thesis.

Special acknowledgements are extended to Dr. J.E.J. Habowsky of the Department of Biology, University of Windsor for serving as a member of my committee and for extending his facilities for the histological preparations, and to Dr. D.E. Schmidt of the Department of Chemistry, University of Windsor for his participation as a member of my committee.

Special thanks are extended to Mr. F. Lukas, Mr. H. Vanderburg and Miss. C. Telasco for their technical assistance; to Miss. O. Christofis and Mr. S. Saravanabavan for their assistance in the preparation of the figures; Dr. P.C. Beers, Dr. F. Huang, Mr. S. Karmiul and fellow graduate students for their encouragement and interest.

Financial assistance from the Province of Ontario and the National Research Council of Canada (Grant No. A-2909 to Dr. A.H. Warner) is greatly acknowledged.
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INTRODUCTION

Wilhelm Roux (1895) stated that the general purpose of developmental physiology is to discover the "forces" which form the shapes. We have to realize that the forces, indirectly or directly, must be brought about by the activities of the cells. Our purpose, therefore, is to reduce the complex morphogenetic events of organ rudiments and of whole embryos into a more simple model, and to ask questions about the cellular forces which interact during primary determination of the embryo. Once this is done we can ask specific questions about the biochemical events which give rise to the embryo.

The theory of early developmental interaction between cells, and of embryonic determination is based to a great extent upon experiments using echinoderm eggs. This embryonic system has, in fact, many advantages when dealing with the cellular basis of morphogenesis, especially as regards to its morphogenetic interactions. These interactions seem to be less complex than those in the vertebrate embryos (Korstadius, 1939). The biochemistry of echinoderm development has been fairly well studied (Gustafson, 1965, 1969) and there exists the possibility that the observations of cellular behaviour may be correlated with the biochemical data. The developing embryo at the gastrula stage has relatively few cells (1,000 - 2,000), and the embryo is quite transparent with cell sheets only one layer thick which facilitates observations as well as interpretations. There is little growth or cell division during the period when the main morphogenetic events occur, and one may therefore disregard
the role of local differences in these activities that may confuse the studies concerning morphogenetic movements. The embryo develops rapidly and the pluteus larva emerges by about 48 hours after fertilization. Chemical agents and microsurgery interfere with development in a predictable way (Gustafson and Wolpert, 1967).

The ability of the sea urchin egg to modify its development in a characteristic way under the influence of defined environmental conditions makes it a useful material for the study of embryonic differentiation, and explains the large amount of research devoted to the study of morphological and biochemical aspects of normal development (Lallier, 1964). The idea of affecting developmental patterns by means of various chemical agents have made these embryos useful for studies of the biochemical nature of differentiation.

Various substances can modify development and produce distinct types of developmental patterns. For instance, chemicals which cause the hyperdevelopment of the endodermal larval structures and/or those which suppress ectodermal development produce vegetalized embryos. On the other hand, chemicals which produce hyperdevelopment of ectodermal structures and/or those which suppress endodermal development produce "animalized" embryos. In weaker concentrations these chemicals lead to the formation of a dorso-ventrally elongated larval type with radial symmetry called "radialized" embryos. All stages intermediate between extreme animalization and vegetalization can also be obtained.

There are only few known vegetalizing agents. The most commonly used is lithium, the effect of which was discovered by Herbst, 1892. Recently, a derivative of pyrazolone, phenazone,
was shown to strongly vegetalize eggs (Lallier, 1959). Chloramphenicol, an inhibitor of protein synthesis, is also an effective vegetalizing agent (Lallier, 1959). Other substances promote vegetalization, but it is often not very pronounced and it generally can be observed only when fragments rather than whole eggs are treated. This is the case with dinitrophenol (Horstadius, 1953). Some amino acids, such as D- and L-p-tyrosine, o-tyrosine, and m-tyrosine in weak concentrations (Fudge-Mastrangelo, 1966), and analogs of amino acids and nucleotides (Gustafson and Horstadius, 1955), favour vegetal development. A number of naturally occurring amino acids also have a certain effect on vegetal development, but the effects are often rather weak. In combination with weak solutions of lithium, that in themselves are not effective, vegetalization is clearly enhanced (Gustafson and Horstadius, 1957).

Tyrosines are synergistic with lithium and 2,4-dinitrophenol but, unlike these agents, the tyrosines do not affect respiration. On the other hand, they have a marked depressive effect on the incorporation of 14C-labelled amino acids into various cell fractions.

Animalizing agents are more numerous. Chemical agents, such as zinc ions (Lallier, 1955b; sulfate-free sea water (Lindahl, 1936, 1942; Runnstrom et al., 1964), o-iodosobenzoic acid (Runnstrom and Kriszat, 1952; Backstrom, 1953), 2-thio-methylcytosine (Gustafson and Horstadius, 1956), thiomalic acid (Lallier, 1952), oxidized lipoic acid (Wolfson and Fry, 1965; Runnstrom, 1956), glucose and other sugars (Horstadius, 1959), and trypsin (Runnstrom and Immers, 1966) produce animalized embryos if applied after...
fertilization and during cleavage. On the other hand, thiocynate produces animalization if applied several hours before fertilization (Lindahl, 1936, 1942; Runnstrom, 1966). Various forms of naturally occurring amino acids including glutamine, reduced glutathione, structural analogs of amino acids, purines and pyrimidines, ascorbic acid and gluco-ascorbic acid also have a certain animalizing or vegetalizing effect (for references, see Gustafson, 1965, and Lallier, 1964). The effect of one agent may be antagonistic or synergistic by subsequent treatment with another agent (Lindahl, 1936, 1942; Gustafson and Horstadius, 1966).

The most active animalizing agents, in increasing order of activity are thiocyanate and iodide (Lindahl, 1936), iodosobenzoic acid (Runnstrom and Kriszat, 1952), thiomalic acid (Lallier, 1952), some sulfonic organic derivatives, such as Evans blue, Niagara blue, etc. (Lallier, 1955b), and zinc ions (Lallier, 1955a). All these agents are able to animalize whole eggs. Theoretically, these agents bring about animalization and vegetalization in a variety of ways by interfering with the structural and biochemical polarity of the egg before fertilization or during cleavage. These agents interfere with the production of 'animal' and 'vegetal' substances that mediate the interactions between cells in different regions probably by affecting metabolic processes such as gene transcription or the utilization of messenger during protein synthesis. Most of the agents concerned exert their specific effects on morphogenesis only if the treatment occurs during early cleavage stages when the fate of the zones of the egg is determined.

An important question that may be asked is 'What endogenous
substance(s) control and regulate normal development"? In an attempt to answer this question, there have been many studies of substances of natural origin which affect egg cleavage. Godlewski (1934) studied the inhibition and cytological alteration produced in sea urchin eggs by extracts of gametes from other species. Heilbrunn et al. (1951, 1954) have shown that extracts of echinoderm ovaries retard or prevent cleavage of sea urchin eggs. Menkin (1956, 1958) has reported that both retarding and accelerating components exist in extracts of sea urchin ovaries, and Wolfson (1959) reported that cleavage of sea urchin eggs is retarded by cell extracts of blastulae, ovaries, testes and gut of sea urchin. This cleavage-retarding factor is heat stable, adsorbable on charcoal, dialyzable and remains after chloroform extraction. In subsequent paper, Wolfson (1960) demonstrated the influence of four sulphhydryl compounds present in the egg extracts of Paracentrotus lividus, on the retardation of cleavage of sea urchin eggs. Berg (1972) observed that the blastocoelic fluid of Lytechinus pictus when applied externally inhibited gastrulation reversibly not only in the same species but also of embryos of other phyla such as that of Mytilus edulis and Ciona intestinalis. After preliminary studies, Berg reported that the inhibitor constituent of the blastocoelic fluid was a complex mixture of proteins. In some unpublished studies, Grayson (1968) made an observation that the culture medium of developing embryos of Artemia salina contained a substance which inhibited cleavage of Strongylocentrotus purpuratus eggs.

