ANTIGENIC ANATOMY OF THE SEA URCHIN

STRONGYLOCENTROTUS PURPURATUS

by

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A THESIS

Presented to the Department of Anatomy
and the Graduate Division of the University of Oregon Medical School
in partial fulfillment
of the requirements for the degree of
Master of Science

May, 1960

APPROVED:

[redacted]

(Professor in Charge of Thesis)

[redacted]

(Chairman, Graduate Council)

ACKNOWLEDGMENTS

Dr. Robert L. Bacon, with his conviction that a graduate student should be allowed to pursue a subject in which he is intensely interested, gave me great freedom in the choice of a topic for investigation. He has been constantly helpful and inspiring and was extremely kind to allow me to investigate an area in which he, too, had had no previous contact. I have him to thank for invaluable experience gained in the study of a field employing new and different techniques.

The financial assistance provided by the Medical School, and by grants from the Medical Research Foundation, the Public Health Service, and the National Science Foundation has made this investigation possible.

I am especially grateful to Mark Becker, Bob Bayly, Philip Keizer, and John Flaxel for their assistance in various technical phases of this work under somewhat less than bearable working hours. Drs. Arthur Frisch, Jack Fellman, Lyle Veazie, and Evelyn Oginsky have given invaluable help and suggestions. I am indebted to Mrs. Robert Hunt for her patience and skill in typing the final copy of the manuscript.

I must gratefully thank my understanding parents for allowing their son to wander home with all sorts of peculiar organisms, and to attend special summer sessions in marine biology. It is through their

patience and encouragement that I was able to look more deeply into those animated curiosities of childhood, and ultimately to study them in college and in medical school. No adequate tribute can be made here -- only a fact may be recognized.

Finally, I wish to express special appreciation to my wife, Betty, who has helped me keep my chin up during times of "laboratory misfortune" but, most of all, during this eleventh hour before the completion of the thesis.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

INTRODUCTION

> . . . that Nature has in these passages [cells of cork] , as well as in those of Animal bodies, very many appropriated Instruments and contrivances, whereby to bring her designs and end to pass, which 'tis not improbable, but that some diligent observer, if helped with better Microscopes, may in time detect . . .

Robert Hooke wrote the above words 295 years ago in his memorable Micrographia and though many diligent observers have been using much better microscopes and other elaborate equipment, we have yet to begin our analysis of nature's contrivances. Every page turned is only a new beginning and, indeed, it seems as though we are nearly moving backward.

The problem of how cells do their work is one of the most fascinating frontiers that is presented to modern man and we approach the task with no less enthusiasm than did Hooke nearly 300 years ago with his very meager understanding of cells.

Immunologic and, of course, many other types of investigations, early led to the suspicion that there is a certain tissue individuality which must be described in terms of both structure and function. That there are different constituents of different tissues in the same organism can be easily shown and has been appreciated for many years as summarized in several excellent general reviews (24,44,63,64, 7, 8). It remains now to show what the exact nature of these

differences is and of what their importance may be to the organism which they comprise.

In a sense, this thesis deals only with an attempt to demonstrate the antigenic structure of sea urchin tissues, and must be considered a morphological approach. However, we shall find that the only way to study and interpret function will be to examine the needs of the being and the processes by which it adjusts to its environment. The needs of any living thing are, of course, centered around certain qualities of life -- one of which is to maintain tissue integrity and reproduce this integrity for posterity. Therefore, the problem of reproducing the structure is, perhaps, the sine qua non in the existence of living things, and the investigation and understanding of structure is of primary importance.

In this paper are described a few preliminary steps toward the solution of the problem of antigenic structure of the tissues in a sea urchin, Strongylocentrotus purpuratus. Only a small fraction of the beginning is presented here and it is hoped that I may continue the work in a year or two.

Essentially, this paper concerns an investigation of the saline soluble antigens of the purple sea urchin. No experiments have yet been directed toward function of the antigens but, rather, this paper deals with "immunomorphology" and will present a few observations on the antigens of the gametes, gonads, gut, and embryos. It is hoped that this work may be utilized by those who may desire to investigate the function of some of the antigens of this urchin.

When experiments were begun in the summer of 1957, agar gel

analysis was not actually in common use and it has only recently become widely employed by investigators all over the world. Several inherent difficulties (i.e., turbidity of the agar, interpretation of results, reproducibility, etc.) have been the chief drawbacks which have retarded its widespread use.

Recent modifications have made gel diffusion a very powerful tool for the study of precipitin systems. These recent modifications include a micro analysis adaptation first presented by Wadsworth (66) and the electrophoretic separation of the antigens which should be attributed to Grabar (16). These recent developments became practical in our laboratory only since the summer of 1959 in the sense that reliance could be placed on the results. Therefore, all my previous work conducted on the urchin problem, while not entirely useless, has been supplanted by newer methods, better analysis, etc. In effect, it has been necessary to "change horses" in the past six months insofar as the immunological analysis is concerned.

An effective procedure for the isolation of sufficient antigenic material had been adopted which employed a 10% concentration of wet tissue, gametes, or embryos in 0.1 molar saline. This homogenate provided apparently good results with the possible exception of the urchin gut. Then, in January, 1960, I decided to concentrate the 10% homogenates to one half their original volume by dialysis and inject this concentrated antigen system. The results of the new technique demonstrated that twice as many antigens could be obtained from gut extracts by simply doubling the concentration and that antigenic analysis could be much more exact with all tissues. Therefore, the

general techniques were again altered and new data obtained, thus protracting the original program to take nearly twice the time originally anticipated. Several procedures that I had hoped to include in the investigation have had to be eliminated and these will be discussed later.

The general application of gel diffusion for the study of antigen-antibody precipitations has become so popular in the past decade that an extensive discussion seems unnecessary here. A thorough review of gel diffusion techniques prior to 1958 has been presented by Ouchterlony (48) and the special adaptation involving electrophoresis has been discussed recently by Grabar (16) and Poulik (56). A synopsis of the many fields of application has been provided by Wodehouse (75).

Briefly, the double diffusion technique (47) involves the simultaneous diffusion of antigen and antibody toward each other in a gelled medium (usually agar) and the formation of specific precipitates where two reactants are present in equivalence ratios. Diffusion of the reactants depends upon various factors including adsorption to gel particles, molecular weight and shape, and to some extent, relative concentration. An adjunct to double diffusion is offered by preliminary separation of the antigens of a mixture by electrophoresis (in the gel) followed by application of antibodies and completion by double diffusion; interpretation of the precipiates thus formed is termed immunoelectrophoretic analysis (IEA).

The original macro methods of Ouchterlony and others have been modified by Wadsworth (66), Crowle (5) and Yakulis (77) with several reported advantages. While older methods have employed about 0.1 ml of

reagents and a development time of approximately one week, these micro techniques can be performed with as little as 0.01 ml of reagents with development in less than two days.

Scheidegger (59) introduced a micro method for IEA employing 0.001 ml of antigen (and 0.005 ml of antibody) in contrast to the original IEA which requires 0.1 - 0.2 ml of antigen. His results (with human serum) are quite comparable to those obtained by the "macro" methods of Grabar.

