THE EFFECTS OF SPECIFIC ANTISERA ON THE CLEAVAGE OF THE SEA URCHIN EGG

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ABSTRACT

Rabbit antisera against sea urchin unfertilized eggs (gelatinous coat removed), fertilized eggs and the gelatinous coat (fertilizin) of the unfertilized eggs have been found to inhibit the nuclear and cytoplasmic divisions of the fertilized egg. Antisera against whole sperm or sperm antifertilizin extracts were not effective in this regard, and absorption of the effective sera with sperm did not remove the blocking antibody of those sera.

Extracts (calcium-free sea water) of whole fertilized eggs containing the ectoplasmic layer material were found to react with anti-egg sera after absorption with sperm, but not after absorption with eggs. This material in this extract has been tentatively described in this thesis as a carbohydrate. One such extract was found to agglutinate the sperm of the species, but not sand dollar sperm. Subsequent extracts have not shown this property.

The respiration of fertilized eggs, blocked by the effective sera, was found to increase over the controls.

The sodium content of fertilized eggs treated with immune blocking serum was not found to differ from the sodium content of eggs in normal serum.

The tension at the surface of unfertilized eggs increases in the presence of anti-unfertilized egg serum, as judged by centrifugation experiments.

TABLE OF CONTENTS

INTRODUCTION

This investigation was originally concerned with the possibility of demonstrating changes in antigenic quality during the development of the sea urchin embryo through the use of antisera made against cleavage stages, blastulae and gastrulae. It was thought that the antisera could be rendered stage specific by means of absorptions if new antigens appeared during development. Similar investigations have been performed with amphibian embryos, and since sea urchin material is in some ways superior to amphibian material for this sort of study, the undertaking of the problem seemed justified. During preliminary experiments, an antiserum made against the 16-cell stage of Lytechinus pictus embryos exhibited an inhibition of the division of 8-cell embryos to the 16-cell stage. The same antiserum was also found to inhibit the cleavage of 2-cell stages to 4-cells, and to inhibit the first cleavage of the fertilized egg. The inhibitions involved both nuclear and cytoplasmic division. It was decided to pursue this problem with the purpose of discovering, if possible, the nature of the block to cleavage and to attempt to identify the source of the antigen responsible for the production of the blocking antibody. In order to provide a background of previous experimental work for the results to follow, the following literature survey is presented.

During the past 50 years, a large amount of literature

has accumulated on the cytotoxic effects of antisera on cells. Earlier studies involved the injection of antiserum into the intact animal, with subsequent observation of the effects on the tissue or organ used as the antigen. Because of the obvious difficulties involved in interpreting the results of such experiments (blood vessel damage and other secondary effects), the tissue culture method, first employed by Harrison (1907), soon became the more popular approach. The course of action of the antisera on various tissues could be followed through the microscope at the cellular level. Observations on the extent and kind of cellular effects have given information about the specificity and mode of action of antisera directed against organs, tumors, tissue extracts and cell particles.

In more recent years embryologists, in attempts to discover the antigenic composition of embryonic tissue, have utilized similar techniques. Immune sera, prepared against adult organs and embryos in various stages of development, have been incorporated into the embryonic on the assumption that when the antigens of the embryo which correspond to those used for immunization appear (or become available to the antibody), an effect on the development of the embryo may be noted. The tissue culture method usually employed for culturing adult tissues involved the explantation of a small sample of the test tissue to hanging drops of normal or immune serum or plasma, and inorganic salts. Embryo extract is sometimes added to insure optimal growth conditions. The

culture of embryos ranges from simply adding sera to the inorganic medium (balanced salt solution) to explantation of the chick blastoderm to an agar block containing the desired sera, in nutrient medium.

I. Effect of antisera on culture of adult normal and malignant tissues.

Lambert and Hanes (1911) were the first investigators to utilize tissue culture techniques in the study of the specificity of cytotoxic antisera. Their study consisted of the injection of rats and guinea pigs subcutaneously with minced mouse and rat sarcoma respectively. They found that after two injections, the plasma of these animals proved unsuitable for the growth of the respective tumors. Lambert (1914) extended these observations. He injected guinea pigs with embryonic rat skin, rat sarcoma and defibrinated rat blood, and obtained almost complete inhibition of growth <u>in vitro</u> of both rat skin and sarcoma in the plasma of the injected animals. Control cultures in normal plasma grew well. Similarly, he found the plasma of guinea pigs injected with either chicken heart or intestine inhibited the growth of both these tissues. Lambert therefore concluded that the cytotoxins produced in the foreign species are not specific for the tissue injected.

Pybus and Whitehead (1929) found antisera produced in rabbits against mouse mammary carcinoma to be toxic to the homologous cells and to mouse heart and kidney cells as well

in serum cultures. The epithelial sheets of the carcinoma and kidney cells were seen to disintegrate within 15 minutes under the action of immune serum, whereas the fibroblasts of the heart were less severely damaged. The authors refer this last fact to the structure of the outgrowth rather than to the specificity of the antiserum.

Niven (1929) described in more detail the effects of a cytotoxic antiserum on cells in culture. She injected minced whole mouse embryos of advanced stages (17 days) intraperitoneally into a rabbit. After 40 injections at intervals of 2-7 weeks the serum was collected and tested on hanging drop cultures of newborn mouse heart. Control rabbit serum characteristically causeddeath of outgrowing fibroblasts after 5-6 hours, as determined by the penetration into the nuclei of neutral red. The toxic effect could be removed by heating at 56° C for 30 minutes. Fibroblasts in unheated immune serum showed signs of deterioration within 5 minutes. Nuclear shrinkage occurred with subsequent pycnosis, regardless of the fixative used, followed by intensification of the nuclear membrane. Mitosis stopped, the chromosomes clumped and the spindle became granular. The cells then became surrounded by a definite pellicle. After 30 minutes, the cytoplasmic constituents were not stainable with either iron-hematoxylin (osmium fixative) or methyl green-fuchsin. Earlier bleedings of the same rabbit gave slower acting, less pronounced effects. The effect of these earlier bleedings was reversed in some cases by transfer to a fresh medium. However the drastic

effects of the final serum were not reversed by transfer. Heating the final immune serum for 30 minutes at 56° C led to a loss of the rapid action of the serum. It was found that this property could be restored by the addition of fresh normal rabbit serum devoid of any drastic, fast acting qualities. Niven concluded complement was required for the intense cytotoxic action of the final serum. The cytotoxic effects were found to be species specific. Rat tissue was harmed to a lesser extent than mouse tissue, and guinea pig tissue in immune serum was not affected to any greater extent than the same tissue in normal serum. The antiserum acted also on cultures of mouse epithelial cells, mouse carcinoma, and a spontaneous mouse adeno-carcinoma as well as on mouse heart fragments. The antibody was found to be absorbable with the homologous antigen, but no further absorption experiments were done. From the cytological data given, the possibility exists that the strong, fast acting serum actually lysed the cells, allowing dispersal of the cytoplasmic constituents and entrance of the antibody into the cells with subsequent effects on the nucleus, chromosomes and spindle.

Other workers have described organ specificity in connection with such cytotoxic activity. Foot (1912) injected rabbits with chicken bone marrow, and observed the inhibition of migration of the marrow cells in immune plasma. Foot thought the sera were organ specific and capable of damaging the bone marrow of other species. Verne and

Oberling (1932) maintained that serum produced against embryonic rat kidney is species specific as well as organ specific. Their sera were damaging to cultures of rat kidney, but not to cultures of other rat tissues or to kidney cultures of other species.

Harris (1948) innoculated rats with grafts of heart from 16 day chicks, day old mice and day old rats (of a different strain than that used for immunization). The effects of the resulting antisera on chick heart, kidney, and spleen, and on mouse and rat heart cultures were studied in plasma hanging drops. The results show species specificity, in that rat anti-chick heart damages only chick heart, rat-anti mouse only mouse heart. Rat anti-rat heart serum showed no effect on rat cells. Rat anti-chick heart damaged cultures of chick spleen or kidney, but to a lesser extent, indicative of organ specificity. In extreme cases, the cellular damage was similar to that described by Niven (op. cit.). The nuclei of the cells became pycnotic, and the staining properties of the cytoplasm were lost.

Grunwalt (1949) made a study of anti-brain sera using tissue culture technique. Rabbits were injected with suspensions of whole chicken brain, the alcohol soluble fraction of whole brain, and the alcohol insoluble residue. The immune sera were tested on cultures of spinal cord of 9 and 13 day old chick embryos, and on heterologous organs of 17 day chick embryos. The outgrowth of nerve fibers from the explant was used as a measure of the effect of the anti-

serum in the case of spinal cord cultures, and cellular proliferation was used in the case of other organs. Grunwalt concluded that only the alcohol fraction is organ specific, and that the sera against whole brain and alcohol insoluble residue are not. All sera were species specific when tested on rat, guinea pig and human embryo spinal cords.

Lumsden (1928) claimed to have produced specific cytotoxins for mouse sarcoma cells in rabbits and rats. Phelps (1937) was unable to confirm this, using the same tumors, and pointed out that the tumor used by Lumsden (no. 63 mouse carcinoma) is so fragile in culture that the addition even of physiological saline is sometimes sufficient to cause cytolysis of the cells. It might be well to point out also that Lumsden's cultures were serum cultures, and that Ludford (1933) has since shown that a number of types of malignant cells do not proliferate as well in serum as on a plasma clot, presumably due to lack of sufficient substratum and lack of ability to adhere to the surface of the coverslip. Harris (1943) utilizing inbred mice and tumors of known genetic background found no evidence of cancer specific antibodies. Cultures of mouse sarcoma, heart, kidney and spleen in rat anti-mouse sarcoma serum were all severely damaged.

It has been found that antibodies against particulate cellular constituents and virus like agents also exhibit cytotoxic properties. Kidd (1946) studied the effect of antibodies produced by rabbits implanted with Brown-Pearce

carcinoma on suspensions of the tumor cells. The antibodies so produced react with a distinctive sedimentable intracellular constituent of the tumor cells. When dispersed tumor cells were incubated with the antibody, no lysis, agglutination or other noticeable phenomenon occurred. However, when these incubated cells were transplanted into a susceptible host rabbit, no growth ensued.

Dulaney and Arnesen (1949) prepared antisera in rabbits against cytoplasmic granules and nuclei from normal and leukemic spleens. The mitochondria, microsomes and nuclei were prepared by differential centrifugation. Leukemic cells, maintained by intraperitoneal transfer in the offspring of brother x sister matings, were incubated with the various sera for two hours and subsequently injected into the host. Anti-normal-cell mitochondrial serum and anti-normal-cell microsomal serum protected only at lower dilutions (90% survivors at 1:200 and 0-1:10 respectively), whereas anti-leukemic-cell mitochondrial serum and anti-leukemic cell microsomal serum protected at considerably higher dilutions (1:500 and 1:200 respectively). Anti-leukemic-cell nuclei serum protected at 1:10 (76% survivors); anti-normal-cell nuclei serum allowed only 12% survivors at this dilution. The number of rabbits used in the production of the above sera was not mentioned. The higher protective titers of leukemic antisera could conceivably have been due to variation in antibody production among individual rabbits.