In an attempt to illucidate the nature of the brine
shrimp cleavage inhibitor, we performed a series of experiments to test its effect upon cleavage. When various Artemia cell fractions were tested using both Strongylocentrotus purpuratus and Lytechinus pictus eggs, most cell fractions had little, if any, effect on cleavage. However, the ascorbic acid sulfate fraction from acid extracts of Artemia salina embryos showed strong retardation of cleavage, whereas some guanine-containing compounds in the same extract showed only a mild retarding effect.

In this study we examined the effect of several acid-soluble compounds on cleavage in sea urchin eggs, and this thesis is the product of this investigation. Of all the acid-soluble compounds tested ascorbic acid sulfate was found to be the most effective compound that produced retardation of cleavage in sea urchin eggs, and this retardation was reversible. Most of the embryos, about 75% - 80%, recovered from the treatment and proceeded to the pluteus stage but were smaller in size compared to control embryos. Radioisotope incorporation studies using $^{14}$C-labelled amino acids indicate that ascorbic acid sulfate treated embryos synthesize proteins and nucleic acids at a lower rate than control embryos. Also, ascorbic acid sulfate appears to have a multiple effect on morphology during cleavage and during the period of primary determination. Although vegetalization seems to be the predominant effect, radialization, and to a small extent animalization, was observed. We speculate that retardation of cleavage is the result of vegetalization produced by the sulfate moiety of ascorbic acid sulfate or by the combination of ascorbic acid and sulfate ions acting separately at different
sites leading to an alteration of mucopolysaccharide metabolism
that may decrease cell adhesion and subsequently alter macromolecular
metabolism during early cleavage.
MATERIALS AND METHODS

Culture of Embryos

The sea urchin, *Lytechinus pictus*, was obtained from Pacific Biomarine Supply Company, Venice, California and kept in a continuously circulating and constant temperature (10°C) aquarium (Aquarium Systems Inc.). Gametes were obtained by injecting 0.5 ml of 0.55 M KCl through the peristomial membrane and inverting the injected animal over a beaker filled with millipore filtered artificial sea water (MPFSW) (Appendix A) containing 30 μg/ml penicillin G and 50 μg/ml streptomycin sulfate. Spawning begins within a few minutes after injection and continues over a period of 5 - 15 minutes. Generally, only ripe gametes are spawned but sometimes a small percentage of eggs in the germinal vesicle stage are released usually towards the end of the shedding period. Only eggs collected from the first few minutes of spawning were used in the experiments. The males were generally allowed to deposit spermatozoa into a dry dish since they keep better when undiluted.

Eggs were washed twice in MPFSW and fertilized with a diluted (1:5000) sperm suspension. After 5 minutes, the eggs were de-jellied by a short treatment in pH 5 MPFSW, washed twice with the standard MPFSW, and harvested by gentle centrifugation (approximately 200 x g) and resuspended in MPFSW. The new fertilized eggs were incubated at 15°C with gentle shaking until needed.
Nucleotide Preparation and Purification

The acid-soluble nucleotides from Artemia salina were obtained by homogenizing the dry dormant cyst in ice-cold N HClO₄ in a Ten Broeck type tissue grinder and the homogenate centrifuged at 15,000 x g for 15 minutes. The supernatant fluid was deacidified by the use of a high molecular weight amine dissolved in an organic solvent (Warner & Finamore, 1967) and the deacidified preparation was fractionated on a DEAE-cellulose column using a linear gradient of NH₄HCO₃, pH 8.6 as previously described (Warner & Finamore, 1967).

The DEAE-cellulose purified nucleotides were further purified by passage through a column of Dowex-50-H⁺ to remove contaminating cations, then neutralized with NaOH.

Preparation and Purification of Ascorbic Acid Sulfate

Ascorbic acid sulfate was prepared according to the method described by Mead & Finamore (1969). Dormant cysts of Artemia salina were ground dry in a motorized motar and then extracted with ice-cold 0.5 N HCl. The extract was centrifuged at 10,000 x g for 60 minutes, and the supernatant fluid filtered and saved. The pH was next adjusted to 2.0 with NaOH and adjusted to contain 0.1 M NaCl then applied to a column of Dowex-1-X2 (Cl⁻). The column was first washed with 0.01 N HCl containing 0.1 M NaCl, then with water until neutral and eluted with 0.5 M NaCl. The eluent was found to contain a mixture of ascorbic acid sulfate and diguanosine tri- and tetra-phosphate. This fraction was desalted using a charcoal column to remove the salts and, after
recovery of the AAS and nucleotides by elution with EtOH:NH₃:H₂O (2:1:2, v/v/v), they were further fractionated on a DEAE-cellulose column using a NH₄HCO₃ system, pH 8.6 (Mead & Pinamore, 1969). The first UV-absorbing compound which eluted was AAS. The column fractions containing the ascorbic acid were pooled, flash evaporated, and passed through Dowex-50-H⁺ column to remove traces of NH₄⁺ and other cations. Finally, the ascorbic acid sulfate was dissolved in MPFSW and stored at 4°C.

**Ascorbic Acid Sulfate Treatment**

After several preliminary experiments, 0.1 mM was chosen as the lowest concentration and 0.5 mM was the highest concentration of ascorbic acid sulfate in the MPFSW that gave consistent results. Moreover, it was observed that 0.3 mM produced optimal retardation without causing too many abnormalities.

In duplicates, the eggs were resuspended in the incubation medium at a concentration of about 400-500 eggs/ml. A mixture of ¹⁴C-amino acids (reconstituted) was added to a final concentration of 0.5 μCi/ml and 20 ml samples were cultured in sterile glass petri dishes with or without ascorbic acid sulfate. In another experiment, sea urchin eggs were incubated in duplicate with the following compounds at 0.3 mM: ascorbic acid sulfate, ascorbic acid, Na₂SO₄, and ascorbic acid + Na₂SO₄. Controls were also run in triplicate. At 0, 1, 3, 5, and 8 hour intervals, 3 ml of the egg suspensions were removed, washed twice with MPFSW, then used for biochemical and/or cytological studies.
Fractionation of Sea Urchin Embryos

The embryos were homogenized with 3 ml of ice-cold N HClO₄ and the homogenate was centrifuged at 15,000 x g for 15 minutes. The acid-insoluble pellet was washed once with 1.0 ml of 0.5 N HClO₄ and centrifuged as before. The acid-soluble fractions were pooled and retained at 0°C for assay of the nucleotide fraction and amino acid fraction as described below.

The acid-soluble fraction was applied to a charcoal column (Activated Carbon, Type S.A., Barnebey-Cheney, Columbus, Ohio) and a sample from the effluent was counted to determine the total non-aromatic amino acids in the total acid soluble fraction. Using a standard of ¹⁴C-reconstituted amino acids as a reference, the percentage of the aromatic amino acids that was retained was estimated and the necessary corrections made from the acid-soluble fraction to estimate the nucleotide pool which was retained, along with the aromatic amino acids, by the charcoal.