The micro IEA technique of Scheidegger (59) has been modified for this work as described below.

The sea urchin has long been a favorite subject for biochemists and embryologists because of the relatively simple anatomy, ease of obtaining specimens and dissecting out tissues or procuring gametes, and because of the remarkable "control" of the development from fertilization to the late larval stages (adults are very difficult to rear and only several are reported in the literature). Extensive knowledge is available on the amino acid metabolism (26,27), the chemical nature of the mitotic apparatus (38,36,37,25,68,69,58,70), the cytoplasmic architecture [for example (19)], the antigens of eggs and early developmental stages (52,49,50,51), the antigens of the sperm (28), the comparative serology of various species of urchins and the nature of hybrid crosses (20) and interordinal crosses with sand dollars (43,9), the reproductive cycles and chemical changes in the gonads throughout the year (14,15), the nature of the cells in the celomic fluid (2) and of the celomic fluid itself including protein changes (15,34), and various other features of the chemistry and experimental embryology [for a review and excellent

bibliography see (21)]. These are several of the reasons why I have chosen to work with the sea urchin and my choice of references above is highly selective to include only those which seem most pertinent to the present paper.

The antigenic anatomy of the sea urchin has not yet been investigated from the comparative standpoint. Several studies referred to above have undertaken to determine the antigenic properties (in various species) of eggs and larval stages up to the pluteus (see above references), sperm (28), the mitotic apparatus (68,69,70) and the gut and the lantern muscle (69,70). Several other papers have appeared but these are among the more significant representatives. Since the various studies are for the most part in differing species and since old techniques of gel diffusion (and other analyses) have been employed in the above work, it is felt that a systematic account of the tissue antigens of one species using modern techniques (especially IEA) is in order, and a preliminary investigation is here reported.

MATERIAL AND METHODS

All antigenic material reported here has been obtained from the common purple sea urchin, Strongylocentrotus purpuratus, which were collected from the region of Depoe Bay, Oregon.

Dissections

Dissections were performed in the following manner. The ambulacral area of the test was broken slightly above the equator so that celomic fluid could be poured out into a beaker. The celomic fluid is centrifuged lightly to sediment the celomocytes which are then prepared in a manner similar to other tissues. Then a careful breaking and picking away of the test was carried in a counter clockwise direction thus exposing the gonads (Fig. 2). Injury to a mature gonad initiated spawning at the injured surface as well as by the gonoducts and the gametes were drawn off with a medicine dropper. Electrical stimulation and KCl injection (21) have proved far less effective than the method described above. Breakage of the test was continued along with careful tearing of the gut mesenteries until the two hemispheres were separated, at which point the top was removed and the gonads and gut dissected free from the test [(23) for a review of urchin anatomy].

The tissues were washed 3-4 times in sea water and the gut was cut longitudinally to provide a long flat sheet of tissue, thus allowing

the removal of gut contents and a peculiar entocommensal turbellarian worm which inhabits the large intestine.

Homogenates

Homogenates were prepared by three methods: trituration with fine sand in a mortar, grinding in a glass tissue homogenizer, or blending with a blade grinder (a Vir-tis #23 homogenizer). Homogenization was continued until microscopic examination revealed nearly complete destruction of cellular architecture. All the homogenates employed here have been prepared in 0.1 molar saline with 10% tissue (wet weight). Since this method of preparation was followed throughout, it was not deemed necessary to do routine protein determinations but several were performed by a biuret procedure which was modified from that of Weichselbaum (67). The homogenates were, in some instances, dialyzed against 0.1 molar saline in the cold and a number of preparations were lyophilized and stored in tightly capped bottles under refrigeration. In all cases the homogenates were clarified by centrifugation at 10,000g's for ½ hour at 5°C. It has been possible to maintain the antigenic responses by freezing the homogenates, and preservatives have not been employed (fresh homogenates have been available nearly throughout the year thus obviating preservatives). Concentration of the extracts to one-half their original volume was achieved by dialyzing against a heavy solution of pyrogallol ("Carbowax" 20M of Union Carbide Chemicals Corporation), according to the method of Kohn (29) until the volume became one-half that of the original solution.

Embryos

Embryos have been reared to the prism stage [stage 19 of Kavanau (27)] by fertilizing the eggs in filtered sea water with 10^{-5} sodium periodate following the methods described by Kavanau (27) and allowing the embryos to develop at 11-12°C in flat Pyrex baking dishes cooled in a constant temperature water bath. At intervals of about 10 - 15 hours the sea water was changed by allowing the embryos to sediment in a separatory funnel, drawing them off the bottom, and adding fresh filtered sea water. Development times, noted at this temperature were, of course, slower than conventional times for 17 - 20°C environments (hatched blastulae at 30 hours, gastrulation complete at 48-50 hours, and prisms with spicules at 70-80 hours). After the proper stage had been reached, the embryos were concentrated by centrifugation out of sea water at 4,000 g's for 10 minutes and then pooling the pellets and centrifuging at 10,000 g's for 5 minutes, transferring to a blender or tube grinder and homogenizing as described above.

Antigens have been of two varieties: those with and those without Freund (13) Adjuvant - Complete (Difco). If no adjuvant was employed, the antigen was injected intravenously as described below. Antigen - adjuvant emulsions were obtained by squirting the antigen into an equal volume of adjuvant and mixing with a #21 guage needle and syringe. When the emulsion maintains a nearly spherical shape when dropped onto water without spreading over the surface, the mixture is ready for injection.

Antisera

Antisera also were of two types depending on the variety of antigen injected (rabbits being the immunizing animals). Antigen - adjuvant mixtures have been the most extensively used preparations. These were administered as subcutaneous injections of 0.25 ml into two different sites at each injection. The dose was repeated in two other sites after two weeks. In the case of intravenous injections (marginal ear vein), a schedule such as the following has been employed: 0.1 ml initially, then doubling doses every other day to 0.8 ml followed by a rest period of 3 weeks, then a challenging dose of 0.4 ml and trial bleeding in 5-8 days.

After one month, the rabbits were trial bled via the ear vein and agar plate studies performed to evaluate the activity of the serum. Antibody titrations were performed on several sera which yielded titers of 1/100 to 1/1000 but titrations were not studied routinely because the results are not dependent on titer but upon the qualitative differences in activity in the agar plates. Investigators using gel diffusion almost universally disregard titers and many feel that low titer antisera (1/100) are frequently better than high titer sera. Since the methods employed in this paper depend upon qualitative interpretations in agar gels, this is the criterion for assessing the "strength" of the antisera. After maximum results were obtained by gel analysis, the rabbits were bled by cardiac puncture and the sera stored in serum bottles under refrigeration (usually frozen after preliminary tests).

Table I summarizes the type of preparation, method of homogenization and injection, and whether or not dialysis and concentration were

employed.