More recently, Imagawa et al. (1954b) investigated the

action of antisera against normal mouse mammary gland, both with and without the mammary gland tumor agent, and of antisera against malignant mammary glands with the agent on cultures of fetal mouse intestine, and cultures of mammary gland cells containing the agent. The antisera were produced in guinea pigs. Degenerative changes (shrinkage of cytoplasm and pycnotic nuclei) were observed only in cultures of cancer cells in antiserum against normal or malignant tissue containing the agent. These antisera were without effect on cultures of fetal intestine. Cultures of malignant tissue and intestine in normal guinea pig serum and in antisera against normal mammary gland without the agent were without noticeable damage. Complement was shown to be necessary for the cytotoxic reaction. That an effect of antiserum against normal tissue without agent is in fact exerted on cancerous cells *in vitro* is indicated by the results of Imagawa *et al.* (1954a). Incubation of tumor tissue in this serum diminished the number of takes upon subsequent transplantation to susceptible hosts, though not to the same extent as sera directed against cells containing the agent. Imagawa (1954a) also reports a heat labile (56° for 30 minutes) cytotoxic factor in normal rabbit serum. The cytotoxic factor was not restored in heated sera by the addition of fresh guinea pig serum.

Law and Malmgren (1951) using rabbit antisera, were unable to demonstrate a difference in growth, in susceptible mice, of mammary cancer cells exposed *in vitro* to sera pre-

pared against mammary gland tumor, with or without agent, or to sera prepared against normal tissue, with or without the tumor agent. The reason for this discrepancy between the results of Imagawa et al. and those of Law and Malmgren is not immediately apparent, though the presence of the cytotoxin in normal rabbit serum may have obscured any differences in number of takes in the experiments of the latter workers. No control data of number of takes after incubation with normal serum were given.

Green et al. (1946) have studied the inactivation of active tumor cell centrifugates presumably containing the mammary tumor agent by antisera against centrifugates of mammary tumor tissue, and heart, spleen, liver, kidney (mixed antigen). The agent was inactivated, as judged again by number of takes in susceptible mice, by antisera against tumor cell centrifugate and not by antisera against the centrifugate of normal tissues. They concluded the virus is of exogenous origin because of the failure of normal tissue particles to induce inactivating antibodies. This is consistent with the results of Imagawa et al. (1954 ab).

Stimulation of cell growth by antisera has been claimed by a number of investigators. Reticulo-endothelial immune serum (REIS), prepared by the injection of spleen and bone marrow into a foreign species, has been claimed to have a stimulatory effect on the reticulo-endothelial system, if used in low concentrations (Bogomolets, 1943). Human REIS, produced in horses, is thought by Bogomolets and other

Soviet workers to have a wide range of therapeutic application by virtue of this stimulation (see Straus (1946) for review). Pomerat and Anigstein (1945) utilized rabbits in preparing antisera against chicken, rat and guinea pig spleen and bone marrow, and evaluated the effects of the antisera on homologous spleen cultures in hanging drop preparations. They noted a marked inhibition of outgrowth in these explants, and a clumping of migrating cells. Pomerat (1945a) also tested the effect of chick REIS on chick heart cultures. It was found that inhibition of outgrowth occurred at higher concentrations of antiserum (1:20). The effect was presumably species specific in that no inhibition of outgrowth of chick heart fragments occurred in rat REIS. There was some evidence of stimulation of growth of chick heart fibroblasts in chick REIS at lower concentrations (1/400 dilution) of antiserum, amounting to approximately a 20% increase over the growth of the controls. Additional embryo extract had to be added to the media before this increase was realized. The antiserum had a complement fixation titer of 1:1200. The effect of rat REIS was tested (Pomerat 1945b) on rat spleen and on Walker rat sarcoma in similar hanging drop preparations. It was found that outgrowths from spleen were inhibited at a dilution of 1:512, whereas the tumor growth was inhibited only at 1:4. Again, outgrowth of these tissues in chicken REIS was comparable with normal serum controls, indicating the species specificity of the effect. Straus *et al.* (1946) studied

the effect of low doses of REIS on the healing of experimentally produced fractures in rabbits. Their findings indicate a more rapid repair of the fractures in animals treated with such low doses of the antiserum, as compared with the rate of repair in animals treated either with higher doses of REIS or normal serum.

Kaliss and Molomut (1952) have reported an enhancement of tumor growth in strains of mice ordinarily resistant to the tumor used. This was brought about by prior injection into the host of rabbit or mouse antisera made against a mixed antigen of tumor, spleen and kidney from the susceptible strain. Marked growth and some takes resulting in the death of the animal occurred if the tumor was implanted a few days after the last of a series of antibody injections. This could conceivably be due to the combination of antibody, present in the circulation, with the implant, thereby changing the normal antigenicity of the tumor for the resistant strain.

II. The effects of antisera on developing embryos.

A number of the preceding papers have shown organ-specific cytotoxic effects of antisera on cells in culture (For a more complete discussion of organ specificity, see Loeb (1945)), indicating that each organ possesses antigenic groups peculiar to it in addition to species antigens. In vitro complement fixation tests (Landsteiner, 1945) indicate a similar situation; that is, though cross reactions occur,

the antiserum reacts at higher dilution with homologous organ cells than with heterologous cells. Recently, Pressman and Sherman (1951), and Bale and Spar (1954) have found that I^{131} labelled gamma globulin from rabbits immunized with various rat organs could be made, upon injection into rats, to localize more strongly in the homologous organ than in the heterologous organs. Prior to injection the antibodies were reacted with homogenates of the homologous organ, followed by elution of the antibody from the antigen-antibody complex at high pH. Since morphological differences in organ structure seem to be paralleled by molecular differences, it became of interest to embryologists to detect when the antigens of a specific organ make their appearance. A number of antigens similar to or identical with adult antigens have been found in the early embryo (review by Tyler, 1955). In some cases these antigens have been found to make their appearance prior to the morphological differentiation of the organ (lens; ten Cate and van Doorenmaalen, 1950; brain, heart and spleen; Schechtman, 1948, Ebert, 1950-51). Other experiments, using embryonic tissue as immunizing antigens, have shown changes in quantity and differences in spatial distribution of antigens as development proceeds (Flickinger & Nace, (1952); Clayton, (1953); Spar, (1953)). Experiments in which embryos were cultured in such anti-organ or anti-embryo sera with the hope of finding more exactly the time of appearance and location of these antigens are described below.

Ebert (1950) explanted chick blastoderms onto agar (method of Spratt, 1947) containing antisera against adult brain, heart and spleen. Schechtman (1948) and Ebert (op.cit.) demonstrated that the early blastoderm (primitive streak) contains antigenic groups occurring also in saline extracts of brain, heart, liver and muscle of newly hatched chicks which are distinct from blood and yolk. It was found that absorption of these sera with either heart, brain or spleen removed precipitins for the embryonic antigens, despite the fact that absorption with heterologous adult antigen does not remove all the precipitins for the homologous adult antigens. The embryonic antigens therefore exhibit a more generalized specificity. It was in order to demonstrate more clearly the specificities of the group of embryonic antigens that the embryos were grown in the presence of the various antisera, the supposition being that each antiserum would affect the development of the embryo in a distinct and specific manner. Embryos were grown also on normal rabbit serum. Explants of head process stage characteristically produced optic vesicles, auditory vesicles, 20 somites, pulsating heart when cultured on medium containing 1:15 normal serum. Anti-heart and anti-spleen sera were not found to differ in their effects in the range 1:15-1:80. Within 10 hours after explantation disorganization of the embryo was observed, characterized by clumping of cells, loss of gross morphological structure. At dilutions of antisera up to 1:200, morphogenesis of the embryo occurred in the complete absence of

growth, yielding normal but dwarfed embryos. Anti-brain sera acted in much the same way as anti-heart and anti-spleen sera, but at higher concentrations. The disorganization of the embryo occurred only at dilutions of 1:15-1:30, and the inhibition of growth was obtained at concentrations of 1:30-1:75. These non-specific inhibitory effects are consistent with the previously mentioned serological studies which revealed a broader organ specificity among the embryonic antigens. However Ebert (op. cit.) continued these studies employing other dilutions of antisera. Dilutions of anti-heart and anti-spleen sera in the range of 1:70 and 1:90, or anti-brain serum between 1:30-1:50 gave more specific effects. Under these conditions, anti-heart and anti-spleen sera affected mainly mesodermal elements, while anti-brain serum acted only on nervous elements. Further, it appeared that embryos grown in the presence of anti-spleen sera developed pulsating hearts, whereas those grown on anti-heart sera apparently did not. Ebert concluded from these studies that organ specific antigens can be detected in the early blastoderm before discrete organs have developed, and further that the antigens are localized in definite areas of the early blastoderm. The possibility that such "specific" effects in dilute antiserum represent beginning "gradient-wise" cytolysis cannot be conclusively ruled out,however.

Flickinger and Nace (1952) carried out similar studies on the embryo of Rana temporaria. Antisera were prepared in rabbits against adult frog serum, ovarian oocyte super-

natant, vitellin and pigment, and supernatant and pigment fractions of hatched tail bud stages. Embryos were cultured in each of these sera. The only specific effects observed were in anti-tailbud supernatant. Gastrulae and neurulae placed in this serum died from a gradient-wise (antero-posterior, dorso-ventral) cytolysis at the early tail bud stage. Embryos placed in this antiserum at early tail bud stage were cytolyzed at that stage, indicating that time of exposure was not a factor. Ambystoma mexicanum embryos were not affected by the antiserum. In view of their finding that all embryonic stages tested contained serum-like combining groups, it is surprising that no effects on development were observed in serum-antiserum. However, lack of such developmental effects can be ascribed to the unavailability of the antigen for reaction in the whole living embryo, whereas in vitro reactions can be observed using fractions of disrupted embryos. This might also be the case with the gastrulae and neurulae in anti-tail bud serum -- i.e. the antigen becomes available for reaction in the epidermis at the tail bud stage, being unavailable for combination with antibody in the preceding stages.

Using Triton alpestris embryos and parts thereof as antigens, and rabbits as antibody producers, Clayton (1953) made a study of the spatial and temporal distribution of embryonic antigens. Antigens arising between blastula and gastrula, between gastrula and neurula, and neurula and tail bud stages were demonstrated by absorptions of anti-

gastrula and anti-tailbud sera with extracts of the preceding stages. Absorption studies of antisera made against ectoderm and archenteron roof revealed antigenic groups characteristic of these germ layers. Intact embryos and tissue explants were cultured in various antisera and in normal serum. The average chance of survival of an embryo in normal serum was 96%. The chance for survival of a gastrula in anti-gastrula serum absorbed with blastulae was not significantly different from the chance for survival in unabsorbed serum (37%), and the chance for survival of blastulae in the same absorbed antiserum was not significantly different from the survival of blastulae in unabsorbed serum (11%), but more than 25% of these surviving blastulae died on reaching the gastrula stage, indicating a stage specific effect. The relatively high mortality of blastulae in anti-gastrula serum was ascribed by Clayton to a difference in location of the blastula antigens--presumably more being available for reaction with antibody than in the gastrula. Explants of ectoderm in anti-ectoderm absorbed with mesoderm, or explants of mesoderm in anti-mesoderm absorbed with ectoderm had a survival of 16%, and a survival of 48% was observed when the antisera were absorbed with homologous tissue.