The acid-insoluble pellet was neutralized with a few drops of N NH₄OAc, incubated with 3 ml ethanol-ether (3:1, v/v) at 40°C for 10 minutes, then centrifuged at 3,000 x g. The insoluble material was washed twice with 1 ml quantities of ethanol-ether and the supernatant fractions were pooled and stored at 0°C. This fraction represents the lipid fraction of the embryo.

The ethanol-ether insoluble fraction was suspended in 3 ml 5% TCA and incubated in a 100°C bath for 15 minutes, cooled and centrifuged at 3,000 x g. The supernatant fluid was saved and the TCA-insoluble pellet was resuspended in 2 ml 5% TCA and the procedure repeated. The supernatant fractions were pooled.
and stored at 0°C. The hot TCA soluble fraction represents the total nucleic acid content (RNA and DNA) of the embryo.

The TCA-insoluble pellet which dissolved in 3 ml 0.5 N NaOH was obtained and stored at 0°C. This fraction represents the total embryo protein. The protein content was estimated by the method of Lowry et al., (1951) using crystalline bovine serum albumin as the standard.

The amount of radioactivity (14C) in each fraction was determined by counting duplicate aliquots in a liquid scintillation system as previously described (Warner & McClean, 1967). All radioactivity was expressed as counts per minute per mg. protein.

Cytological Studies

Samples at 0, 1, 3, 5, and 8 hour stages were washed twice in MPFSW and fixed in Bouin's; Zenker's acetic acid fixatives overnight. The DNA in some specimens was localized using the Feulgen reaction as described in Appendix B whereas other specimens were treated with hematoxylin and eosin as described in Appendix C.
RESULTS

Treatment of Sea Urchin Embryos with Acid Soluble Nucleotides

In some preliminary experiments, newly fertilized sea
urchin eggs were treated with nucleotides obtained from the acid-
soluble fraction of dormant cyst of *Artemia salina* fractionated
by DEAE-cellulose as described in the materials and methods.

The fractions containing the CMP, and AMP plus UMP
were found to have no observable effect on morphogenesis. However,
ocasionally the latter fraction was found to contain a small
amount of ascorbic acid sulfate, and in those cases, cleavage
of the sea urchin embryos was retarded by this fraction. The
GMP fraction has a very mild inhibiting effect on cleavage and
the embryos appeared slightly smaller in size. The ADP and ATP
fractions, unlike GMP seemed to facilitate development, probably
by enhancing oxidative phosphorylation. When the GDP, GTP,
and Gp₃G (diguanosine triphosphate) fractions were tested in
separate experiments, it was observed that all of the nucleotides
had a very mild effect in retarding the rate of cleavage. In
fact, the controls after 3 hours were at the 3rd cleavage stage,
whereas the treated embryos were at the 2nd cleavage stage.

The Gp₄G (diguanosine tetraphosphate) fraction also
produced a mild retarding effect on cleavage. Using Gp₄G, it
was observed that the eggs became rounded-up and produced a thick
hyaline layer. Surface blebs were observed in some treated embryos
but these were reabsorbed eventually. The number of blebs
induced by Gp₄G was much greater than the number induced by ascorbic
acid sulfate, and the effect of Gp4G was only temporary and reversible. (Compare Plates I, II, & V.)

The Effect of Ascorbic Acid Sulfate on Early Morphogenesis of Sea Urchin Embryos

From the results of the present study it is clear that ascorbic acid sulfate has a positive retarding effect on cleavage in sea urchins. Also, the hyaline layer on the surface was thicker in the AAS treated embryos than in controls. (Compare Plates I & V.) Although blebs usually appeared at the initial stages of cleavage, they eventually disappeared as the embryos rounded-up. It should be noted, however, that the translucent cytoplasm becomes opaque following fertilization. (Plate IV.) Most of the morphological changes seemed to be confined to the peripheral layer or cortex adjoining the plasma membrane. During the time that the controls undergo 3-4 cleavages, the treated embryos appeared to remain "dormant". Some, however, enter the first cleavage stage. A few treated embryos showed incomplete furrows. (Compare Plates I, III, IV, V, VI, & VII.) On closer examination using phase contrast, it appeared that some of the rounded embryos were not totally "dormant", and that they had undergone normal nuclear divisions without the accompanying cytoplasmic divisions leading to 4, 6, 8, or 16 nuclei per cell. (Plates VIII, IX, X, & XI) Also, after 5-8 hours post-fertilization the AAS treated embryos seemed to show signs of recovery. (Plates V, X, XII, XIII, & Table 1) Some of the embryos had undergone 2-3 cell divisions, while others formed quadrupartition (4 cells), or octopartition
(8 cells) all at once. This phenomenon was also observed by Ikeda (1965) in Anthocidaris crassipina when the eggs were exposed to 26°C for 25 minutes. The 2-cell stage seemed to be skipped entirely in the next division and the embryo divided into 8 cells all at once. Yuyama (1971) observed quadripartition in Arabica punctual and Lytechinus variegatus eggs that were treated with 0.1 M 2-mercaptoethanol. This chemical also caused premature splitting of centrioles prior to their duplication. (Compare Plates I, VI, VII, VIII & X).

In some of the treated sea urchin embryos 4/6, or 8 monoasters were observed. (Plate XI) The possibility of polyspermy was ruled out by the fact that the spermatozoa suspension was very dilute (1:5,000) and five minutes after the addition of spermatozoa, the egg suspension was washed twice with MPFSW and resuspended in MPFSW. Since the cortical changes begin immediately after fertilization, making the cytoplasm opaque, there could have been some polyspermy unobservable, but this phenomenon was not noticed. In addition, the controls showed no multinucleate eggs or monoasters.

After fertilization of Lytechinus eggs the embryo required about 5 hours to reach the 32-64 cell stage (blastula stage). In contrast, most of the AAS-treated embryos reach only the 2-3 cell stage, and a good proportion of the eggs still had not completed the 1st cleavage. After 8 hours in AAS the vegetalized, animalized, and radialized embryos could be distinctly recognized. The animalized embryos had more uniform blastomeres compared to control embryos and their nuclei appeared to be larger. The animalized embryos constitute about 10-15% of the total population. (Plate XIV).
The radialized embryos were elongated and had fewer cells than the animalized embryos. They showed radial symmetry, and there appeared to be more of these embryos (approximately 20-30%) than the animalized ones. (Plate XV) The vegetalized embryos had fewer blastomeres than control embryos and the blastomeres were generally larger than those in the same region of control embryos. The larger blastomeres probably represent the vegetal region, whereas the other blastomeres represent the vegetalized animal region. These embryos constituted about 50-60% of the total population. (Plate XVI) Occasionally, exoblastulae with evaginated endomesoderm were also seen having various odd shapes. (Plate XVII)