Ultracentrifugation

A Spinco Model L preparative ultracentrifuge has been employed to separate the 3.5 and 7S particles from the 20S particle as described by Kane and Hersh (25). Since the procedure was followed exactly as outlined by these authors, no more mention will be made here (see discussion of physicochemical properties of egg proteins below).

Agar

Two types of agar were required, one for IEA and one for the macro and micro methods. A 1% solution of Difco Bacto agar was prepared in (1) veronal buffer, pH 8.6, $\mu = 0.075$ or $\mu = 0.0375$ for IEA, or (2) 0.1 molar saline. These two types of agar will be referred to as buffered agar and saline agar respectively. Buffered agar (for IEA) and saline agar (for micro and macro plates) was, if necessary, then centrifuged while hot to remove any particulate materials and remelted for application to the plates.

Preservatives in the Agar

When the remelted agar has cooled to about 45°C, preservative can be added to any agar described above. For the material reported in this paper, streptomycin sulfate (Merck) in 0.01 and 0.1% concentration in the agar and Merthiolate (Lilly) in a 1:10,000 concentration have been employed on occasion, but agar without preservatives is superior

(see discussion).

Plates

Both the micro and IEA methods employ 2x3 inch micro slides carefully washed with detergent, rinsed in distilled water, and labelled on the underside with a diamond pencil. One ml of the appropriate hot agar solution was placed on each slide and distributed evenly over the surface after which the slides were placed in a hot oven (about 100°C) to dry for 10-15 minutes (this provided a "bottom layer" of agar that obviates subsequent filling of the wells). The agar-coated slides, while still hot, were placed flat in a tray so that the remaining hot agar could be poured in over them to a depth of 2 mm -- this provided agar of even thickness and eliminated meniscus effects along edges of the plates. A "trough-form" (see below) was placed on top of each IEA plate while the agar was still hot and the agar of both types of plates allowed to harden at room temperature for about ½ hour. After this time, the trough-forms were removed from the IEA plates, each plate was cut out and removed from the tray, and a steel tube 3 mm in diameter was used to cut wells in the agar. See Fig. 1a for plate dimensions.

The glass box used for the macro method was constructed from 3x3 inch clear glass plates, forming the top and bottom, and four 1x3 inch micro slides held in place by vinyl tape for the sides (the top was attached in such a way that it swung open by a tape hinge on the back 1x3 slide -- Fig. 1e). A small sponge attached to the top plate serves as a humidifier to keep the agar moist. Eight ml of saline agar was added from a 10 ml pipette and allowed to harden for ½ hour as a bottom

layer. Penicylinders (porcelain or steel) which are 8 mm in diameter were placed on the bottom layer of agar to serve as well-forms (Fig. 1c) and then eight more ml of hot agar were added and allowed to harden. Finally, the forms were removed.

Fixed forms may be employed for all three methods but, in our laboratory, we find that such a diversity of size and spatial relationship of wells is required that forms are not practical. The IEA trough-forms (Fig. 1d) are an exception, since these are so easy to make and are seldom altered. In this paper, only one size of trough-form has been employed. This was constructed from a 1x3 inch micro slide which was cut in half to provide two pieces 1x1.5 inches; the two halves were kept 13 mm apart by a block of lucite to which the slides were attached by tape. The trough-forms should be treated with a silicone product to lessen capillarity of hot agar on the glass.

Filling and Development of Plates

A syringe and needle were used to deliver the reactants to the appropriate reservoirs. For purposes of comparison, Fig. 3a and Fig. 3d are diagrammed in Figs. 4a and 4b respectively and the A, B, and C wells contain antigens, while the D and E wells (or troughs in IEA) contain antibodies. The 8 mm wells contain 0.10 ml, the 3 mm wells 0.01 ml, and the IEA troughs 0.10 ml of solution. After filling the wells of micro and macro plates, they were "developed" in a humid atmosphere at room temperature for 1-5 days. When electrophoresis of the antigens in IEA was completed, the current was discontinued and antibodies were placed

in the troughs, after which the plates were developed in a humid atmosphere for 1-3 days.

Agar Electrophoresis

Veronal buffer pH = 8.6 and μ = 0.075 or 0.0375 has been most effective. The choice of buffer depends on the antigen under investigation and is somewhat experimental though the 0.0375 ionic strength is probably best (with higher voltages). Both the agar and the cell contain the same buffer. A standard paper electrophoresis apparatus can be employed with the method of IEA described; we used the Spinco Durrum cell and Duostat (provided with the Model R system for serum proteins) and found the equipment very satisfactory. The Durrum cell was set up as for paper electrophoresis and the wicks were placed in their holders (the paper strips and their holders were, of course, omitted). In this cell, there is a distance of exactly 3 inches between negative and positive wicks so that the plate is held in place on top of the plastic wick holder, and the wick establishes good electrical contact when pressed against the agar of the plate (Fig. 1f). Constant voltage was selected on the Duostat to provide 70-100 volts per cell; this yielded a current of about 5-8 ma per plate. Electrophoresis was carried out for about 2 hours with the material reported here and the antiserm was then added to the appropriate trough immediately afterward to prevent extension of the precipitation arcs into the region of the trough. Voltage, buffer, and electrophoretic time depend, of course, on the solutions under investigation and must

be established by experiment. The most rapidly moving urchin antigen migrates 9-10 mm toward the anode in 2 hours at 70 volts, 6 ma, and ionic strength = 0.075.

Paper Electrophoresis

Electrophoresis on paper was performed with the veronal buffer described above ($\mu = 0.075$) in a Spinco Durrum type cell containing 8 paper strips run at 16 ma constant current and 350 volts for 20 hours. The strips were then stained with bromphenol blue and scanned with an Analytrol.

Photography

A light box containing two 100 watt frosted light bulbs in an aluminum-foil lined wooden box as pictured in Fig. 1g is highly satisfactory. A piece of black felt was supported on an asbestos pad above the light bulbs to provide a dark background with diffuse bright light passing through an aperture at the top of the box. The box was modified from a design of Dr. Sheldon Segal at the Rockefeller Institute. The agar plate to be recorded was placed over this aperture and a picture was taken with a 35 mm single lens reflex camera having a close-up bellows attachment. Pictures were recorded on Kodak Microfile film and developed in Kodak D-11. We have confirmed the finding of Hunter (22) that high contrast film is superior for photographic records.

Actually, it is often the case with this simple device that bands or arcs not readily visible to the eye can be clearly shown on a

negative. Enlargements may be made (as for this paper) but analysis is best performed from the negative. The actual plate may be used as a "negative" and an enlargement made directly, but we have not used this technique. Hunter (22) and others have described various lighting arrangements for plate observation and photography.

Pictures were taken at least once every day until no new bands or ares appeared. It is easily observed by those who follow plate development day by day that there really is no such thing as a "mature" plate because some precipitates may form early and disappear before the major number of precipitates form. Therefore, a "mature" plate may show fewer antigens (or antibodies) than are actually present in the solution. In this paper, the term "mature" refers to a plate which shows no new precipitates.