Stimulation of the growth of embryonic organs by the homologous anti-organ serum has been reported by Weiss (1939, 1947). Autolyzed suspensions of adult chicken liver, kidney and muscle were injected into guinea pigs. The resulting antisera were injected into chick eggs, ranging from 60 hours

to 8 days incubation. Only the results for the oldest group, sacrificed on the 20th day of incubation, are available. Although the total weight of injected embryos averaged considerably below that of the controls, those embryos receiving anti-liver serum showed an increase in liver weight of 10% above normal. Embryos receiving anti-kidney serum showed an increased weight of the metanephros of about 10% above those receiving anti-muscle or anti-liver sera, and an increase of about 1% over normal embryos. Embryos receiving anti-muscle serum showed no increase in liver or kidney weight. No normal sera were injected. These results are opposed to the results of Ebert (1950a) on the chick, and opposed to most of the results observed when antiserum and homologous tissue are cultured together. The stimulation of the reticulo-endothelial system by REIS, as yet largely unsubstantiated by tissue culture methods, remains the only parallel case.

The classical phenomena observed when cells are mixed with antibody include agglutination, swelling, opsonization, lysis, as well as the phenomena of growth inhibition, loss of proliferative power and changes in staining properties described, under the general heading of cytotoxic effects, in the papers presented above. Zinsser (1939) has stated:

> The resultant reactions which may be observed with this sensitized antigen (agglutination, precipitation, complement fixation, bactericidal phenomena, bacteriolysis, opsonization, or sensitizing effects in the anaphylactic sense) are determined not by differences in the nature of the antibodies with which the antigen

> has united, but rather by the physical state of the antigen itself, the nature of the co-operative substances (alexin, leucocytes, tissue cells) and by the environmental conditions under which observations are made.

This statement has been called the unitarian view. Thus, precipitation and agglutination are seen to be a function of antigen particle size, and inhibition of migration or cell division and lysis may be a function of cell constitution and whether or not the reaction of cells and antiserum involves complement. The central core of this view is now well substantiated by the general body of immunological investigation (Zinsser et al.(1939), Boyd, (1947)), though cases in which the quality of the antibody determines the type of reaction observed (e.g. "univalent" antibodies) occur. The words "cytotoxic serum" or "blocking serum" will therefore be used in the unitarian sense in this thesis when referring to the effect of antisera on cells in culture.

MATERIALS AND METHODS

I. Preparation of antigens.

Eggs and sperm of Lytechinus pictus or Strongylogentrotus purpuratus were obtained by KCl injection (Tyler, 1949a) or by dissection of the gonads. Preparatory to injection into the rabbit, the unfertilized eggs were freed of their gelatinous coat by treatment with acid (pH 3.5) sea water (Tyler, 1949b), washed three to four times with fresh sea water and suspended in either 1% NaCl or sea water. The final nitrogen concentration was adjusted to 200-300 micrograms (µgm) of nitrogen per cubic centimeter. Kjehldahl nitrogen determinations throughout the experiments were done by the digestion method of Boell (1945) and the distillation and titration method of Ballentyne and Gregg (1947). Demembranated fertilized eggs and early cleavage stages were obtained by removing the fertilization membrane with a fine pipet 1-5 minutes after insemination, or by exposure to a 1 molar solution of urea for 30 sec. directly following insemination. The latter procedure was used for the tougher membranes of S. purpuratus, while the former sufficed for L. pictus eggs. These antigens were diluted with sea water to a final nitrogen concentration of from 300 to 1000 micrograms per cc. Mesenchyme blastulae and gastrulae were harvested by centrifugation, washed, and diluted to a final concentration of 200-250 µgm nitrogen per cc. All antigens were frozen in 1 cc lots at -15°C until injected.

Antisera were also supplied by Dr. Tyler. For the preparation of these, the following antigens were used. Whole sperm were centrifuged lightly and washed with sea water to remove seminal fluid. A 3 to 10% suspension in sea water was made on the basis of packed volume of sperm, and a given volume injected. Antifertilizin from sperm was prepared from sperm washed as above followed by acidification to pH 4.5. After centrifugation, the residue was discarded and the supernatant solution dialyzed against distilled water. The material was then precipitated out of solution at pH 3.0, and the supernatant discarded. The precipitate was brought into solution at pH 8.2 and the salt concentration adjusted to 1% by the addition of NaCl. The nitrogen content by Kjehldahl determination was 45-75 µgm per cc. Gelatinous coat (fertilizin) was obtained by acid treatment of unfertilized eggs. The supernatant sea water containing the fertilizin was alkalinized with 1 M NaOH (40 ml per liter of solution) and the resulting precipitate collected by centrifugation and 1/20 the original volume of neutral 3.3% NaCl added, and the pH adjusted to 3.5-4.5. The solution was then dialyzed against 3.3% NaCl, followed by cold precipitation in 5/4 volumes of 95% EtOH. The precipitate was dissolved in 3.3% NaCl, the pH adjusted to 7-8. This final solution, used as the fertilizin antigen, contained from 32-58 µgm Nitrogen per cc, depending on the preparation. The egg residue following fertilizin extraction was frozen-thawed, blended for one minute and centrifuged for 1 minute at moderate speed. The supernatant was used as

the antigen, and contained approximately 99 µgm nitrogen per cc.

II. Preparation of antisera.

Albino rabbits were bled either from the marginal vein of the ear or by cardiac puncture for pre-injection serum, which was collected by allowing the blood to clot at room temperature for one hour and at 5°C over night. The clot and cells were centrifuged down and the clear serum either frozen immediately in serum bottles, or dialyzed vs. sea water and frozen (-15° C). No serum was used on eggs before dialysis. One week following the pre-injection bleeding, the injection schedule was started. This consisted of intravenous injections of 0.5 cc antigen alternated with subcutaneous or intraperitoneal injections of 1.0 cc. Injections were given on alternate days for 10-12 injection series. Animals were bled 5-7 days following the final injection by cardiac puncture. The injection procedure used by Dr. Tyler was essentially the same as described above, with additional bleedings taken 10-15 days after the last injection.

III. Titration of antisera.

Serial dilutions of the resulting antisera were mixed with freshly shed eggs of either L. pictus or S. purpuratus, or with the sperm of these species. The highest dilution causing precipitation membranes to appear on the gelatinous coat of the eggs, and the highest dilution of antiserum agglutinating sperm was recorded.

IV. Heated sera.

A few sera were heated to 100°C (15 min.), 70°C (15 min.) and 70°C (3 min.) to test for the thermolability of the substance causing the effect on the eggs. The sera were sealed in vials to prevent loss of carbon dioxide and placed either in 70°C constant temperature bath, or in a flask of boiling water equipped with a reflux condenser. Heating aliquots of one immune and one normal serum to 56° C for 30 min to inactivate complement was also done to ascertain the role of this group of proteins in the reaction of antiserum with eggs.

V. Absorptions.

A number of sera were absorbed with either sperm or demembranated fertilized eggs. Earlier absorptions with sperm were done by adding undiluted semen (1-2 drops per cc serum) to the sera and allowing ½ hour for reaction at 15°C. The sera were used without further absorption if sperm agglutination tests proved negative. It was subsequently noted that during the repeated centrifugations required to rid the absorbed serum of sperm (especially normal serum, in which the sperm are quite active), antifertilizin was released into the serum as evidenced by the appearance of a precipitation membrane on the jelly of unfertilized eggs when they were added to absorbed serum. If the serum-sperm suspension was heated briefly (40°C for 1 minute), the sperm were centrifuged down easily, usually the first time, and this difficulty was thereby avoided in later tests. The sperm were also washed two or three times with sea water in later ab-

sorptions to rid them of seminal fluid. Sera absorbed with fertilized eggs were mixed for ½ hour with an equal volume of demembranated, washed fertilized eggs or early cleavage stages (1 cc eggs and 1 cc serum, usually). This was followed where necessary with a second absorption employing half the original amount of eggs.

VI. Ring Test.

A calcium-free sea water extract of Lytechinus pictus fertilized eggs, containing the material of the ectoplasmic layer, was dialyzed against distilled water, precipitated at pH 2.8, washed and resuspended in 3.3% NaCl at pH 9.0. After adjusting to pH 8.0, the material was layered over sera in 3 mm tubing and reactions read after one hour at 19°C.

VII. Culture of embryos.

Removal of the fertilization membrane was done with a 20 cc syringe fitted with a 20 gauge needle, since the use of enzyme treatments or urea treatments could alter the surface specificity. Culture of fertilized eggs was in embryological salt cellars. Two drops of undiluted serum and two drops of egg suspension were mixed in a dish rimmed with vaseline. After addition of eggs, the dishes were sealed by a glass cover plate, and the cultures subsequently observed through the cover with a binocular dissecting microscope. When photographs were taken, the cover was temporarily removed and the eggs in the dish photographed under the low power (100 x) of a compound microscope.

VIII. Biochemical methods and ultracentrifuge studies.

The respiration of fertilized eggs in normal and immune sera was followed in a conventional Warburg apparatus using flasks of approximately 4 cc capacity. Egg suspensions were made in 0.01 M glycylglycine buffer, pH 8.0 (Tyler & Horowitz, 1937).

The sodium content of eggs treated with normal or immune serum was determined on packed eggs, after removal of an exactly known amount of serum, by the method of Stone and Goldzieher (1949). The method involved converting sodium to the insoluble sodium-zinc-uranyl acetate, followed by the development of a colored uranium complex with 30% H_2O_2. Optical density was read in a Coleman spectrophotometer at 445 mμ.

For ultracentrifuge studies, unfertilized eggs of S. purpuratus were freed of their gelatinous coat by acid sea water treatment and placed in antiserum against the residue (after fertilizin removal) of unfertilized eggs of S. purpuratus. After varying lengths of time, the eggs, along with those in normal serum, were layered over 1 molal sucrose and centrifuged in a Kirk-type micro-ultra centrifuge at various forces to determine the change in tension at the surface produced by antiserum treatment.

IX. Fixation and staining of eggs.

Fertilized eggs, after varying lengths of exposure to normal or immune sera, were flattened and fixed between coverslips in Bouin's fluid after the method of Tyler (1946 a). This was followed by staining in Delafield's hematoxylin.

RESULTS

I. Development of eggs in normal and immune sera.

The eggs of both species developed normally in normal serum, at the usually used 1:1 dilution, until the blastula stage. Development from this point on was characteristically abnormal. Gastrulation was seldom observed, and in the typical case, the embryos formed stereo-blastulae, in which the blastocoele was filled with primary mesenchyme cells, and died. This was most probably due to an amino acid effect, caused by the degradation of serum proteins by sea water bacteria and also probably by enzymes and amino acids liberated from damaged eggs. Hörstadius and Gustafson (1948) have noted animalization of embryos grown in serine and lysine, and Tyler (1953) reported obtaining stereo-blastulae in glycine solutions stronger than 10^{-3} M. Because of this effect, cultures were discarded at twenty-four hours after fertilization (mid-blastula).