In many cases the treated embryos showed signs of total or partial recovery by 8-10 hours incubation and became rounded up and underwent normal cell divisions. The exoblastulae and the radialized forms probably did not recover. In the radialized and vegetalized embryos the Feulgen reaction showed marked differences in the nuclei compared to non-treated embryos. The treated embryos showed either no Feulgen reaction or at best only a mild Feulgen reaction up to the fifth hour. Mitotic figures were seldom seen in the vegetalized larvae at this stage. (Compare Plates XV, XVI, XVIII, & XIX) With the passage of time, most of the treated embryos (except those treated with 0.5 mM AAS and those which were exogastrulae or other abnormal forms) showed "complete" recovery and proceeded to the pluteus larval stage although these embryos were smaller in size. (Plates XX & XXI) In summary, cytological observations point to a multiple of morphological effects produced in the sea urchin embryos by ascorbic acid sulfate.
Effect of Ascorbic Acid Sulfate On The Rate Of Cleavage

Table I shows percentage of cleaved, non-cleaved, and abnormal embryos produced by ascorbic acid sulfate at the different concentrations tested. In general, the rate of cleavage during the first 1½ hours seems to be higher in the treated embryos compared to control embryos. However, by the 3rd hour of incubation there is a noticeable decline in the rate of cleavage in all the treated embryos. The 0.2 mM and 0.3 mM ascorbic acid sulfate treated embryos seem to follow similar trends of cleavage. In both cases, the rate of cleavage is retarded by about 36% whereas in the 0.5 mM ascorbic acid sulfate treated embryos cleavage is inhibited by approximately 66% compared to control embryos. By the 5th hour, the embryos treated with 0.2 mM and 0.3 mM ascorbic acid sulfate began to recover (by 18%), whereas those embryos treated with 0.5 mM ascorbic acid sulfate showed no signs of "recovery". As the incubation period increased, the majority of the 0.2 mM and 0.3 mM ascorbic acid sulfate treated embryos recovered from the effects of the compound and approached the level of recovery found in the embryos treated with 0.1 mM ascorbic acid sulfate. However, the 0.5-mM ascorbic acid sulfate treated embryos also showed some recovery (about 39%) during the latter period (5-8 hours).

Thus, the morphological studies indicate that the higher the concentration of ascorbic acid sulfate used in the incubation medium, the greater is the percentage of retardation of development (cleavage). The rate of recovery is inversely proportional to the concentration of the ascorbic acid sulfate tested. The higher the concentration, the longer is the time required
for recovery and lower the percentage of recovery. Also, it appears that the effect of AAS on cleavage is reversible within the concentration limits used in these experiments.

Effect of Ascorbic Acid Sulfate on the Incorporation of $^{14}$C-Amino Acids into Embryo Proteins

According to Kavanau (1954), there are three major periods of intense protein synthesis in the sea urchin embryo. The first rise corresponds to the first wave in hexose monophosphate shunt enzymes, viz. glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Backstrom, 1959b, 1963b), which show a strong increase during cleavage stages, and to a lesser degree, to changes in the mitochondrial population (Gustafson & Lenique, 1955). During early development the respiratory control is closely associated with the rate of protein synthesis which shows a peak during cleavage followed by a marked increase at the onset of gastrulation. These patterns were obtained with embryos showing a high degree of synchrony of development.

In order to understand the effect of AAS on cleavage at the macromolecular level, we examined the uptake of $^{14}$C-amino acids reconstituted into proteins at various stages of early development, and compared these data with those on the uptake into the amino acid pool. In the 0.1 mM AAS treated embryos the initial uptake was approximately 15% lower than the control embryos during the first few hours and decreased to about 19% by the 8th hour. In the 0.2 mM AAS treated embryos the initial uptake was 14% lower than the controls and declined to 30% by the 8th hour.
In the 0.3 mM AAS treated embryos the initial uptake was only 5% lower than the controls but decreased to 32% of the controls by the 8th hour. In the 0.5 mM AAS treated embryos there was a sharp decline in the initial uptake which was 39% lower than the controls and decreased to about 50% the level of the controls by the 8th hour. In general, the uptake of $^{14}$C-amino acids into proteins by the embryos treated with AAS was approximately 10-50% lower than the uptake into the proteins of control embryos during the period of development studied. There was one exception to the above and that was in the 0.2 mM AAS treated embryos. These embryos showed an increase in $^{14}$C-labelled protein after 3 hours incubation, an observation which is inconsistent with other data. The results of these experiments are shown in Figures I - IV.

If we examine the specific activity of the amino acid pool, we see a different picture. The pool in the 0.1 mM AAS treated embryos appears similar to the control embryos. On the other hand, in the 0.2 mM, 0.3 mM, and 0.5 mM AAS treated embryos the amino acid pool follows a similar pattern. The specific activity of the pool of the treated embryos appears slightly higher than that of the control embryos from the beginning and increases by about 5-15% by the 8th hour. This increase in specific activity of the pool and the decrease in the rate of incorporation of the $^{14}$C-amino acids into proteins suggests that ascorbic acid sulfate treatment decreases the overall protein synthesis in the AAS treated embryos. This decrease is not due to the non-availability of the precursor substances but probably due to some other factors. The cytological
observations on the rate of cleavage corroborates the findings of the $^{14}$C-amino acids incorporation into the proteins of the treated embryos. The results of these experiments are shown in Figures I - IV.

The Effect of Ascorbic Acid Sulfate On Nucleic Acid Metabolism

Nucleic acid synthesis goes "hand in hand" with protein synthesis, and both of these processes are very closely related. The 0.1 mM, 0.2 mM, and 0.3 mM AAS treated embryos show a more or less similar trend in the $^{14}$C-amino acids uptake and incorporation into the nucleic acids. Initially it was observed that the percentage of uptake of $^{14}$C-amino acids was, on the average, about 20% lower than that in control embryos and decreased to about 40% by the 8th hour. In the 0.5 mM AAS treated embryos, there is a still greater decrease (about 50-55%) in the $^{14}$C-amino acid uptake compared to control embryos throughout the period of development studied. In general, the uptake of $^{14}$C-amino acids and the incorporation into nucleic acids by the AAS treated embryos was approximately 20-55% lower than that in the control embryos during the period of development studied. The fluctuation observed at the 3rd and 5th hour may be associated with the mitotic cycle. (Holter & Zeuthen, 1957)

The specific activity of the nucleotide pool follows a similar pattern to that of the amino acid pool. In the 0.1 mM AAS treated embryos the specific activity of the nucleotide pool is similar to the control embryos. In the 0.2 mM, 0.3 mM, and the 0.5 mM AAS treated embryos the specific activity of the pool
is initially approximately 5% higher than the control embryos and gradually increases to about 10-15% by the 8th hour.

In summary, the incorporation of $^{14}$C-amino acids into newly synthesized proteins as well as into the newly synthesized nucleic acids is significantly less in the ascorbic acid sulfate treated embryos compared to the control embryos of the same stage. On the other hand, the nucleotide and amino acid pools appear to have the same specific activity in the treated embryos as well as in the control embryos. These results are shown in the Figures 1-IV.