Staining

After development in a humid atmosphere, the plates were washed for 1 day in saline to remove non-specific materials not involved in the precipitation reactions. Then the plates were washed in 2% acetic acid for 2 hours, rinsed in distilled water 1 day, and dried by placing a wet filter paper over the agar to aid evaporation, which may be hastened by means of a dry-air blower. After the agar was dried, the precipitates were stained with amido black 10B (amido Schwarz or buffalo black), which is reported as a superior stain with rabbit precipitins, following the method recommended by Grabar (16), or merely stored, unstained, in a micro slide box for permanent records.

Rehydration of the gel brings back the precipitate patterns.

Records

Records of all antigens, injected animals, antisera, and plate reactions were kept on heavy cards. Photographic negatives were labelled as to reaction card number and date and then stored in envelopes attached to the card. Reaction cards have been filled out for nearly 200 immunoelectrophoretic analyses (3 antigens and 2 antisera for each analysis), 72 rabbits have been injected, and over 100 homogenates have been prepared. Sample cards for record keeping are provided in the appendix.

RESULTS

The fact that gel diffusion analysis depends upon many factors (such as the denaturation of antigenic properties in homogenates, sensitivity of the immunizing animal, difficulties encountered in preparing plates, etc.), requires a rather unique way of presenting results. Many studies have been performed on various antigens and antisera and it would be impossible to include photographs of all plates (over 200). Various antisera to each antigen homogenate have been studied as to their response when reacted against the homogenate.

Due to the fact that many forms of gel analysis are available, it seems worthwhile to present a comparison of the IEA method with the ordinary double diffusion methods, both macro and micro. The macro method of Ouchterlony was used extensively in the beginning phases of this investigation and was eventually replaced with IEA and I wish to demonstrate the rationale for dispensing with the double diffusion techniques.

Comparison of IEA with the Macro and Micro Double Diffusion Methods

The results of this separate study are shown in Figure 3a, b, c, d, and f and Fig. 4. For purposes of comparison (Fig. 4), the A, B, and C wells contain antigens -- egg, blastula, and ovary respectively -- while the D and E wells (or troughs in IEA) contain antisera --

anti-egg and anti-ovary respectively.

Since a new micro method of ordinary diffusion (without electrophoresis) is becoming popular, the following results are presented largely for the purpose of showing that one must compare the various techniques with respect to each antigen-antibody system because, at least with sea urchin material, the ordinary micro methods should be mentioned only for condemnation.

In the following comparisons, the designation "A-D" refers to reactions between well A and well (or trough) D, while "B-D" refers to the reaction between B and D and so on. Lower case letters have been assigned to bands and arcs which are thought to correspond regardless of the method of analysis (Fig. 4).

IEA. The IEA photographs shown in Fig. 3a and 3b were recorded after about 48 hours of development. Figure 3a shows IEA with buffered agar containing no preservative while 3c shows the effects of streptomycin (0.1%). A summary of IEA with the precipitation arcs labelled is shown in Fig. 4a. It can be seen readily that streptomycin in this concentration (and to a lesser extent in the 0.01% concentration employed but not pictured) markedly inhibits diffusion and combination of the reactants. Merthiolate inhibits precipitations slightly (not shown).

In the A-D area, 5 arcs are seen in all 3 plates, but in B-D only Fig. 3a shows 6 arcs while 3c exhibits five. The most striking overall contrast is seen in the B-E region which shows the 6 arcs of B-D in Fig. 3a and only 4 in 3c (also note the extreme inhibition in Fig. 3c). By reference to Fig. 4 and the IEA photographs, it is seen that only

the cathode end of the d arc splits into 2 separate arcs (d and d'). Area C-E demonstrates 4 arcs (Fig. 4a) one of which (e) does not appear in Fig. 3a due to loss of detail in the photographic process; also, more inhibition is seen in the preservative-agar of 3e. IEA plates are mature in 1-3 days.

Macro. Figure 3b is the same plate as that shown in 3d but taken 24 hours earlier to demonstrate several bands not apparent in the latter photograph. It is clear that the overall development of bands is far inferior to the IEA technique, especially in the B-D and B-E regions. As seen from Fig. 4b there appear to be 7 possible bands between A and D; however, it is quite possible that band f may not represent an actual precipitation band but merely a diffuse area of extension from nearby bands.

The C-E and B-E areas show a "c" band which is identified by its time of appearance and diffusion rate in comparison with a similar band in the A-D and B-D regions.

Effects due to preservatives are not pictured but revealed diffusion inhibition as noted for IEA. Plates are mature after about 3-5 days.

Micro. The photograph of Fig. 3f reveals a striking paucity of band formation (20 hours of development), and preservatives produced further inhibition. Photographs taken every few hours confirmed the fact that the micro method failed to show the same number of bands as seen with either the macro or IEA methods. For example, in the A-D area of Fig. 3f we see only 4 bands (b, c, d, and e) while in Fig. 3a we see at least 5; furthermore, in the B-D region of the micro plate we see only 3 bands while we see 4 definite bands in macro and 6 in the IEA

plates. Plates are mature in 10-36 hours.

Major Comparative Analysis of Urchin Antigens

It seems that the most effective way to present the results of studies performed on the tissue and embryonic antigens is to construct a chart which contains a summary of the reactions between any one antiserum and the various homogenates (Fig. 5). Antisera are placed in positions corresponding to the vertical lines (troughs), while antigen homogenates are placed in the circular wells and the precipitation lines between indicate the position of the reactions as projected from negatives by a photographic enlarger. The results, then, may have been obtained from one or more separate reaction plates. Therefore, it is necessary to refer to a reaction card number (Table II) and comment on the buffer, voltage, and electrophoretic time employed to give the reader some notion of the factors involved in each reaction (several cards may be referred to in constructing these results).

Fig. 5 (A to D) is arranged such that the rows (horizontal) show the reactions of the 6 types of sera to each of 2 alternating homogenates. The next row shows reactions of the 6 sera with 2 other alternating antigens, etc. For purposes of simplifying reference to any particular reaction, the horizontal rows are labelled with a capital letter and each reaction area is referred to with a number (1 to 12).

The code employed in labelling the antiserum troughs and antigen wells is as follows:

R = egg

V = ovary

Q = sperm

T = testis

G = gut

EC = prism larvae

C = celomocytes

CF = celomic fluid (free of cells)

E = esophagus

S = small intestine

L = large intestine

3.5 = 3.5S particle

7 = 7S particle

20 = 20S particle

In order to attempt some discussion of antigen homologies between different tissues, it is expedient to study the horizontal rows A to D vertically rather than horizontally. Therefore, I have given labels to all of the precipitation ares formed between an antiserum and the homologous antigen system. The reader may make his own interpretation of antigen homologies by comparing the labelled diagram of the homologous reaction with the various antigens in the vertical columns. For instance, row A contains egg and ovary solutions as antigens and A_1 and A_2 show the reaction between egg and ovary with anti-egg serum.