It may be well to note here that during titration of the sera, normal serum controls occasionally caused precipitation membranes to appear on the gelatinous coat. The reaction was obtained with a number of normal sera when the ionic strength of the medium was lowered by mixing equal parts serum and isosmotic 1 M sucrose. This procedure was used by Perlman (1954), in a study of the effects of antisera on unfertilized sea urchin eggs, as a means of intensifying the reaction of

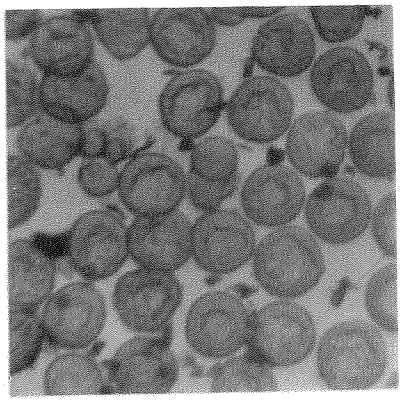
the antiserum with the eggs. The observed effect of this procedure on the reaction of normal serum with the gelatinous coat of the eggs indicates that reactions brought about by this means are very probably non-specific. Since all the present tests were run at the ionic strength of sea water, no difficulty was experienced with most normal sera. The higher salt concentration apparently weakened considerably, or abolished, the reaction of such naturally occurring heteroagglutinins with the gelatinous coat.

Development in anti-sperm serum or in anti-antifertilizin serum paralleled development in normal serum, except in a few cases where a slight retardation occurred (Figure 1). The retarding antibody was absorbable with homologous sperm. A summary of the results with anti-sperm and anti-antifertilizin sera is presented in Table 1. The number following the letters in the serum code indicates the number of the rabbit, and the subscript of that number, the bleeding. A "C" or "NS" in place of the subscript number indicates a control preinjection bleeding. The control serum was used with the immune serum of the same rabbit wherever possible, but if the control bleeding was not available, use was made of a supply of dialyzed normal rabbit serum. Development to early or mid-blastula was observed in all the control sera used in each individual test.

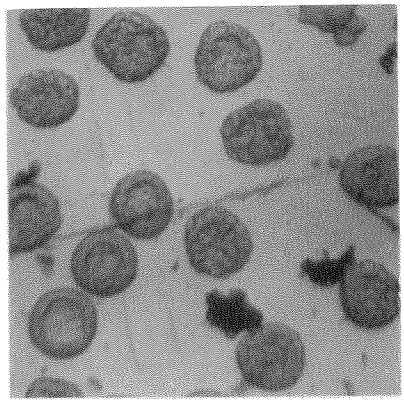
In contrast to the results with anti-sperm sera, fertilized eggs placed in anti-gelatinous coat sera, anti-unfertilized egg sera (gelatinous coat removed), or in antisera

Figure 1.

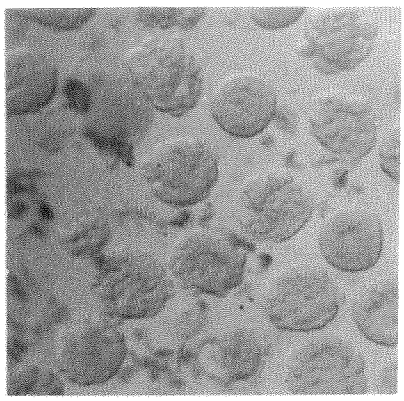
The development of Lytechinus eggs in normal serum and in antisera against whole sperm and antifertilizin.



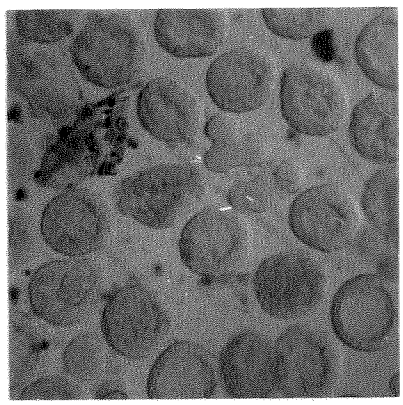
Normal serum.



Anti-whole sperm serum.



Anti-antifertilizin serum.



Anti-antifertilizin serum, sperm absorbed.

Table 1

Immune Sera

No. of Expt's	Serum	Against	Titer-homol. Egg	Titer-homol. Sperm	Absorb with	Test against	Stage at 24 hours
1	LPA1	Lytechinus antifertilizin	1/4	1/512		Lytechinus 2 cell	16 cells (10 hrs)
1	LPA1	"	1/4	1/512		"	64 cells
1	LPA1	"			Lytechinus sperm	"	mid blastula
2	$LPA1_2$	"	0	1/16		"	early blastula
2	$LPA1_2$	"			Lytechinus sperm	"	mid blastula
1	LPA2	"	1/4	1/4		"	early blastula
1	$LPA2_2$	"	1/4	1/4		Lytechinus one cell	"
2	E42B	"	0	1/128		Lytechinus 2 cell	"
1	E42B	"			Lytechinus sperm	"	mid blastula
1	$SPA2_3$	Strongylocentrotus antifertilizin	0	1/64		"	early blastula
1	$SPA3_3$	"	0	1/512		"	early blastula
1	$LPWS1_2$	Lytechinus sperm	0	1/8		"	early blastula

Table 1 (Cont'd)

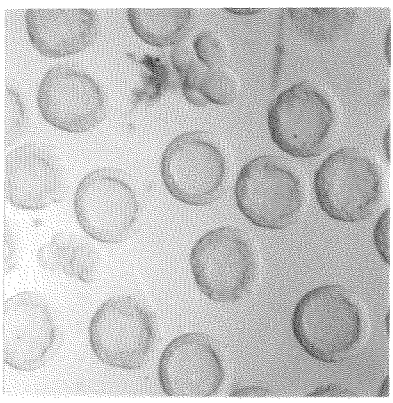
No. of Expt's	Serum	Against	Titer-homol. Egg	Titer-homol. Sperm	Absorb with	Test against	Stage at 24 hours
2	$LPWS1_3$	Lytechinus sperm	0	1/64		Lytechinus 2 cell	mid blastula
2	$LPWS1_{123}$	"	0	1/8		Lytechinus 2 cell	early blastula
1	$SPWSL_1$	Strongylocentrotus sperm	0	1/1024		Lytechinus 1 cell	"
1	$SPWS1_2$	"	0	1/1024		"	"
1	$SPWS1_3$	"	0	1/256		"	"
1	$SPWS2_2$	"	0	1/256		"	"
1	$SPWS2_3$	"	0	1/1024		"	"
2	I II III V	"	0	1/1024		"	"
1	I II III V	"			Lytechinus sperm	"	mid blastula
Control sera							
2	LPA1C	pre-inject.	0	0		Lytechinus 2 cell	early blastula
1	LPA1C	"			Lytechinus sperm	"	"
1	LPA2C	"	0	0		"	"
2	LPWS1C	"	0	0		"	"
1	SPWS1C	"	0	0		Lytechinus 1 cell	"

against demembranated freshly fertilized eggs and eggs in cleavage stages developed only slightly beyond the stage at which they were added to the antisera. Eggs placed in these antisera directly following fertilization failed to cleave. Eggs placed in the antisera at the telophase of the first cleavage division exhibited resting nuclei in both blastomeres when the next division should have occurred. In most experiments, the blocked eggs looked normal for 4-5 hours even though no nuclear or cytoplasmic divisions took place. After this time, they exhibited varying signs of cytolysis. Absorption of the sera with the sperm of the species did not remove the blocking antibodies, but they could be removed by absorption with fertilized eggs or eggs in cleavage stages freed of their fertilization membranes. This is shown in Figure 2. The results obtained with antisera made against unfertilized eggs, fertilized eggs and the gelatinous coat are summarized in Table 2. The coding of the sera is the same as described previously for Table 1.

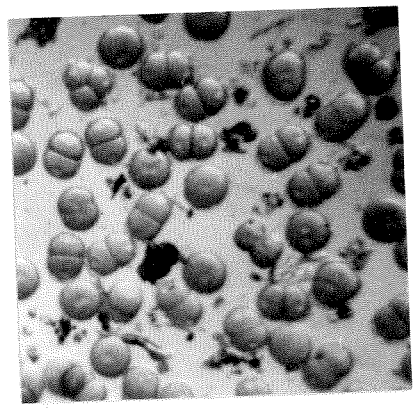
To ascertain the possible effect of complement in the blocking reaction, an anti-fertilized egg serum ($LPF1_2$ -- anti-Lytechinus fertilized eggs) and the corresponding pre-injection control serum were heated to 56° C for thirty minutes. Following this treatment, they were tested, along with unheated sera as controls, on Lytechinus fertilized eggs. The eggs were added to the sera in first division telophase (cleavage furrow just complete on 50% of the eggs). The eggs in heated and unheated immune serum did not cleave

Figure 2.

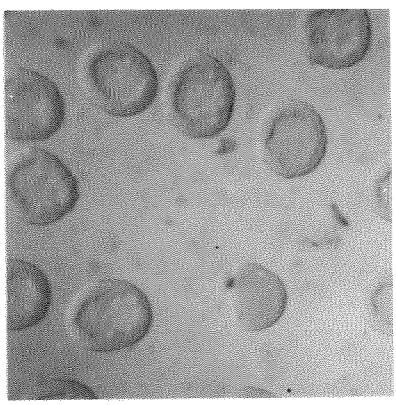
The development of Lytechinus eggs in normal serum and in anti-16 cell stage serum.



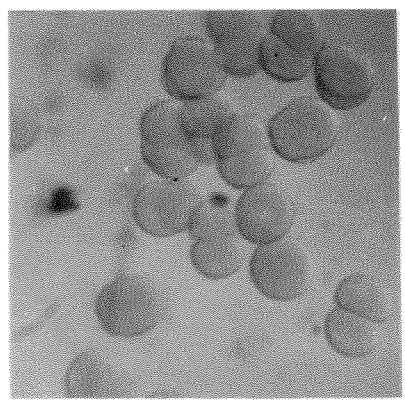
Normal serum.



Anti-16 cell serum.



Anti-16 cell serum, 16-cell absorbed.



Anti-16 cell serum, sperm absorbed.