The Effect of Ascorbic Acid Sulfate Treatment on Lipid Metabolism

The $^{14}$C-amino acid uptake and incorporation into the lipid fraction does not appear to follow the same trend as that of the nucleic acids and the proteins. Initially there is a marked suppression (approximately 50%) up to the 5th hour of development in $^{14}$C-amino acid incorporation into the lipid fraction in the ascorbic acid sulfate treated embryos. However, by the 8th hour of development all the ascorbic acid sulfate treated embryos but for those treated with 0.5 mM ascorbic acid sulfate, appear to have reached the level of $^{14}$C-amino acid incorporation into the lipid fraction of the control embryos. The initial suppression in $^{14}$C-amino acid incorporation into the lipid fraction is in agreement with the cytological observation of cleavage in the ascorbic acid sulfate treated embryos in which some embryos are multi-nucleate. This is probably the result of the lack of synthesis of new cell membranes which would account for the low level of $^{14}$C-amino acid uptake and incorporation into the
the lipid fraction of the ascorbic acid sulfate treated embryos. Thus from the results it appears that ascorbic acid sulfate seems to have a general inhibitory effect on lipid metabolism during the early phase of the treatment. However, the effects of ascorbic acid sulfate on lipid metabolism are complex and further experiments are needed to elucidate these effects.
DISCUSSION

The origin of polarity is an unsolved problem, particularly that of the dorsoventral axis of the egg. The nature of the polar differences is another obscure problem (Gustafson, 1965; Runnstrom, 1966). Experiments on the effect of removal of part of the inner cytoplasm of unfertilized sea urchin eggs support the view that animal-vegetal polarity is somewhat related to the cortex (Hörstadius et al., 1950). This conclusion was made from experiments by Runnstrom, Lindahl and Pease (see Gustafson, 1965) with various sea urchin eggs where the organelles and inclusions of the egg were stratified by centrifugation. The occurrence of a yellow ring, as well as the effect of surface-active agents, suggested that polarity is somewhat related to the architecture of the lip-protein layer of the cortex. Lallier (1958) has suggested that animalization and vegetalization result from changes in the configuration of proteins. Animalizing agents such as zinc ions and polysulfonic dyes have an affinity for protein in the more vegetal part of the embryo, whereas vegetalizing agents such as lithium or phenazine appear to act by disturbing the hydrated layer of protein molecules in the animal region of the embryo. Furthermore, the observation that chloramphenicol, an inhibitor of protein synthesis, exerts vegetalizing effect (Lallier, 1961) emphasized the importance of protein synthesis in the process of embryonic determination and differentiation, particularly as it pertains to the dorsal-ventral region of the blastula.
In the present study, ascorbic acid sulfate treatment of newly fertilized eggs of *Lytchenius pictus* produced a marked suppression of protein synthesis during early development. There was approximately a 10-50% decrease in the incorporation of $^{14}$C-amino acids into proteins by the embryos treated with ascorbic acid sulfate in comparison to the control embryos maintained under identical conditions. The specific activity of the amino acid pool, on the other hand, was very similar to that of the control embryos early in development (up to 3 hours), but later increased by about 5-15%. It appears that the amino acid pool is maintained at a relatively steady rate during early development and that ascorbic acid sulfate does not interfere appreciably with the mechanism of uptake of amino acids into the pool.

The decrease in $^{14}$C-amino acid incorporation into protein as a result of ascorbic acid sulfate treatment could be due to interference in synthesis at the transcriptional level or at the translational level. In the sea urchin egg there appears to be a large store of 'masked mRNA' (informosomes) of maternal origin (Spirin et al., 1966) in a sequestered state and the mRNA's from these particles are gradually liberated by the action of proteases (Mano & Nagono, 1966; Mano, 1966) during early differentiation and at hatching. This mRNA directs the synthesis of microtubular proteins (Raff et al., 1972) and nuclear proteins, (Keades & Gross, 1969) during early development. Therefore, it appears unlikely that ascorbic acid sulfate is acting at the level of transcription.
It has been established that unfertilized sea urchin eggs contain polysomes, but that they are much less active (15 times less) in protein synthesis than those of fertilized eggs (Piatigorsky and Tyler, 1970). The reason for this observation appears to be due to the presence of an inhibitor substance on the ribosomes in unfertilized eggs which prevents the formation of initiation complexes using polyuridylic acid as the template (Metafora et al., 1971). A similar observation has been made using ribosomes and polysomes from brine shrimp embryos (Huang, 1973). Perhaps ascorbic acid sulfate interferes with the activation of the ribosomes or polysomes during early development and consequently the rate of protein synthesis remains low in treated embryos.

The observed reduction in protein synthesis in embryos treated with ascorbic acid sulfate is consistent with cytological observations using light and phase-contrast microscopy. In the treated embryos the rate of cleavage was found to be about 15-40% lower than in control embryos. The presence of 8-16 monoasters and multi-nuclei in some embryos without accompanying cytoplasmic divisions are further evidence in support of the argument that ascorbic acid sulfate probably interferes with microtubular protein synthesis. In addition, the formation of the mitotic apparatus during cleavage appears to be affected by the ascorbic acid sulfate treatment.

In embryos treated with ascorbic acid sulfate, $^{14}$C-amino acid incorporation into the nucleic acid fraction is approximately 20-55% lower than in control embryos, whereas the specific activity
of the nucleotide pool is similar in both treated and untreated embryos. These findings suggest that although ascorbic acid sulfate does not interfere with the mechanism of uptake of $^{14}$C-amino acids into the nucleotide pool, it affects nucleic acid synthesis in a manner similar to that of protein synthesis. However, it remains to be determined whether ascorbic acid sulfate interferes with DNA or RNA synthesis, or both.

Giudice, G., (1973) suggested that in the absence of intercellular contacts a "signal" originating in the membrane is responsible for turning off DNA synthesis. O'Melia (1972) showed that, although the permeability and time course of uptake of RNA precursors of animalized and control embryos are similar, animalized embryos incorporate only 50% of the precursors into newly made RNA compared to control embryos. Perhaps the sulfated polysaccharides that are extruded from the vegetal cells in treated embryos play a role in lowering the adhesion between cells and in the development of pseudopodial activity (Immers, 1961b). The sulfated mucopolysaccharides may cover the cell surfaces forming a sheath around them, thus decreasing intercellular contacts and turning off nucleic acid syntheses.

The rate of lipid synthesis and/or rate of lipid interconversion increases after fertilization (Obiri, 1964). No data are presently available on the timing of this increased synthesis (Epel et al., 1969), but from the results obtained in the present study, lipid metabolism, and in particular the synthesis of new cell membranes, seems to be affected by ascorbic acid sulfate during early development of sea urchin embryos. In particular, there is a significant decrease (40-50).
in $^{14}C$-amino acid incorporation into the lipid fraction during the first 4-6 hours of development. However, the amount of $^{14}C$-amino acids incorporated into the lipid fraction changes during development such that most ascorbic acid sulfate treated embryos achieve the level of incorporation present in control embryos between the 6th-8th hour of development. The initial decrease in $^{14}C$-amino acid incorporation into lipids and the lack of synthesis of new cell membranes in some embryos is consistent with the observation that many multinucleate embryos exist among the ascorbic acid sulfate treated population, probably as a result of failure in the synthesis of cell membranes in each cell cycle.

In certain cases phospholipid synthesis appears to be sensitive to inhibition of DNA synthesis (Pasternak, 1973). However, preliminary experiments using enucleated fragments of sea urchin eggs suggests that, unlike protein (Denny and Taylor, 1964) or RNA (Craig, 1970) synthesis, phospholipid synthesis requires an intact nucleus. Development of eggs in the presence of hydroxyurea (Keads and Gross, 1969; Keads et al., 1969) limits the incorporation of valine into proteins after 3 hours incubation, while the incorporation of choline into lipids and thymidine into DNA is diminished somewhat earlier. In *Lytechinus pictus* synthesis of phosphatidylcholine appears to be more sensitive to hydroxyurea than is the synthesis of protein (Pasternak, 1973). The inhibition of lipid synthesis may be due to some link between the synthesis of DNA and phospholipid (Bergeron, 1971) during cleavage.