If it is desired to see the reaction between anti-egg serum and gut antigens in an effort to understand which gut antigens might also be contained in the unfertilized egg, one would look at the reaction in area C_2 and compare this with the precipitates in area A_1. Then, if it seems necessary to compare the relative electrophoretic mobilities of an antigen-antibody arc in the two reactions, one would refer to Table II and look under the "A" row (horizontal index) and follow down to "1" in the vertical index to find the desired information about egg vs anti-egg. Then Table II is investigated for the C_2 area to reveal the nature of the electrophoretic separation employed in that instance. If two card numbers are referred to in Table II, the first one mentioned is that employed in making the sketch in Fig. 5.

Since a detailed presentation of the results of gel diffusion analysis follows, it is now appropriate to mention several precautions to be taken into consideration. There are many variables which can have profound effects on immunologic studies. Since the individual susceptibility of the rabbits is a possible variable, one cannot rely completely on a detailed analysis of the antisera. Neither can one rely completely on analysis of the antigenic solutions because there are always the possibilities of protein denaturation, seasonal differences, individual urchin differences (though pooled tissues were usually employed), concentration factors, etc.

Therefore, one must realize that reciprocal reactions must be performed. For example, if one desires to study the antigenic similarities between gut and egg solutions, he should perform reactions

with anti-egg serum and gut antigens and also with anti-gut serum and egg antigens. It is readily appreciated by consulting Fig. 5 that the reciprocal reactions are not always similar, and this is not necessarily due to the investigator; however, the rabbit cannot be blamed for faulty experimental techniques.

Antigen homologies may be strongly suggested by investigation of Fig. 5 with respect to Table II, but proof of the homologies must await the use of specific absorptions, coalescence of the arcs on plates set up appropriately, and regular Ouchterlony plates to demonstrate coalescence or spur formation [see Ouchterlony (48) for a review of such analytic methods].

It can readily be appreciated by inspection of Fig. 5 that 6 antisera have been studied with respect to their reactions with each of 8 homogenates. The manner in which the diagram is represented, and the nature of relationships that can be established between the antigen and antibody solutions in IEA present analytical problems. An absolute correlation between precipitates in one reaction area with those of another is not possible. However, if an homologous antigen-antibody pattern can be established, then any homogenate may be examined for the presence or absence of antigens similar to those for the described pattern. Thus, if homogenate A reveals 5 antigens with the homologous antiserum and homogenate B contains only 3, it is evident that A and B have 3 antigens in common.

The results of the comparative tissue analysis are presented below by considering the reaction between one antiserum and each of

the 8 extracts investigated. A specific designation of an antigen in an homogenate refers to the corresponding labelled antigen of an homologous reaction with that particular serum unless otherwise stated.

Anti-egg Serum. The minimum number of antigens present in egg homogenates is evidenced by their reaction with anti-egg serum (Fig. 5, area A_1). These five precipitates are labelled a, b, c, d, and e. Antigen e migrated strongly toward the anode, while antigen a migrated toward the cathode; the other antigens migrated very slightly. Essentially, the same reaction was observed with ovary homogenates (area A_2). Sperm extracts showed one arc near the antiserum trough that was single and another arc nearest the antigen pit which was a "humped" diphasic arc. A diphasic arc suggests the presence of 2 antigens with similar immunologic properties but different electrophoretic mobilities. Testis homogenates demonstrated 4 antigens which probably correspond to a, b, d, and e of area A_1. An interesting result appeared when the antiserum was reacted with embryo extract (area C_1). Seven precipitates were formed in area C_1, a fact which demonstrated that the egg extract must have elicited a response in the rabbit which could not be observed unless the embryonic antigens were employed for analysis. The significance of this finding will be discussed below, but I wish to point out here that the a, b, c, d, and e antigens do appear, along with 2 new ones, in the C_1 area. Gut extracts showed 4 arcs which probably correspond to the b, c, d, and e antigen of the egg. Celomocyte extracts contained 1 antigen in common with the egg which probably corresponded

to either the d or e egg antigen (area D_1). Celomic fluid was seen to possess two antigens (area D_2) which probably corresponded to the g and either the d or e antigen of the egg.

Anti-ovary Serum. The ovary contains at least 7 antigens as presented in Fig. 5, area A_3 which represents the reaction between ovary and anti-ovary serum. These antigens have been labelled a, c, d, e, f, g, and h; a b antigen is suspected but has not yet been observed. Egg homogenates were seen to contain 5 ovary antigens which likely represent an absence of the anodic h antigen which was present in the ovary. The c, d, and e antigens of egg were easily identified; however, either the f or g antigens of ovary may represent a "b" antigen which forms next to antigen a but closer to the antigen pit and can be detected easily in area A_1. Testis extract appeared to possess all the antigens also found in ovary solutions. An additional antigen is suggested by the presence of a diphasic arc in area B_3, but this latter antigen could also be present in ovary and be masked by an inadequate electrophoresis. No reaction was observed between sperm extracts and anti-ovary sera. Gut homogenates demonstrated 3 separate arcs (area C_3), at least one of which was diphasic, representing 2 antigens. Therefore, gut appeared to share at least 4 antigens with the ovary, which probably correspond to antigens c and e and a diphasic f or g. Embryo extracts demonstrated quite clearly that the prism larvae share 5 antigens with ovary and these may correspond to the c, d, e, f, and a or g antigens. The ovary antiserum reacted with 1 antigen in celomic fluid (area D_3) which is

likely d or e. Celomocytes share 2 antigens with ovary (area D_4).

Anti-sperm Serum. Sperm extract reacted with anti-sperm serum (Fig. 5, area B) demonstrated 3 antigens a, b, and c. Only the b antigen migrated significantly with electrophoresis. Egg homogenates revealed at least two antigens in common (area A_5) and a third was suggested by the diphasic nature of one arc. Ovary extracts gave results similar to those obtained with eggs (area A_6). Area B_6 shows the reaction with testis extracts, and area C_5 the reaction with embryo extracts, both of which again demonstrated 3 antigens. Gut homogenates contained only 2 antigens present also in sperm which likely correspond to the a and c antigens (area C_6). Celomocytes (area D_5) and celomic fluid (area D_6) both showed the presence of one essentially non-migrating antigen, but the celomic fluid had the additional b antigen seen in sperm extracts.

Anti-testis Serum. With anti-testis serum, the testis extracts demonstrated 6 antigens (a, b, c, d, e and f in area B_7). A very similar pattern has already been noted in area B_3 between testis and anti-ovary serum. It is seen in area A_8 that the testis antiserum revealed only 2 or 3 antigens in ovary homogenates; this is not what one might expect from the reaction shown in area B_3. This antiserum revealed only 2 definite antigens in common with eggs (area A_7) but showed 3 antigens in sperm extracts (area B_8). Curiously, embryo extracts appeared to share only 1 antigen with anti-testis serum (area C_7) while gut demonstrated four common antigens (area C_8). Celomic fluid contained 2 antigens which likely correspond to the c and d or e antigens of testis (compare area D_7 with B_7). Celomic fluid

possessed 2 antigens in common with testis (area D_8); neither of which migrated significantly during electrophoresis.