Table 2

Immune sera

No. of Expt's	Serum	Against	Titer-homol. Eggs	Titer-homol. Sperm	Absorb with	Test against	Stage at 24 hrs.
1	$LPF1_1$	Lytechinus fert. eggs	?	?		Lytechinus 1 cell	1 cell
4	$LPF1_2$	"	1/2	0		Lytechinus 2 cell	2 cells
2	$LPF1_2$	"	"	"		Lytechinus 1 cell	1 cell
2	$LPF1_2$	"			Lytechinus sperm	Lytechinus 2 cell	2 cells
4	$LP16l_2$	Lytechinus 16 cell	1/2	1/2		"	2 cells
2	$LP16l_2$	"	"	"		Strongylocentrotus 2 cell	64-128 cells
1	$LP16l_2$	"	"	"		Dendraster 2 cell	16-32 cells
1	$LP16l_2$	"			Strongylocentrotus sperm	Lytechinus 2 cell	2 cells
2	$LP16l_2$	"			Lytechinus 16 cells	Lytechinus 2 cells	mid blastula
3	$LPEH1_1$	Lytechinus unfert. eggs	1/2	1/8		Lytechinus 2 cells	2 cells

Table 2 (Cont'd)

No. of Expt's	Serum	Against	Titer-homol. Eggs	Titer-homol. Sperm	Absorb with	Test against	Stage at 24 hrs.
1	$LPEH1_1$	Lytechinus unfert. eggs			Lytechinus sperm	Lytechinus 2 cells	2 cells
1	$LPEH1_1$	"			Lytechinus fert. eggs	Lytechinus 2 cells	early blastula
2	LPF1F2	Lytechinus fertilizin	1/32	1/2		Lytechinus 2 cells	2 cells
1	LPF1F2	"	"	"		Lytechinus 1 cell	1 cell
1	LPF1F2	"			Lytechinus sperm	Lytechinus 2 cell	2 cell
1	$SPF1_2$	Strongylocentrotus fert. eggs	?	?		Lytechinus 2 cell	16-64 cells
1	$SPF1_2$	"			Lytechinus sperm	Lytechinus 2 cell	16-32 cells
1	SPF12	"			Lytechinus fert. eggs	Lytechinus 2 cell	early blastula
1	$SPEH1_2$	Strongylocentrotus unfert. eggs	1/16	?		Lytechinus 2 cell	"
1	$SPEH1_2$	"	"	"		Strongylocentrotus 1 cell	early cleavage
1	$SPF1_4$	Strongylocentrotus fertilizin	0	1/64		Lytechinus 2 cell	2 cell

Table 2 (Cont'd)

No. of Expt's	Serum	Against	Titer-homol. Eggs	Titer-homol. Sperm	Absorb with	Test against	Stage at 24 hrs.
1	$SPF1_4$	Strongylocentrotus fertilizin			Lytechinus sperm	Lytechinus 2 cell	2 cell
1	$SPF2_4$	"	0	1/128		Strongylocentrotus 1 cell	1 cell
Control Sera							
5	LPF1NS	pre inject.	0	0		Lytechinus 2 & 1 cell	mid blastula
4	LP161NS	"	0	0		"	"
4	LPEH1NS	"	0	0		"	early blastula
1	LPEH1NS	"			Lytechinus sperm	"	"
1	LPEH1NS	"			Lytechinus fert. eggs	"	"
2	LPF1F2C	"	0	0		Lytechinus 2 cell	"
2	SPF1NS	"	1/4	1/8		Lytechinus 2 cell	"
1	SPF1NS	"	?	?	Lytechinus sperm	"	"
1	SPEH1NS	"	0	0		Lytechinus 2 cell Strongylocentrotus 2 cell	"

beyond the two cell stage, and cytolyzed after five hours in the antiserum. The eggs in heated and unheated normal serum developed to the early blastula stage, at which time the cultures were discarded.

Other samples of the same sera were heated to 100° C for 15 minutes, to 70° C for 15 minutes, and to 70° C for three minutes. Denatured, precipitated proteins were removed by centrifugation. Those eggs placed (10 minutes after fertilization) in antiserum heated to 100° C for 15 minutes developed to top swimming mesenchyme blastulae. Eggs in antiserum heated to 70° C for 15 minutes produced multinucleate single cells which were cytolyzed after 24 hours, while eggs placed in antiserum heated to 70° C for only three minutes cytolyzed as single cells after four hours in the antiserum. Eggs in heated normal serum developed to top swimming mesenchyme blastulae in all cases.

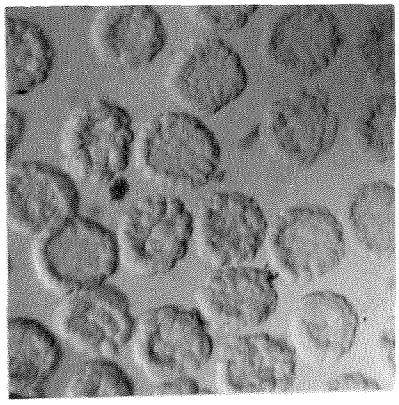
As can be seen from Table 2, there is also a certain degree of species specificity attached to the reaction. As, for example (Figure 3), the decreased severity of the block when S. purpuratus eggs were used in place of Lytechinus eggs with anti-fertilized egg serum made against Lytechinus eggs.

II. Precipitation tests.

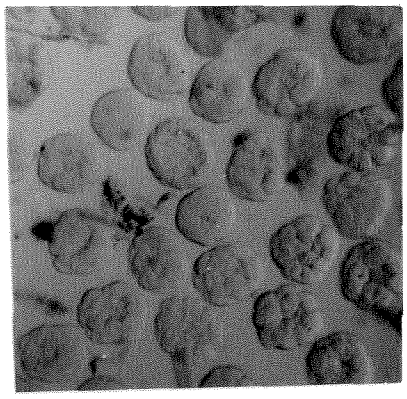
Since a dark irregular outline on the surface of the ectoplasmic layer was consistently observed on eggs in blocking antisera, it was decided to test whether or not a calcium-free sea water extract of this material (see METHODS

Figure 3.

The development of Strongylocentrotus eggs in normal serum and in antiserum against Lytechinus 16- cell stage.



Normal serum



Anti-16 cell serum

section for preparation) would react with antisera in a ring test. The results appear below in Table 3.

Table 3

RING TESTS WITH ECTOPLASMIC LAYER EXTRACT

anti-L. pictus cleaving eggs	++
" sperm absorbed....	+
" 16-cell absorbed..	-
anti-unfertilized eggs, without jelly.....	+
anti-unfertilized eggs, without jelly, sperm absorbed.....	+
anti-unfertilized eggs, without jelly, 16 cell absorbed.....	+
normal serum.....	-
saline.....	-

As can be seen from the table, positive reactions occurred only when the serum was still effective (as judged from previous tests) in inhibiting cleavage, and were not abolished by absorption of the antisera with sperm. The material in this extract also gave a positive anthrone test, but a negative biuret test, indicative of carbohydrate. The ultraviolet absorption spectrum (Beckman spectrophotometer) showed no maximum between 220 and 300 millimicrons. The extract was observed to agglutinate sperm of L. pictus, but not of Dendraster excentricus. Subsequent extracts have not shown this agglutinating property, however.

III. Cytological details.

Aside from the grosser features of the inhibition observed in the living state under relatively low magnification,

fixed-stained preparations gave more precise information about the effect of blocking antisera on nuclear and cytoplasmic activity. Two types of experiments were performed. Eggs were put into inhibiting antiserum at a given stage, and samples fixed at intervals, starting immediately after the eggs were placed in the antiserum. After staining, the amount of progress made by blocked cells in a given length of time was determined by comparison with unfixed normal serum controls. The second type of study involved transferring eggs from sea water to antiserum at intervals. When the transfers were made, a second sample was fixed and stained to determine the stage at which the eggs were placed in the antiserum. After an arbitrary length of time in the antiserum, usually an hour, the eggs in antiserum were fixed and their progress compared with the sample taken at the time of addition. The first type of study gave a measure of the time required for the antiserum to act, while the second gave information as to whether or not there were differences in the susceptibility of different stages to the action of the antiserum. The proportion of eggs and antiserum was adjusted to give maximal inhibitory effect, comparable to that observed in the living cultures. For example (experiments of the first type) eggs placed in anti-16 cell serum (Lytechinus) 3 minutes after fertilization progressed to the fusion of the pronuclei in one hour, at which time the control eggs in normal serum were in metaphase of the first cleavage division. One-half hour later, those in im-

mune serum were still in the fusion nucleus stage, while the controls were in pro-metaphase of the second cleavage division. Apparently a gradual slowing down and stopping of mitotic activity occurs in the immune serum. Experiments of the second type also indicate that mitosis is inhibited about 1/2 hour after addition to antiserum, regardless of the stage the eggs are in at the time of addition. Eggs (again using anti-16 cell serum) were placed in antiserum 10, 30, 40, and 50 minutes after fertilization and at the first cleavage. Control samples were taken as each addition was made. The eggs were allowed to remain in the antiserum for one hour, and their stage was then determined. As the time of addition became later after fertilization, a later stage was reached in the antiserum. These results can be found in Table 4.

Several experiments of the first type were done on eggs in two cell telophase in order to see if any cyclical breakdown and reformation of the nuclear membrane occurred in antiserum, giving the false impression of a complete block to nuclear activity. Eggs placed in anti-Lytechinus 16 cell serum and fixed at intervals over a period of five hours gave no indication of any such activity. Occasional monasters were observed in the cytoplasm, however it is not possible to say from the stained preparations whether these were carried over and retained from the first cleavage or were being abortively produced in attempts to carry on the normal division sequence. In some cases diasters were observed indicating

Table 4

THE EFFECT OF ADDING EGGS TO ANTISERUM AT VARYING TIMES AFTER FERTILIZATION

Time after fertilization	Stage when placed in antiserum	Final stage reached after 1 hour in antiserum
10 min.	Egg pronucleus only visible	Metaphase of first division
30 min.	Egg and sperm pronuclei visible side by side	Metaphase of first division
40 min.	Fusion of pronuclei and few metaphase figures	Single cell with 2 nuclei
50	Telophase and anaphase of first division	Two cells with interphase nuclei

the latter alternative. Slides of eggs in normal serum showed mitotic figures of the second division at a time when the eggs in immune serum exhibited interphase nuclei. It is presumed from the results on uncleaved eggs that a second division would occur in inhibiting antiserum were the eggs put in at a later time in the mitotic cycle (prophase or metaphase rather than the previous telophase), or in more dilute antiserum.

IV. Respiration of fertilized eggs in normal and immune sera.

Fertilized eggs (0.5 cc of 5% suspension, in 0.01 M glycylglycine buffer) were placed in the main chamber of ca. 4 cc vessels, and 0.3 cc of normal or immune serum were placed in the side arm. The center well contained 0.05 cc of 10% NaOH. These conditions, though giving a relatively low rate of oxygen uptake by the egg buffer mixture, were found to give fairly good blocking of the eggs when the appropriate antiserum was added from the side arm. A greater amount of eggs would have been desirable, however since the capacity of the side arm limits the amount of serum to be added, the concentration of eggs was of necessity low. It was also noted that dilution of a blocking mixture of eggs and antiserum with an equal volume of sea water does not lessen the effect. This fact was made use of in selecting the concentration of egg suspension used in the flasks. The vessels were equilibrated for 20 minutes at 18°C before readings were taken. Following equilibration, readings were taken of the respiratory rate of the eggs in glycylglycine

buffer. After three readings, taken 10 to 20 minutes apart, the side arms were tipped and the readings continued for another hour or more. At the end of the run, the contents of the flask were removed and examined microscopically to determine the cleavage stage. The pH of the buffered mixture was checked using a glass electrode or indicator paper. Experiments in which control eggs failed to cleave, or in which pH was greater than 8.0, were discarded. A typical curve for respiration of eggs in anti-sperm serum, anti-fertilized egg serum and normal serum will be found in Figure 4. It can be seen from these curves that the total oxygen uptake in anti-fertilized egg serum is nearly 2.5 times the total uptake in either normal or anti-sperm serum. As can be seen in Table 5, eggs in blocking antisera consistently show higher total oxygen consumption than eggs in normal serum or anti-sperm serum, which usually show about the same total consumption. An exception to this generalized picture is found in experiment XXXVII. Anti whole sperm serum ($LPWS2_3$) caused a significant increase in oxygen consumption when added to the eggs. It is also evident that there was a retarding action of this antiserum on the eggs, of a sort similar to that shown in Figure 1.