After the initial suppression of protein, nucleic acid and lipid synthesis, there appears a period of recovery after 5 hours.
The recovery of these embryos may be facilitated by the synthesis of some enzymes, including sulfatases, at the cell-membrane level which break down ascorbic acid sulfate into ascorbic acid and sulfate moieties. In support of this idea Fedecka-Brumer et al. (1971) have shown that aryl sulfatase is synthesized or activated during early development of sea urchin embryos and suggested that this enzyme is probably associated with sulfate transport or sulfated mucopoly saccharide metabolism. In another laboratory Bond (1972) has studied the mode of uptake of ascorbic acid sulfate during the growth phase of human fibroblasts in tissue-culture using $^{35}$S-ascorbic acid sulfate. He reported that $^{35}$S-ascorbic acid sulfate is not assimilated appreciably during the rapid growth phase but mature fibroblasts utilized $^{35}$S-ascorbic acid sulfate rapidly, incorporating $^{35}$S into both high and low molecular weight compounds. In contrast, he found that the mechanisms of $^{35}$S-sulfate uptake differed from that of ascorbic acid sulfate uptake and suggested that ascorbic acid sulfate is involved in sulfation. Immers (1961), using autoradiography to study the uptake of $^{35}$S-sulfate added to the sea water culture medium, found very slight or no incorporation at all in the early post-fertilization period. It seems that the amount of sulfated polysaccharides already present in the sea urchin egg is sufficient for the first cleavages and new synthesis of the compound occurs only during later development (blastula stage), when $^{35}$S-sulfate is incorporated into the blastocoelic gel and the hyaline layer of the embryo. Furthermore, parallel staining with Hale reagent showed that in earlier stages of development the sulfate group of the acid polysaccharides in the surface gel are unmasked (Immers, 1961.) From parallel incorporation of $^{14}$C-labelled amino
acids and $^{35}$S-sulfate Immers (1961) suggested that acid polysaccharides may be the compounds that are primarily formed during development and perhaps function in protein synthesis.

The incorporation studies and the Hale reaction showed many interesting details (Immers, 1961). The incorporation of sulfate groups is paralleled by an incorporation of amino acids. On the basis of this observation Immers suggested that the mucopolysaccharides play some role in protein metabolism, perhaps as acceptors of newly synthesized proteins or as inhibitors of proteolytic enzymes. These functions could be of importance for the animal and vegetal regions. In support of this idea it is known that low levels of sulfate in the culture medium brings about animalization, but results in some abnormalities in the nuclei of the animal region (Immers, 1961b; Runnstrom et al., 1964). Sulfate may also play a role in the formation of the vegetal substances (embryonic inductors) active during the period of primary determination of the germ layers.

The content and distribution of ascorbic acid in sea urchin embryos has been studied by Backström (1956, 1957) in normal and in vegetalized or animalized embryos. Ascorbic acid exists either in a bound form (ascorbigen) or in a free form. The bound form quickly disappears during cleavage. The free ascorbic acid content fluctuates during the blastula stages and during gastrulation but increases thereafter. However, the increase depends mainly on the accumulation of ascorbigen. In the vegetalized embryo, the ascorbic acid content is somewhat lower than in the control embryos before hatching. After hatching the bound ascorbic acid quickly increases and rises above the level of the controls. In the animalized embryo, the
ascorbic acid content decreases for the first few hours, then increases and attains values higher than the controls during late blastula stages and at hatching. After hatching, no ascorbigen accumulates in the animalized embryo and the ascorbic acid content decreases slowly. These observations show that the bound ascorbic acid accumulates during the differentiation of the endomesoderm.

The study of the distribution of ascorbic acid in sea urchin embryos by Eranko's silver impregnation method confirms the relationship observed between the accumulation of ascorbic acid and the development of the endomesoderm in these embryos (Backstrom, 1957). The apparent morphogenetic effects of ascorbic acid and its anti-metabolite, glucoascorbic acid, support the idea that the accumulation of ascorbic acid is connected with endomesodermal differentiation. Indeed, ascorbic acid potentiates the vegetalizing effect of lithium (Runnstrom, 1956), whereas glucoascorbic acid promotes the animalization of isolated animal halves (Gustafson and Horstadius, 1955). Backstrom's results from later stages of development indicate a gradient in the ascorbic acid content directed towards a vegetal trend of development. The older the embryo the greater the accumulation of ascorbic acid in their vegetal parts. The high ascorbic acid content of the vegetal region and low content of the animal embryo are in good agreement with the findings that glucoascorbic acid, an ascorbic acid analogue, causes animalizing tendencies (Gustafson and Horstadius, 1955).

Tissue culture studies by Jeffrey and Martin (1966a,b) and Reynolds (1967) have demonstrated the requirement for ascorbic acid
in the synthesis of bound hydroxyproline (i.e., collagen) by isolated embryonic chick bone rudiment grown in a chemically defined medium. Jeffrey and Martin (1966a, b) demonstrated that in the presence of puromycin (to inhibit protein synthesis) incorporation of $^{14}$C-proline into bound hydroxyproline was inhibited to a greater extent than incorporation into bound proline and concluded that incorporation of proline into peptide linkage preceded hydroxylation. Furthermore, in vitamin-C depleted bones prelabeled with $^{14}$C-proline, the addition of ascorbic acid to the medium stimulated the formation of peptide-bound $^{14}$C-hydroxyproline. Since this effect occurred even in the presence of puromycin it was concluded that ascorbic acid stimulated hydroxylation of peptidylproline which was formed prior to the introduction of puromycin during the prelabeling period with $^{14}$C-proline.

In a preliminary experiment, to confirm whether ascorbic acid sulfate had the same biological effect as ascorbic acid and whether it was the ascorbic acid or the sulfate moiety that was responsible for the retardation we tested ascorbic acid, $\text{Na}_2\text{SO}_4$, and ascorbic acid plus sulfate and compared these findings with those using ascorbic acid sulfate only. Ascorbic acid seemed to stimulate mildly both protein and nucleic acid syntheses in comparison to ascorbic acid sulfate (which suppressed both protein and nucleic acid synthesis). Sulfate alone had a very mild stimulatory effect on protein synthesis while it suppressed markedly nucleic acid synthesis in comparison to ascorbic acid sulfate. A combination of both ascorbic acid and sulfate in equal proportions had a somewhat stronger but variable retarding effect while that of ascorbic acid sulfate was
more reproducible and the effect relatively milder. Thus the biological activity of ascorbic acid sulfate appears to be somewhat different from that of either ascorbic acid or sulfate or their combination. This finding could be attributed to the structure of the ascorbic acid sulfate (2-O-sulfonato-L-ascorbic acid) molecule and the high energy that is required to transfer the sulfate pKₐ of 2.75) from carbon-2 which are significant in determining the biological activity (Bond et al., 1972). Furthermore, Bond et al. (1972) have suggested that ascorbic acid sulfate may function in sulfate metabolism and that the ascorbate is required for proper sulfation of mucopolysaccharides. In support of this hypothesis, other investigators have claimed that oxidative transfer of sulfate from ascorbic acid sulfate occurs and suggests that a high-transfer-potential sulfate is formed by the oxidation (Ford and Ruoff, 1965; Chu and Stauwwhite, 1968; Mamma, 1968; Mamma and Verlangieri, 1971).