Anti-gut Serum. An unexpectedly large number of antigens was observed in the homologous reaction with gut material (area C_{10}); the 8 antigens were labelled a, b, c, d, e, f, g, and h. The antigenic complexity of gut extracts makes comparisons with other tissues difficult. Egg homogenates shared 3 antigens as seen in area A_9, and ovary extracts also appeared to share only 3 (area A_{10}). Both sperm (area B_9) and testis (area B_{10}) showed only 2 common antigens. The anti-gut serum demonstrated only 1 antigen in the embryo extracts (area C_9). Celomocytes (area D_9) seemed to share 3 antigens, 2 of which migrated poorly while 1 migrated rapidly toward the anode. Celomic fluid contained 2 antigens (area D_{10}): 1 of which migrated slightly toward the cathode and 1 migrated strongly toward the anode.

Anti-embryo Serum. Embryo extracts contained at least 10 antigens (area C_{12}) which were labelled a through j. These antigens showed a wide range of electrophoretic mobilities. Egg extracts (area A_{12}) demonstrated 5 antigens which probably correspond to antigens a, f, g, d, and e of the embryo; it is noted that no embryonic c antigen was represented in egg extracts. Ovary homogenates (area A_{11}) revealed the a antigen and at least 4 others which were difficult to identify, but probably included the f, d, and e antigens; the c antigen was not seen in the ovary extracts. Testis extracts produced 3 arcs, 1 of which was diphasic, suggesting 4 separate antigens (area B_{11}). Sperm extracts (area B_{12}) revealed 2 antigens which probably correspond to the d and i antigens of the embryo. Gut

homogenates showed 5 antigens (area C_{11}) which likely include the g or h, e, f, and a antigens. Celomic fluid contained a rapid g or h antigen and a prominent non-migrating antigen (area D_{11}), while the celomocytes showed the presence of 2 antigens which migrated poorly, if at all, during the electrophoresis. The complex antigenic structure of the prism embryos had no counterpart in any other homogenates investigated.

Staining

In my experience, washing the plates in distilled water and fixing the precipitates with 2% acetic acid often helps with visualization of the arcs or bands. Staining appears to offer no special advantage with this precipitation system; unstained plates show as much detail as the same plates after staining. However, I have used only one stain and a number of others might be employed (e.g., azocarmine, thiazine red, etc.).

Special Analysis of Gut Antigens

Since whole gut extracts contain 7 antigens, it was desired to determine whether any of the antigens were confined to one of the 3 divisions of the GI tract (esophagus, small intestine, and large intestine). The results of this study are presented in Fig. 5E. It was found that all 3 divisions mentioned above showed the same reactions indicating that, with the antiserum and gut extracts employed, there is no regional antigenic difference in the GI tract.

Different Extraction Methods

This subject has not been investigated in sufficient detail for adequate analysis. However, it is desirable to mention that the procedure used in preparing antigen solutions does effect the results obtained by IEA. It has been found that, in general, a blendor provides better homogenization than the other methods employed. The effects of homogenization and dialysis are currently under investigation but it is suggested at this point that fresh, blended, non-dialyzed preparations are superior for plate analysis.

The original concentrations of protein in extracts were 2-5 mg/ml and presumably adequate immunologic response was observed with such solutions. Then it was observed that concentrating the antigen homogenates to twice their original protein concentration greatly enhanced the response. Therefore, a new series of experiments was begun to study the various homogenates after concentration of the extracts. Comparative studies are not presented here but the gut antigen analysis illustrates the efficacy of concentration of the extracts. Non-concentrated gut extracts induced the production of 3 detectable antibodies in the rabbit while concentrated (2x) homogenates induced the production of 7 detectable antibodies.

Ultracentrifugal Patterns

Figures 5F and 3e illustrate the comparison of anti-egg serum with 3 ultracentrifugal fractions obtained from 0.1M KCl extracts of the homologous egg material. These results demonstrated that the

preparations were not immunologically pure and that the 7S particle (calcium insoluble protein) contains at least 2 antigens which are probably the d and e antigens seen in area A_1 of Fig. 5.

Paper Electrophoresis

Numerous attempts at paper electrophoresis of the various urchin extracts employing phosphate, borate, and veronal buffers have been largely unsatisfactory. A lengthy discussion of these results is not warranted in view of the poor resolution obtained. Essentially, concentrated ovary solutions have shown 3 definite peaks on paper electrophoregrams (Fig. 3g) which correspond grossly to the IEA patterns (A_3 of Fig. 5) showing 3 major areas of antigen migration (one strongly anodic, several mildly anodic, and one to several weak cathodic moieties). Results with egg homogenates are also shown in Fig. 3g which suggest a gross similarity to the egg IEA (A_1 of Fig. 5).

DISCUSSION

Techniques

The general effectiveness of various techniques employed in this work, and several important modifications presented by others, will be discussed below.

Immunoelectrophoresis has become very widely used in the past several years and a popular account has been presented by Williams (71). Many modifications of the original IEA techniques (16, 71, 72, 73) have evolved which include the use of antigen-specific stains (16, 45), micro methods (59), different gels (4, 56), and 2 dimensional electrophoresis with discontinuous buffers (61, 56).

Immunoelectrophoretic analysis of new antigen systems must be performed with several different voltages and electrophoretic times. Two antigens may demonstrate the same mobilities in 1 experiment, while under different conditions (i.e., greater voltage and/or longer electrophoresis), they may show 2 different mobilities. A diphasic arc such as the e-f arc in area B_7 of Fig. 5 represents 2 antigens which have different electrophoretic mobilities but induce the formation of similar antibody populations when injected into a rabbit. Had the electrophoresis been carried out at a lower voltage and shorter time, the diphasic arc would have been monophasic, thus

suggesting a single antigen.

Choice of a method of gel analysis must be based on experimentation to select the most appropriate procedure with greatest resolving power. The experiments which were employed in the selection of a procedure for this work are discussed below.

By consulting the accompanying Figs. 3 and 4 and the section on results, it is evident that the amount of information gained from the 3 methods (IEA, macro, and micro) is greatest when electrophoresis of the antigens is first performed in the agar gel. This electrophoresis not only provides a more distinct pattern of bands in most cases but also supplies information on the migration of the individual antigens to further distinguish them from each other. The method described here for IEA was particularly useful in that the electrophoresis cell may be placed in a refrigerator during the entire operation if any reactants are heat labile. Drying of the agar during electrophoresis was minimized since the cell was covered.

Since the micro method evidenced such poor resolving power with the urchin antigen-antibody systems, it was discarded in favor of the macro technique when IEA could not be performed. In my experience, the micro technique was useful only for a rapid, easily set up screening procedure, but has insufficient resolving power for a critical analysis.

The macro technique was quite useful but required large volumes (0.1 ml) of antigen and required a week or longer to develop to "maturity." However, it is obvious by comparison of Figs. 3a and 3d that the b band of region A-D in the macro method was not seen on the

IEA plate.