The maximum increased rate of respiration in blocking antisera consistently occurred 20 minutes after the introduction of the antiserum. After 20 minutes, the rate commenced to fall, leveling off at a somewhat higher value than the controls 35-40 minutes after the maximum was reached.

FIGURE 4

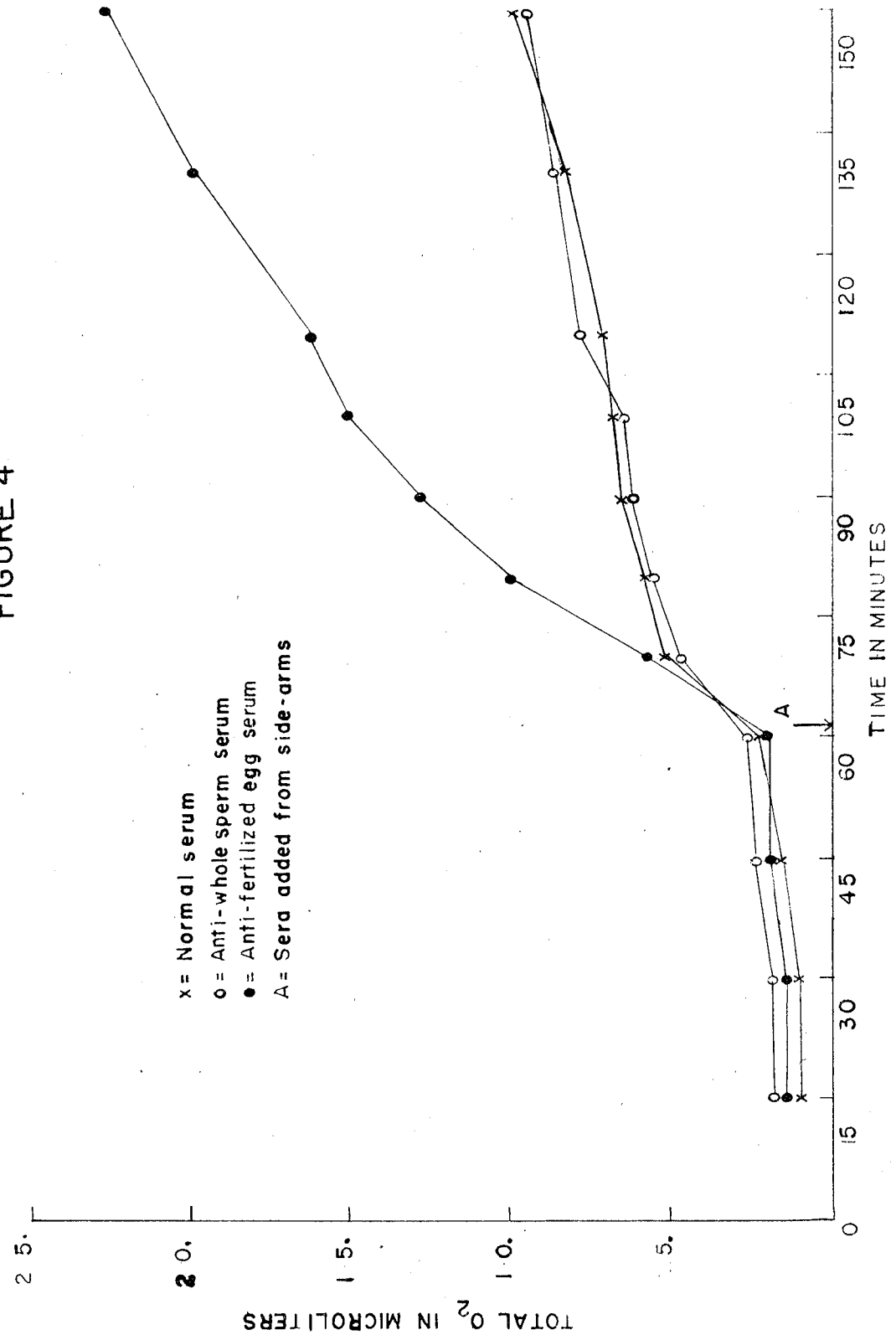

x = Normal serum
o = Anti-whole sperm serum
● = Anti-fertilized egg serum
A = Sera added from side-arms
TOTAL O_2 IN MICROLITERS
TIME IN MINUTES
25.
20.
15.
10.
5.
0
15
30
45
60
75
90
105
120
135
150
A

Table 5

TOTAL OXYGEN CONSUMPTION OF EGGS IN VARIOUS NORMAL AND IMMUNE SERA

Expt. No.	Test eggs	Serum	vs.	Total O_2(ul) $\times 10^{-1}$	Stage at end time
XXV (2 hrs)	L.pictus	LPF1F2	fertilizin	2.9	1 cell
	"	$LPF1_2$	fert. eggs	2.0	1 cell
	"	LPF1NS	pre-inject.	0.7	8-32 cells
XXXVI (2hr30')	"	$LPF1_2$	fert eggs.	2.25	1 cell
	"	$LPWSI_3$	wh. sperm	0.95	8-16 cells
	"	LPF1NS	pre-inject.	0.95	8-16 cells
XXXVII (2hr30')	"	$LPWS2_3$	wh. sperm	2.05	4-8 cells
	"	$LPWS1_3$	wh. sperm	1.3	16 cells
	"	LPX1NS	pre-inject.	1.5	16 cells
XLII (2 hrs)	"	$LPWS1_3$	wh. sperm	0.60	8 cells
	"	$LPEH1_1$	unfert. res.	1.80	1-2 cells
	"	LPF1NS	pre-inject.	0.50	8 cells

The time course of the rate increase in anti-sperm serum $LPWS2_3$ was slightly different. The maximum was reached in 15 minutes, followed by a decrease to the level of the controls after 40 minutes. The slight increase in respiratory rate in non-blocking normal serum or anti-sperm serum, seen in Figure 4, was not always obtained. No correlation was found with particular sera, since the same sera used on different days were inconsistent in this respect. Since the thermobarometer indicated a positive pressure change in the vessel at the first reading after tipping the side arms in experiments showing this increase, it is thought that the transitory rise in rate of oxygen consumption (return to normal after 10 minutes) in these sera was an artifact. This artifact might have been the result of rapid warming of the small vessels when removed from the bath for tipping the contents of the side arms with subsequent "sticking" of the manometer fluid of the thermobarometer at a high level.

In an attempt to determine whether or not a non-specific mitosis-inhibiting agent might yield similar results, sodium lauryl sulfate was tested. A final concentration of 0.024% after addition of the substance to the main chamber from the side arm blocked cleavage without immediate cytolysis, and gave no increased respiratory rate.

V. Sodium Content of eggs in immune and normal serum.

In an effort to see if a generalized permeability change, which might account for increased oxygen consumption, was

brought about by the action of the antiserum, it was arbitrarily decided to analyze eggs in normal and immune blocking sera for sodium content. The unfertilized egg contains very little sodium (Rothschild & Barnes, 1953). Assuming the amount of sodium in fertilized eggs is comparably low, any permeability change caused by antiserum might be detected as an increased amount of sodium inside the eggs. Since there is a relatively large amount of sodium in sea water, it was necessary to remove an exactly known large amount of supernatant serum from the eggs after treatment, and to measure the amount of sodium in the remaining residue after cytolysis with distilled water and precipitation of proteins with 10% trichloracetic acid. The eggs of S. purpuratus were used for these experiments. In practice, 1.5 cc of blocking anti-unfertilized egg serum (eggs after removal of the gelatinous coat) were mixed with 2 cc of a 2.5% suspension of demembranated fertilized eggs approximately 45 minutes after fertilization. Exax Hopkins vaccine tubes were calibrated and employed as the reaction vessels because the eggs could be packed in the capillary after incubation with the serum and their volume determined, and because the calibrations allowed removal of nearly all the supernatant serum to a known volume. The tubes were Desicoted to insure against liquid remaining on the sides of the tube. After two hours at 18°C with continuous rotation, all but 0.03 ml of the supernatant was removed. Egg volume (after light centrifugation for one minute) was 0.025 cc. Glass distilled

water was added to cytolyze the eggs, followed by 1.0 cc 10% TCA. After centrifugation and filtering, 0.5 cc of the supernatant was analyzed by the procedure indicated in the METHODS section. A series of standards were run with the unknowns, which were run in triplicate. The results of this experiment are given in Table 6. Over this range of concentrations, the standard curve follows a straight line relationship fairly well when optical density is plotted as a function of concentration. The results in Table 6 do not indicate any difference in sodium content of eggs treated with either normal serum or with blocking immune serum. Previous experiments similar in design to the one above but employing anti-fertilizin serum and other methods for removal of supernatant serum gave similar results, but the above experiment is considered more accurate because of the more accurate determination of the volume of serum removed. Observation of the standard values indicates that an increase in sodium concentration corresponding to 100 mg% could have been detected. Under the conditions used for measurement, an increase of 100 mg% corresponds to a 35% increase in sodium permeability, assuming the non-solvent volume of the eggs to be about 20%, and assuming the eggs normally contain no sodium.