The detection of p-nitrophenyl sulfate as an oxidation product of isopropylidene ascorbic sulfate and p-nitrophenol in an aqueous solution indicates that a high-transfer-potential sulfate had been found (Robinson, 1962). Robinson estimated the ΔG of hydrolysis of p-nitrophenyl sulfate equal to -15kcal/mole, which is comparable to that for 3'-phosphoadenosine-5'-phosphosulfate.

In general ascorbic acid sulfate appears to decrease the rate of protein synthesis, nucleic acid synthesis and the synthesis of cell membranes in sea urchin embryos during early development. The effect is reversible and the embryos are able to overcome the effect of the chemical by the 5th hour of development even in the presence of ascorbic acid sulfate. The exact mode of action of
ascorbic acid sulfate is not clear. It appears very likely that hydroxylation of amino acids and sulfation of mucopolysaccharides may be the probable mechanisms by which ascorbic acid sulfate is able to produce retardation of cleavage and vegetalization of embryos of the sea urchin, Lytechinus pictus.
SUMMARY

1. In a preliminary experiment newly fertilized sea urchin eggs were incubated with different nucleotides obtained from the acid soluble fraction of the dormant cyst of *Artemia salina*. Diguanosine tetraphosphate (Gp₄G) produced a mild inhibition of cleavage while diguanosine triphosphate (Gp₃G), GTP, GDP and GMP produced none or very little retardation on the rate of cleavage compared to control embryos. CMP, AMP, and UMP had no observable effect; in contrast ADP and ATP facilitated development.

2. Ascorbic acid sulfate at all concentrations tested (0.1-0.5 mM) inhibited cleavage in 15-40% of the sea urchin, *Lytechinus pictus*, embryos compared to the control embryos.

3. ¹⁴C-amino acid incorporation into sea urchin embryo proteins decreased approximately 10-50% in embryos treated with ascorbic acid sulfate compared to control embryos.

4. ¹⁴C-amino acid incorporation into sea urchin embryo total nucleic acids decreased approximately 20-55% in embryos treated with ascorbic acid sulfate compared to control embryos.

5. Uptake of ¹⁴C-amino acids into the amino acid and nucleotide pools appear to be relatively unaffected by the ascorbic acid sulfate treatment.

6. There is a significant decrease (40-50%) in ¹⁴C-amino acid incorporation into the lipid fraction in the first 4-6 hours of sea urchin development. This finding is consistent with the observation that many multinucleate cells exist within embryos treated with ascorbic acid sulfate probably as a result of failure in the synthesis of cell membranes in each cell cycle.
7. The presence of numerous monoasters in some treated embryos lends further support to the argument that ascorbic acid sulfate probably interferes with microtubular protein synthesis and affects the formation of mitotic apparatus during cleavage.

8. Ascorbic acid sulfate also produces a significant number of "vegetalized", "animalized" and "radialized" embryos. The mechanism of induction of these embryos is not known but it may be related to the interference in protein synthesis in various regions of the embryo.

9. The retardation effect on early morphogenesis by ascorbic acid sulfate appears to be reversible in most cases and the embryos seem to be able to recover from the effects of this compound with time of incubation even in the continued presence of ascorbic acid sulfate. The mechanism of recovery from the ascorbic acid sulfate treatment is not clear but perhaps the embryo secretes an enzyme(s) which leads to the breakdown of ascorbic acid sulfate in the medium.
<table>
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1 Values as percentage of total embryos.
FIGURE I

The effect of ascorbic acid sulfate (0.1 mM) on incorporation of $^{14}$C-amino acids into developing embryos of the sea urchin, *L. pictus*: a) amino acid pool; b) total proteins; c) acid-soluble nucleotide pool; and d) total nucleic acids (DNA and RNA).

( ) control embryos and (X--) ascorbic acid treated embryos.
FIGURE II

The effect of ascorbic acid sulfate (0.2mM) on incorporation of 14C-amino acids into developing embryos of the sea urchin, *L. pictus*. a) amino acid pool; b) total proteins; c) acid-soluble nucleotide pool; and d) total nucleic acids (DNA and RNA). ( ) control embryos and (x--x) ascorbic acid treated embryos.
FIGURE III

The effect of ascorbic acid sulfate (0.3 mM) on incorporation of $^{14}$C-amino acids into developing embryos of the sea urchin, *L. pictus*. a) amino acid pool; b) total proteins; c) acid-soluble nucleotide pool; and d) total nucleic acids (DNA and RNA).

(--_______--) control embryos and (X------X) ascorbic acid treated embryos.
The effect of ascorbic acid sulfate (0.5 mM) on incorporation of 

\(^{14}C\)-amino acids into developing embryos of the sea urchin, \textit{L. pictus}. 

- a) amino acid pool; b) total proteins; c) acid-soluble nucleotide pool; and d) total nucleic acids (DNA and RNA).

(_______) control embryos and (x--x--) ascorbic acid treated embryos.
FIGURE V

The effect of various concentration of ascorbic acid sulfate on the incorporation of $^{14}C$-amino acids into the lipid fraction from *L. pictus* embryos.

(—) control and (x—x) treated embryos.
PLATE I  Control *L. pictus* embryos 3 hours after fertilization at 15°C. The majority of the embryos have completed the third cleavage. 500X MAG. Live embryos.

PLATE II  The effect of diguanosine tetraphosphate (0.3 mM) on *L. p pictus* embryos 3 hours after fertilization at 15°C. Surface blebs are still observable at this stage. The hyaline layer is not as thick and there are a larger number of embryos that have undergone the first cleavage compared to the ascorbic acid sulfate treated embryos. Mitotic activity is absent or low compared to controls. 3200 X MAG. Live embryos; phase contrast.
PLATE III  The effect of ascorbic acid sulfate (0.3 mM) on *L. pictus* embryos after 3 hours at 15°C. stage. Very few embryos have completed the first cleavage. The majority are still "dormant" while others show incomplete furrows, and have become rounded after absorption of the blebs. In control embryos cleavage begins at 90 minutes at 15°C. Compare with Plates 1, 1V and VI. 500 X MAG. Live embryos.

PLATE IV  The effect of ascorbic acid sulfate (0.3 mM) on *L. pictus* embryos after 3 hours at 15°C. The embryos have become rounded and the blebs have been reabsorbed in all but one case. Dark, dense granules can be seen around the periphery of the cortical region and an incomplete furrow is visible in one of the embryos dividing the animal region into two incomplete blastomeres. In later stages the latter embryo would give rise to three blastomeres, two vegetalized animal halves and the uncleaved vegetal half. Cytoplasmic constrictions and superficial furrows are also seen in the other embryos. No mitotic activity is observable in any of the embryos at this stage. 2000 X MAG. Live embryos.
PLATE V

The effect of ascorbic acid sulfate (0.1 mM) on *L. pictus* embryos after 1½ hours at 15°C. The use of phase contrast shows the thickened hyaline layer surrounding the plasma membrane. 2800 X MAG. Live embryos; phase contrast.