The conclusions of Crowle (5) and Yakulis (77) that the micro method is equal and often superior to the macro method for resolution may be true for their antigen-antibody systems. However, it was clearly shown in the present paper that such was not the case with sea urchin extracts that have so far been investigated. This emphasizes the fact that has been suggested by many workers that several different methods of analysis should be investigated and critically compared before one particular procedure is wholly relied upon. Also, it is not necessarily true that staining of the precipitates is always indicated and will always reveal bands not seen before staining.

The use of agar preservatives and their possible mode of action deserves some place in this discussion.

The mode of action of streptomycin has been investigated by many workers and has been suggested by some to involve a linkage with nucleic acids (e.g., 6, 35). Kout and Kubickova (30) have recently established that the drug can react with antibodies to prevent the agglutination of red blood cells; we may be dealing with a similar type of reaction in the precipitation reactions described above. Merthiolate is extensively used as a preservative and this general use might be discouraged by results obtained in our laboratory and by the work of others. Pressman and Grossberg (57) showed that Merthiolate can inhibit antibodies which are formed in response to antigens (haptens) related to the substituted benzoate group and, though this is probably a specific competition, the role of Merthiolate as a

general inhibitor must be considered. Since Merthiolate is a mercurithiobenzoate ($C_2H_5HgS-C_6H_4COONa$) quite closely related to p-chloromercuribenzoate, it is conceivable that we may be dealing with the formation of a mercaptide, thus covering otherwise active sulfhydryl groups. The mode of action of such inhibitions, if real, must await further study.

Excellent separation of the antigens cannot always be obtained by electrophoresis in 1 direction with only 1 buffer. Therefore, in order to achieve better separations it may be necessary to electrophorese the antigens in agar prepared with 1 buffer, cut out the agar, place it in contact with a fresh agar sheet containing a second buffer, and then run the electrophoresis in a direction perpendicular to the first separation. This latter technique has been developed by Smithies and Poulik (61, 56) who suggest that better resolution may be obtained if the antigens are initially separated by 1 or 2 dimensional electrophoresis in starch gel, followed by IEA.

Consden and Kohn (4) have developed an interesting modification of gel diffusion and IEA in which a cellulose acetate film replaces agar. Their techniques reduced the quantities of reagents required, diminished the time of plate development, and eliminated the disadvantage of extension of precipitate zones into the region of an antibody trough or antigen well.

Agar diffusion techniques have also been employed in radiobiology. Perlmann and Hultin (53) described a method for the incorporation of ^{14}C into antigens and the subsequent production of an "agar autoradiogram"

which looked identical to a homologous double diffusion analysis. Such a modification might offer significant advantage to special studies in radiochemistry.

Fluorescein-labelled antibodies could, theoretically, be employed in gel diffusion studies to demonstrate otherwise invisible precipitates. To the author's knowledge, this method has not yet been reported but was first suggested to him by Bernard Pirofsky of the Medicine Department of this Medical School. The use of anti-rabbit globulin to demonstrate non-precipitating antigen-antibody complexes has been studied (45) and was found to reveal otherwise undetectable precipitates. Concentration of the antigens or antibodies is of profound importance to the formation of a precipitate as mentioned previously in connection with gut extracts. Useful adjuncts to antigen concentration might be the employment of fluorescein antibodies and anti-rabbit globulin.

Agar and starch electrophoresis have, in general, given better resolution of protein systems than either paper or free boundary electrophoresis. Beaven (1) described a method for spectroscopic recording of agar electrophoregrams which might be employed in studies such as those reported here.

The effectiveness of the use of adjuvants has been emphasized by Freund and many other workers (12, 13). Smaller amounts of antigen may be employed if combined with adjuvants, a fact of distinct advantage when working with the small volumes obtained from embryo cultures. As mentioned previously, the titer of antisera was not routinely

determined but was generally between 1/100 and 1/1000, suggesting that the adjuvants employed were effective because only ½ to 2 mg of antigen were injected. Wright (76) described a convenient method for the determination of antiserum titers by gel diffusion which could be employed where necessary.

Lyophilization did not appear to affect the homogenates which have been investigated (Table I). However, it was observed that dialysis diminished the resolution of precipitate patterns and also that a blender provided better homogenization than either tube grinding or triturating with sand.

Major Analysis of Tissue Antigens

The various extracts are discussed below from the standpoint of the maximum number of antigens which the extract shared with each of the other homogenates. Adult tissues (gonads, gut, celomic fluid, and celomocytes) are considered first and then the gametes and developmental stages will be discussed. The term "homologous reaction," which is used several times below, refers to a reaction between an antigen and an homologous antiserum (e.g., ovary antigen vs anti-ovary serum).

Ovary. The reaction between ovary and anti-ovary serum demonstrated a minimum of 8 antigens in that tissue. The egg appeared to share 6 antigens with ovary while sperm shared only 3 antigens. Testis extracts, curiously, demonstrated 7 antigens in common with the ovary. This close relationship of ovary and testis was suggested only by use of anti-ovary serum; therefore, this result must be highly questioned

since anti-testis serum showed only 2 antigens with ovary extracts. Though several different tests confirmed this finding, the author is yet very dubious of the reaction and it is here stressed that the relationship deserves further study.

Gut extracts possessed at least 4 antigens in common with ovary; however, it was also demonstrated that the a antigen of ovary extracts was not present in the gut. This will be discussed below in connection with gut antigens.

A very interesting and possibly significant finding is demonstrated with embryo vs ovary studies. Area A_3 of Fig. 5 reveals a g and h antigen in ovary vs. anti-ovary serum. This antiserum revealed only 1 cathodic antigen when reacted with prism extracts indicating that 1 of the antigens (either g or h) was not found in the embryo. It was also noticed in studies with anti-embryo serum, that the g and h antigens of the embryo extracts (area C_{12}, Fig. 5) were entirely missing in extracts of ovary, egg, and testis. This suggested that the embryo antibodies were directed against more specific embryonic antigens and would not react with the g and h antigens present in ovary extracts. However, the ovary antibodies did react with 1 of the anodic embryo antigens. This suggested that the less specific ovary antibodies were unable to distinguish between the ovary and embryo antigen.

Alternative interpretations of the apparent discrepancy between the 2 antisera should be presented. It is possible that concentration factors prevent demonstration of the antigens. It is also possible that the anti-embryo serum did not react with the anodic antigen of ovary, but this is not likely because the antiserum showed a strong

reaction with 2 anodic embryo antigens. Finally, it is possible that the anodic embryo antigen shown with anti-ovary serum is a non-specific reaction and represented an artifact. In conclusion, it is the author's opinion that the first interpretation was the most likely.

Ovary shared 2 antigens with celomic fluid and also 2 antigens with celomocytes. Hyman (23) pointed out that the hemal (vascular) system extends into the gonads and that the celomic fluid and celomocytes can therefore be transported into the interior of the gonad. The genital (aboral) sinus carries the vessels into the upper pole of the gonad and is demonstrated clearly in Fig. 2 as a Y-shaped structure medial to the gonoduct. Therefore, it was not surprising to observe the celomic fluid and celomocyte antigens in gonad extracts.