Table 6

SODIUM CONTENT OF EGGS TREATED WITH NORMAL AND IMMUNE SERUM

Sample	OD	%trans.
normal serum	0.42	37.8
normal serum	0.46	34.7
normal serum	mistakenly overdiluted with distilled water	
immune serum	0.49	32.1
immune serum	0.48	36.0
immune serum	0.43	36.9
Standards		
50 mg% Na	0.18	66.0
100	0.24	57.0
150	0.44	36.3
250	0.63	23.5

VI. Effects of immune serum on the tension at the surface of unfertilized eggs.

A combination of antibodies with the surface of the egg would be expected to change in some way the properties of the surface. This possibility was investigated by means of centrifugation experiments. Unfertilized eggs of Strongylocentrotus purpuratus were freed of their gelatinous coat with the acid sea water treatment, since it was desired to have a uniform surface of the eggs throughout the experiments. They were placed in antiserum made against unfertilized eggs after removal of fertilizin by similar treatment. Similarly treated eggs were added to the same amount of normal serum. After one hour, the eggs were layered over 1 molal sucrose and centrifuged at various speeds for various lengths of time to determine if there were any change in the tension at the surface of the eggs due to the presence of antiserum. Early experiments indicated that eggs in normal serum were more easily pulled into light and heavy halves than were those exposed to immune serum. Conditions were then standardized so that the increase in strength could be expressed in quantitative terms. Three drops of a concentrated egg suspension were added to four drops of either normal or immune serum, and allowed to stand at 18°C for one hour. After this time, they were layered over the sucrose and centrifuged at 16 pounds pressure in the Kirk-type microcentrifuge for 20 minutes. At the end of this time, eggs in normal serum characteristically were breaking into light and heavy

halves, while those in immune serum stratified only. After this observation on small aliquots of the sample, the eggs were returned to the centrifuge for 20 minutes at 20 pounds pressure. At the end of this period, eggs in normal serum were splitting into quarters, while those in immune serum were splitting into halves. Though the endpoint is not as clear as one would like, fairly good agreement between experiments was obtained, using 50% split-50% elongate and ready to split as the stopping point. In two separate experiments, this point was reached at the pressures indicated above for normal and immune serum. Since a calibration curve for this centrifuge was available, relating pounds of air pressure to rpm, a calculation of the additional force required to separate eggs into light and heavy halves after treatment with immune serum was possible. Twenty pounds pressure corresponds to 24000 x g, and 16 pounds to 16900 x g, a difference of 7100 x g, or an increase of approximately 40% in the force necessary to pull the eggs into light and heavy halves after treatment with antiserum.

DISCUSSION

From the results presented in the introductory section, it is apparent that anti-organ sera are capable of detecting organ antigens in adult and embryonic tissues using both in vitro serological methods and tissue culture methods in which the specificity of the antiserum is judged by its ability to produce cytotoxic effects on the cultured cells. Antisera made against embryos are also capable of detecting antigenic differences between successive embryonic stages, again utilizing serological methods with soluble extracts as antigens, or observations on the cytotoxic effects of the antisera on cultures of embryos.

The present study indicates that antisera prepared in rabbits against sea urchin fertilized and unfertilized eggs, fertilizin, sperm and sperm antifertilizin have, through tests of the cytotoxicity of the antisera on cultures of fertilized eggs and through serological tests, revealed differences in the antigenic properties of eggs and sperm. It was observed that only antisera against eggs and egg components are effective in blocking the cleavage of the fertilized egg, whereas anti-sperm sera and anti-antifertilizin sera have not shown this property. Further, it was observed that where anti-sperm sera (or anti-antifertilizin sera) showed a delaying effect on the cleavage of the eggs, this effect was absorbable with sperm. Absorption of the various

anti-egg sera with sperm did not remove their inhibitory antibodies, whereas absorption with fertilized eggs will remove the blocking antibodies of these antisera.

It is concluded therefore that the antigens similar to those found on the surface of the sperm, where absorption reactions presumably take plaee, are not concerned with the production of the inhibiting antibody. This does not preclude that antigens in the interior of the sperm are not of a type capable of causing the production of an inhibiting antibody. There may be insufficient material of this antigenic type in thesperm as compared with the eggs to cause antibodies to be produced against it. As Tyler (1955) has pointed out, the complete absence of an antigen cannot be proved by serological methods.

Since absorption of anti-unfertilized egg sera, or anti-fertilized egg sera (anti-cleavage stage sera also) with fertilized eggs or cleavage stages removed the inhibiting antibody, and since the ectoplasmic material extract reacted in a ring test with unabsorbed sera only, it seems probable, barring contamination of the test antigen with material from inside the egg, that this substance contains antigenic groups similar to those inducing the blocking antibody, and that these groups are also present in the unfertilized egg (and perhaps elsewhere in the fertilized egg).

The fact that fertilizin preparations of considerable purity (electrophoretically homogeneous) are effective in causing the production of blocking antibody is of interest.

The question of the identity of fertilizin antigenic groups and the antigenic groups of the fertilized and unfertilized egg arises. There appear to be three possibilities which must be distinguished; 1) The fertilizin antigen responsible for the effect is different from those found elsewhere in the eggs which have a similar effect. 2) The fertilizin antigen and the egg antigen are identical at least as far as their immunological properties are concerned. 3) The fertilizin antigen is different from the effective antigens of the remainder of the egg, and the observed inhibition in anti-fertilizin serum is due to contamination of the original antigen with material from inside the eggs.

If possibility (1) is the case, the following absorption experiments should give these results; a) Absorption of anti-fertilizin serum with fertilizin should remove the inhibitory antibody. b) Absorption of anti-fertilizin serum with fertilized eggs (ectoplasmic layer) should not remove the inhibiting antibody. c) Absorption of anti-egg serum with fertilizin should not remove the effective antibody. d) Absorption of anti-egg serum with fertilized eggs should remove this antibody. If possibility (2) is true, the above absorptions should remove the block under the conditions of a, b, c, and d. In the third case, such an impurity might be suspected if a large amount of fertilizin were required to remove the effective antibody from anti-fertilizin or anti-egg sera. Dr, Tyler has performed some experiments absorbing anti-fertilizin serum with fertilizin.A partial overcoming of the block was found using relatively

large amounts of fertilizin solution, indicating the possibility at least of such an impurity. To date, no further exploration of this question has been done. The possibility also exists that the fertilizin remaining on the surface of the eggs after acid treatment (postulated to be present since eggs so treated are fertilizable and will agglutinate on addition of antifertilizin solution) was responsible for the inhibitory antibody. Since the ectoplasmic layer apparently contains antigenic groups similar to those causing the production of inhibiting antibody, it seems worthwhile to speculate on the similarity of this substance to fertilizin. It may be recalled that an experiment in this thesis indicated that ectoplasmic material from fertilized eggs possessed sperm agglutinating capacity. Motomura (1953) has also observed a sperm agglutinating substance obtainable from fertilized eggs previously washed to remove gelatinous coat material -- a substance which may also be derived from the ectoplasmic layer. These observations point to the possible similarity of the two surfaces, and therefore to the possibility of structural or antigenic similarity of the substances. The two substances may be similar chemically also, though this is not a requisite for antigenic similarity, since Landsteiner (1945) has shown with proteins coupled to haptenic groups that it is the combining groups which must be similar and not necessarily the whole molecule. Chemical tests on the ectoplasmic layer extract indicate the presence of carbo-

hydrate, but not of protein. Tyler (1949b) has shown fertilizin to belong to the class of substances known as mucopolysaccharides. More sensitive tests may indicate the presence of protein in the ectoplasmic extract also. The solubilities of the two substances appear to be different. Fertilizin, as previously indicated, is soluble at acid pH, whereas the ectoplasmic material is insoluble at low pH and soluble in the range pH 9.0-9.8. Caution must be exercised in interpreting the solubility data of substances of this sort, since Kraus (1949) has shown that fertilizin, like hyaluronic acid, co-precipitates with proteins at low pH in a mucin clot reaction. The possibility of co-precipitation with protein contaminants of the ectoplasmic layer must be eliminated before the solubility above is taken to be the true solubility of the substance.

In the present work, as in the experiments described in the earlier literature, the question of causal relationships, between organ or embryonic antigens and morphogenesis or between surface antigens and the cleavage process, arises. It does not seem to this writer thatthe action of antibodies on specific organs, developing organs or cleaving eggs reveals more than the presence of reacting antigens. The secondary phenomena of the antigen-antibody combination are in themselves inhibitory and therefore one cannot decide whether the specific block of an essential antigen has been accomplished or whether such secondary effects as lysis etc. are responsible for the inhibitions of morphogenesis and

cleavage observed. The production of univalent or incomplete antibody by photoxidation offers a possible means of avoiding at least agglutination phenomena and possibly other secondary effects. This procedure has been used by Tyler (1946b) in a study of the fertilizing capacity of antiserum treated sperm where it was necessary to remove the agglutinating activity of the antiserum without destroying its specificity. That sera so treated retain their original specificity is evidenced by their ability to combine with the antigen (sperm) and inhibit the agglutinating action of the unoxidized antiserum.

The observed increase in respiration of blocked eggs finds no parallel in the previous literature. For example, Haurowitz and Schwerin (1940) studied the respiration of pigeon erythrocytes in the presence of immune rabbit serum and active and inactive complement, yielding lysis and agglutination respectively. Glucose was also present in the medium (0.2%). No increase or decrease in oxygen comsumption was noted in either case. Warren (1945) found no increase or decrease in lumination intensity of agglutinated luminous bacteria in rabbit antisera. Sevag and Miller (1948), studying the respiration of E. typhosa (strain O-901) cells in the presence of immune serum and active or inactive complement found that intact sensitized cells consumed oxygen at the same rate as the controls. However, in the presence of active complement, the cells lysed with an accompanying increase in respiration (1.4 fold), which was

followed by a decrease to $\frac{1}{4}$ the rate of the controls after 180 minutes. Glucose was present in the medium. Harris (1948) measured the oxygen uptake of Salmonella in the presence of agglutinating serum and glucose, and found no increase or decrease of respiration over a wide range of antiserum concentrations. Thunberg tests measuring the rate of reduction of methylene blue by the organism in the presence of a number of substrates and antiserum and normal serum also failed to indicate differences in rate of reaction. Nowinski (1948) investigated the possibility of a stimulation of respiration by reticulo-endothelial immune serum (REIS) acting on rat spleen slices, and by anti-chick brain serum acting on chick brain homogenates (1949). A slight inhibition of respiration was obtained with REIS, but no effect of anti-brain serum on the oxygen uptake of brain homogenates was observed. MacDonald (1949) obtained similar results with REIS using the Thunberg technique for dehydrogenases.

That the increased respiration of sea urchins eggs in blocking antiserum cannot be explained on the basis of increased permeability to oxygen in the presence of antiserum is indicated by the work of Amberson (1928). Amberson found that the respiration of fertilized Arbacia eggs was constant over a wide range of oxygen tensions (20-220 mm Hg), indicating that permeability is not a limiting factor in the respiration of fertilized sea urchin eggs. As an alternative, it might be suggested that since the eggs have

relatively little sodium and large amounts of potassium compared to sea water, any alteration of the egg surface allowing potassium to leave the cells or sodium to enter might be expected to cause an increase in respiration since osmotic work must be done to maintain such concentration gradients. Rothschild and Barnes (op. cit.) pointed out that if sodium were actively transported out of the eggs, and no active work were being done to maintain the differences of potassium and chloride concentrations inside and outside the eggs, Donnan equilibrium would require that the ratio of potassium inside to potassium outside be equal to the ratio of chloride outside to chloride inside. They further show that their data favor this hypothesis over the hypothesis of active transport of either potassium or chloride, but that the evidence was insufficient to warrant any definite conclusion. No alteration in the sodium content of the eggs in immune serum was detected in the present work, but the possibility exists that the eggs were able, by virtue of their increased respiration, to exclude excess sodium entering the eggs during the period of incubation with the sera.