PLATE VI

Control *L. pictus* embryos 1½ hours after fertilization incubated at 15°C. Majority of embryos have completed the first cleavage. Mitotic figures are distinct and the hyaline layer is much thinner than in the ascorbic acid sulfate treated embryos. Compare with PLATE V Positive Feulgen reaction. 2800 X MAG. Feulgen reaction.
PLATE VII. The effect of ascorbic acid sulfate (0.5 mM) on *L. pictus* embryos after 3 hours at 15°C. A large number of spherical surface blebs are still visible. Most embryos are irregular in shape and not too many have become rounded. One of the embryos in the centre has divided into three blastomeres, two arising from the animal region while the vegetal region has not undergone division. Compare with PLATES I & III. 500 X MAG. Feulgen reaction.

PLATE VIII The effect of ascorbic acid sulfate (0.3 mM) on *L. pictus* embryos after 8 hours at 15°C. The embryo on the left has not undergone cytokinesis while the nuclei have undergone 4-5 divisions. The nuclei are arranged peripherally along the cortex while a few are scattered in the cytoplasm. The controls and the embryo on the right have progressed to the mid-blastula stage. 2000 X MAG. Feulgen reaction.
PLATE IX

An early blastula of *L. pictus* 5 hours after fertilization. The cell partitions are distinctly visible. The rim of the blastula has a single row of cells, the nuclei arranged as in PLATE X but only one per cell. 2800 X MAG. Feulgen reaction.

PLATE X

The effect of ascorbic acid sulfate (0.3 mM) on *L. pictus* embryos after 8 hours at 15°C. The cytoplasm is devoid of any partitions and the nuclei are arranged peripherally. Compare with PLATES VIII and IX 2800 X MAG. Feulgen reaction.
PLATE XI: The effect of ascorbic acid sulfate (0.3 mM) on *L. pictus* embryo 8 hours after fertilization at 15°C. Monoasters (about 12-16) are arranged along the periphery but some are scattered in the central region. Only a few of these embryos were observed. **2800 X MAG.** Hematoxylin and eosin stained embryo.

PLATE XII: Normal *L. pictus* embryos 8 hours after fertilization at 15°C. The majority of the embryos are in the mid-blastula stage. **500 X MAG.** Live embryos.
PLATE XIII  The effect of a low level ascorbic acid sulfate (0.1 mM) on *L. pictus* embryos 8 hours after fertilization at 15°C. Most of the embryos are in the early blastula stage. Compare with PLATE XII. 500 X MAG. Live embryos.

PLATE XIV  Animalized embryo of *L. pictus* with blastomeres of uniform size produced by 8 hours in ascorbic acid sulfate (0.3 mM) at 15°C. The large nuclei are Feulgen positive and show a high level of mitotic activity compared to the rest of the embryo. Compare with PLATE VIII. 2000 X MAG. Feulgen reaction.
PLATE XV  Induction of a radialized *L. pictus* embryo after treatment with ascorbic acid sulfate (0.3 mM) for 8 hours at 15°C. The embryo shows a radial symmetry and it is elongated on the dorso-ventral axis. The blastomeres of the vegetal region are larger in size. The nuclei give a weak Feulgen reaction and there is very little mitotic activity at this stage. Compare with PLATES VIII & XIV 2000 X. MAG. Feulgen reaction.

PLATE XVI  Formation of a vegetalized embryo of *L. pictus* after 8 hours incubation in 0.3 mM ascorbic acid sulfate at 15°C. This embryo shows the larger vegetalized blastomeres at the animal region. The nuclei generally give a weak Feulgen reaction and there is very little mitotic activity at this stage. Compare with PLATES VIII, XIV, & XV 2000 X. MAG. Feulgen reaction.
PLATE XVII  Production of an exo-gastrula embryo of *L. pictus* after 8 hours incubation with 0.3 mM ascorbic acid sulfate. The endomesoderm has become evaginated and the archenteron appears as an irregular mass of cells. Compare with PLATES VII, VIII, & XV 2000 X MAG. Feulgen reaction.

PLATE XVIII  Blastomeres from a control embryo of *L. pictus* showing high mitotic activity and positive Feulgen reaction. Metaphase, anaphase, and telophase stages are seen. Compare with PLATES II, V, & VI. 5000 X MAG. Feulgen reaction.
The effect of ascorbic acid sulfate (0.3 mM) on *L. pictus* after 8 hours of incubation at 15°C. This "animalized" embryo contains nuclei which give a positive Feulgen reaction. A high mitotic rate is seen in the entire embryo.

Compare with PLATES XV, XVI, & XVII 2800 X MAG. Feulgen reaction.
PLATE XX
Control *L. pictus* embryo after 24 hours incubation at 15°C. (Late gastrula). The archentron is bent and has fused with the presumptive mouth ectoderm which has undergone invagination. Loose mesenchyme cells are also seen in the coelome. 800 X MAG. Live embryos; phase contrast.

PLATE XXI
Development of *L. pictus* embryos after 24 hours in ascorbic acid sulfate (0.3 mM). The embryos have recovered from the effects of ascorbic acid sulfate and are similar to the controls but smaller in size. Otherwise, they appear completely normal and healthy. In the bottom right are seen some debris from dead exo-gastrulæ. 800 X MAG. Live embryos; phase contrast.
APPENDIX A

Composition of Artificial Sea Water (from Tyler, 1953). The "Sea Water" has proved effective, not only for prolonging the life of the sperm and for improving fertilization and early development, but also for maintaining adult animals for long periods of time in a closed aquarium system.

<table>
<thead>
<tr>
<th>Stock Solution</th>
<th>Proportions</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.55 molar NaCl</td>
<td>1000 volumes</td>
</tr>
<tr>
<td>0.55 molar KCl</td>
<td>22 volumes</td>
</tr>
<tr>
<td>0.37 molar MgCl₂</td>
<td>195 volumes</td>
</tr>
<tr>
<td>0.37 molar Na₂SO₄</td>
<td>103 volumes</td>
</tr>
<tr>
<td>0.37 molar CaCl₂</td>
<td>35 volumes</td>
</tr>
<tr>
<td>0.55 molar NaHCO₃ (to pH 8)</td>
<td>ca 6 volumes</td>
</tr>
</tbody>
</table>
APPENDIX B

Feulgen Reaction

Zenker's acetic fixed specimens were (a) hydrolized in 1 N HCl at room temperature for 1 minute, (b) hydrolized in 1 N HCl at 60 degrees C for 8 minutes, (c) rinsed sections in 2 changes of distilled water, (d) transferred sections to Schiff's reagent for 2 hours in the dark, (e) rinsed for 10 minutes each in 3 successive changes of SO2 water, (f) washed in tap water for 5 minutes, (g) dehydrated in alcohol, and (h) cleared in xylene and mounted in permount.

Controls of pleuteus larval stages were treated along with the experimental slides. The DNA material both in the nucleus and cytoplasm showed bright pink staining.
APPENDIX C

Hematoxylin and Eosin Staining

Bouin's fixed material was washed several times with 70% ethanol until the yellow colour was removed. The slides were washed in decreasing concentrations of alcohol to tap water and stained in hematoxylin (Harris) diluted 1:1 with distilled H₂O. The slides were stained for 3 minutes, destained in 0.25% HCL for 30 seconds, washed in tap water for 5 minutes, dehydrated in alcohol, stained with 0.5% eosin in 90% ethanol for 10 seconds, dehydrated in alcohol, cleared in xylene, and mounted with permount. The nuclei stained purplish-blue while the cytoplasm also showed a granular-purple light stain against the red-pink eosin background.
LITERATURE CITED


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