The increased tension at the surface of the unfertilized egg after treatment with anti-egg serum is indicative of the strengthening of the egg surface due to the presence of antibody. This is most easily visualized if the individual antibody molecules are assumed to be combined with more than one point on the surface of the egg. This assump-

tion could be tested by utilizing univalent antibody. If the assumption is correct, no increase in tension at the surface would be expected in the presence of univalent or incomplete antibody.

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APPENDIX

The Use of Chrome-hematoxylin-phloxine as a Stain for Neurosecretory Elements of the Crayfish Central Nervous System.

Minor Problem Report by John W. Brookbank

The secretion of hormones by nerve cells is a relatively recent discovery in the crustacea. In past years, the sinus glands of the eyestalks have been considered the primary source of hormones in this groups of animals (see reviews by Brown, 1944 and 1948). In more recent years, physiological and morphological data support the theory that the sinus gland is a storage release organ, formed by the coming together of a large number of fiber tracts from neurosecretory cells located elsewhere in the eyestalks and possibly also in the brain and thoracic ganglia (Bliss et al., 1954; Enami, 1951). One of these groups of neurosecretory cells, the X-organ (first described by Hanström in 1931), has been shown by a number of experiments to be the source of at least one hormone involved in the inhibition of molting during intermolt periods. Briefly (see Bliss et al. 1954 for summary and references), it was found that bilateral eyestalk removal, a procedure known to accelerate molting, could be duplicated by X-organ removal, but not by sinus gland removal. In addition, Passano (1951) has observed droplets of material coursing along the nerve fiber between the X-organ and the sinus gland in phase contrast microscope studies. The above

evidence, coupled with the observation (Bliss and Welsh, 1952) that the sinus gland is acellular and hence unlikely to produce secretions of any sort, indicates strongly that the X-organ-sinus gland system of decapod crustacea is analogous to the intercerebralis-cardiacum-allatum system of insects and to the hypothalamo-hypophyseal system of vertebrates (Scharrer and Scharrer, 1944).

The chrome-hematoxylin-phloxine (CHP) stain of Gomori (1941) has been used to differentiate neurosecretory cells from other nerve cells in sectioned material by a number of workers (Scharrer, 1952 (insects); Bliss and Welsh, 1952; Bliss et al., 1954). In such preparations, the cells of the decapod X-organ were seen to be filled with blue-black granules which surround a pink nucleus containing dark chromatin strands and a bright red nucleolus. The axons of these cells contained pink or lilac material, the blue-black or purple material reappearing in visible form in the endings of these fibers in the sinus gland. Bliss et al. (1954) have extended the application of this stain to the brains of two species of decapods (Gecarcinus lateralis and Cambarus virilis), as well as to other areas of the eyestalks of these species, and found cells in both locations of staining quality similar to the cells of the X-organ. The function of these nerve cells has not been determined physiologically in the manner described for the X-organ, but they have been described as neurosecretory by Bliss et al. because of the similarity of their appearance, after staining with CHP, to the cells of the X-organ.

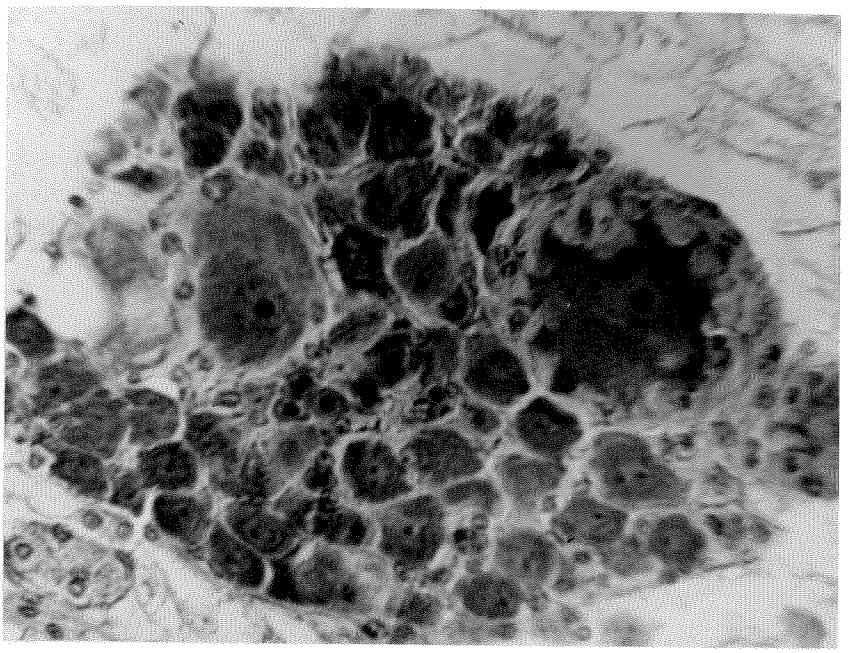
At the suggestion of Dr. Wiersma, similar preparations were made of the brain, eyestalks and abdominal ganglia of Cambarus clarkii using the CHP stain. Cells (here designated type 1) of the type described by Bliss et al. (1954) were found in various locations in the brain (Figure 1) corresponding roughly to the regions marked out by the above workers for Cambarus virilis. Areas presumably corresponding to their areas B2 and B3 were found between the central area of the brain and laterally occurring structures (see Figure 2) which appear to be neuropile (but which could, from their histological appearance, also be glandular) rather than on the extreme lateral surface of the brain. These cells of type 1 ranged in size from 10 to 40 micra. Other cells (type 2), with inconspicuous perikaryon and fiber processes were found in the lamina ganglionaris of the eyestalks and lateral to the above mentioned "neuropile" (see Figure 2), but were absent from the abdominal ganglia. These type 2 cells may conceivably be internuncial cells. The type 1 cells of the abdominal ganglia (2nd, 3rd and 4th) stained in a manner identical with the type 1 cells of the eyestalks and brain, and were in the same size range (Figure 3).

In view of the fact that all cells of the brain above 10 micra in diameter stain as neurosecretory (type 1) cells, the problem of the whereabouts of the cell bodies of the medial giant nerve fibers of this animal arises. Allen (1894) was of the opinion that these large fibers arose in the ventral portion of the lobster brain from 2 large cells. Johnson,

(1926), working with Cambarus, was also of this opinion, and Wiersma (1947) has confirmed and extended Johnson's results. It seems therefore inescapable that one pair of these 40 micron type 1 cells represents the cell bodies of motor fibers of known function, and that therefore the CHP method is not selective for exclusively neurosecretory elements. The same argument can be applied to the abdominal ganglia in connection with the cell bodies of the 1st, 2nd and 3rd root giant fibers, all of which originate from cells in the abdominal ganglion from which the fibers emerge. No neurosecretory function has been described for the abdominal ganglia, though their motor functions have been studied (Wiersma, 1947).

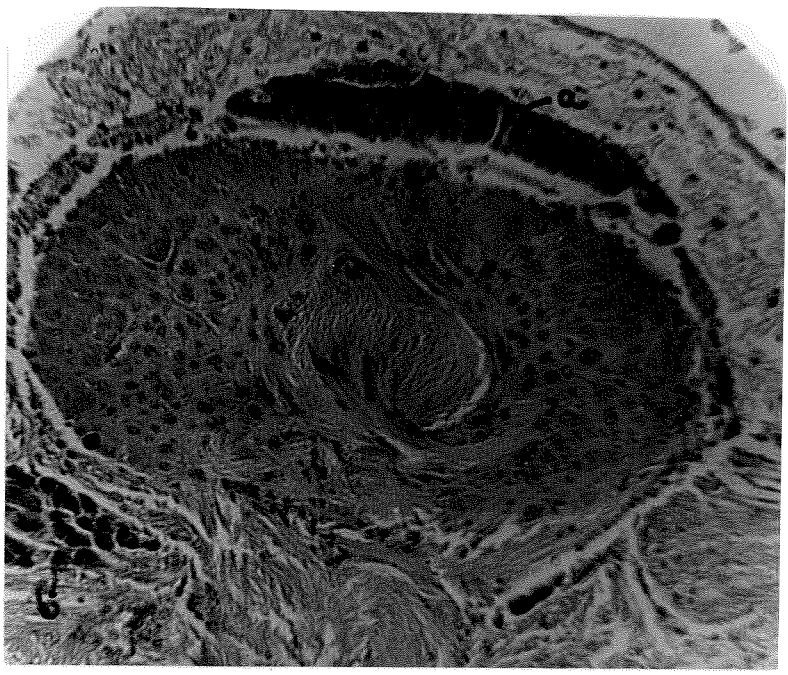
It may well be that the amount of secretion by these cell bodies necessary for the maintenance of their respective fibers completely overshadows other more specific secretory products (e.g. hormones) which may insome cases (X-organ) be produced. Alternatively, the cells staining as type 1 cells with the CHP stain may all have a dual function - the conduction of impulses and the transport of hormones, in which case the neurosecretory system of the crayfish and other decapods would be very extensive indeed. It seems, therefore, that additional physiological experiments are necessary before one can state that the type 1 cells of the crayfish brain and abdominal ganglia are neurosecretory.

Figure 1.



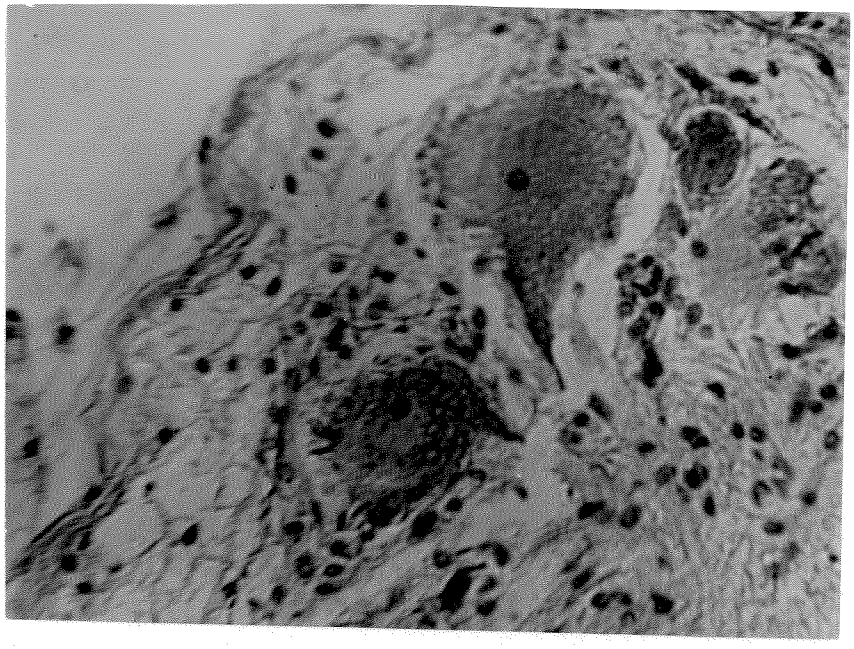
Type 1 cells of the anterior region of the brain (X 700)

Figure 2.



"Neuropile" area of brain. (X 300)
a) type 2 cells
b) type 1 cells

Figure 3.



Two type 1 cells of the 2nd abdominal ganglion (X 760).

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