Went (69) studied the reaction between anti-egg serum and ovary antigens (S. purpuratus) and showed 5 antigens in common which is consistent with the work reported here. The mitotic apparatus (discussed below) contains 2 antigens, both of which are located in the ovary according to Went.

Testis. Six testicular antigens were demonstrated in the homologous reaction with testis antiserum (area B_7 of Fig. 5). The discrepancy noted between the homologous reaction and that with testis vs anti-ovary serum, which revealed 7 antigens, has already been discussed above in consideration of ovary antigens. Eggs were seen to share 4 antigens with testis while sperm extracts contained only 3; however, it should be pointed out here that sperm extracts exhibit only 3 antigens in the homologous reaction (see discussion of sperm below). Gut extracts were seen to contain 2 antigens in common with the testis.

Celomic fluid gave the same pattern that was observed with other antisera, i.e., 2 antigens, while celomocytes also contained 2 antigens in common with testis.

Testis antisera also demonstrated the very interesting phenomenon with embryo extracts that was discussed above for ovary antigens. It was noted (Fig. 5, area C_8) that an anodic embryo antigen was demonstrated with anti-testis sera. However, the anti-embryo serum did not react with an anodic antigen from testis extracts (area B_{11}). Again, the author suggests that a possible explanation for this phenomenon might be that the embryo antibodies are more specific, reacting only with the embryo antigen. Testis antibodies might be less specific, thus being capable of reacting with either the testis or embryo anodic antigen.

Went (69) demonstrated 2 testicular antigens with anti-egg serum (S. purpuratus). He also showed that at least 1 mitotic apparatus protein is present in the testis.

Gut. It is evident that the gut exerts strong antigenicity in concentrated solutions. Several of the 7 gut antigens were demonstrated in all homogenates investigated. Four gut antigens were seen in egg and ovary extracts. Sperm and testis shared 2 slowly migrating antigens with gut. Celomocytes shared 2 non-migrating and 1 anodic antigen in common with gut extracts which represented the strongest correspondence between the celomocyte antigens and any other extract: this might be expected since the gut wall is heavily laden with celomocytes.

Gut homogenates reacting against anti-embryo serum demonstrated an anodic a antigen common to both extracts. However, testis, ovary,

and egg antibodies did not detect this anodic antigen in gut extracts, nor did anti-gut serum exhibit this antigen with any other extracts. This suggested that the embryo shares a gut antigen not found in any other tissue including eggs and sperm. Therefore, the prism larva contains an adult gut antigen not present in either the egg or the sperm.

The nature of these antigens is left to speculation but a few findings deserve mention here. One of the most interesting features of the present work is that the esophagus did not appear to be antigenically distinct from the rest of the GI tract (Fig. 5E). This seems strange if one assumes that the esophagus contains few, if any, digestive enzymes (food remains in the esophagus only a short time). Lasker and Giese (34) reported the presence of an amylase and a protease in the small and large intestine; these segments also showed several other carbohydrate enzymes in lesser concentration. It is not known whether or not the gut enzymes are antigenic. However, if the esophagus contains no digestive enzymes, the results presented here suggest that the antigenicity of the gut is not attributable to these enzymes in view of the antigenic similarity of the 3 GI tract segments.

The studies of Went and Mazia (69, 70) failed to demonstrate any antigens whatsoever with anti-egg serum. The results reported here show very clearly (Fig. 5, C_2) that anti-egg serum exhibits 4 antigens in common with gut extracts.

Celomic Fluid. It is interesting that there were 2 antigens in the celomic fluid after removal of the clot by centrifugation. These antigens were also present in egg, ovary, sperm, testis, gut and embryo extracts. One would not expect this fluid to share antigens with all

tissues investigated, especially the gamete extracts.

Giese et al. (15) reported that fresh celomic fluid formed long fibrous clots soon after removal from the urchin. Treatment of the clotted fluid with TCA produced no precipitate but did reveal 2 peaks with paper electrophoresis. Therefore, there is agreement between the work of Giese et al. and the present paper.

Kohler and Metz (28) studied sperm extracts (see discussion below) and noted that sperm antisera showed no reaction with celomic fluid (A. punctulata). This was, assuredly, not the case with S. purpuratus, since there were 2 sperm antigens present in the celomic fluid of this species.

Why are sperm and egg antigens found in celomic fluid?

It will be of interest to analyze the results of the injection of celomic fluid and celomocyte extracts into rabbits. These antisera will soon be available.

Celomocytes. Three antigens were found in celomocyte extracts. These 3 antigens were demonstrated with anti-gut serum. Other antisera revealed antigens as follows: egg and sperm=1 and ovary, testis, and embryo=2.

The nature of the celomcytes has been investigated by Boolootian et al. (2), who described the properties of 7 different types of cells. No attempt has been made here to localize the various antigens to a cell type. The author wishes to point out that the eleocyte celomocytes contain echinochrome pigment which is presumably bound to protein. Possibly the protein moiety is antigenic and could be isolated

to determine whether it is a protein also found in other tissues.

The possibility exists that the "celomocyte" extracts employed here did not represent true celomocyte antigens, since the fibrous clot of the crude celomic fluid is included with the extract. This clot material may represent a specific celomic fluid protein.

Sperm. Sperm extracts were originally shown to contain 3 antigens in the homologous reaction (Fig. 5, B_5). However, experiments performed very recently (after preparation of Fig. 5) clearly showed that, in reality, sperm contained 5 antigens by IEA. Unfortunately these results were not available in time for incorporation in Fig. 5 and preceding sections of this paper.

Egg, ovary, testis, and embryo extracts were seen to share 3 antigens with sperm, and gut demonstrated 2 common antigens. Celomocytes shared only 1 antigen, while celomic fluid demonstrated 2 in common.

Kohler and Metz (28) studied the sperm of A. punctulata and found by gel analysis that there were 3 or 4 sperm antigens. They noted also that egg and celomic fluid extracts did not contain any sperm antigens using tube precipitation and absorption methods. The present work on S. purpuratus identified 5 sperm antigens, 2 of which were found in celomic fluid. And three sperm antigens were found in egg extracts, thus contradicting previous work.

Pernot (55) investigated guinea pig sperm with IEA. He revealed 11 antigens in the semen and 7 antigens on sperm tails, while the work of Kohler and Metz on sea urchins revealed only 1 antigen on sperm

tails by gel analysis.

The extensive literature concerning antifertilizin, fertilizin, etc. studies is beyond the scope of this paper [see (65, 50, 51) for adequate reviews].

Egg. Eggs were seen to contain a maximum of 5 antigens in the homologous reaction (Fig. 5, area A_1). Ovary antiserum evidenced 6 antigens present in eggs while the egg antiserum showed only 5 antigens in ovary extracts. Since more than a dozen anti-egg sera were employed in this work, it is likely that the discrepancy was not an artifact of observation. Possibly a concentration effect, or a poor electrophoretic separation, could account for the observed differences.

Sperm extracts shared 3 egg antigens while gut contained 3 or possibly even 4 antigens in common. Four egg antigens were noted in testis extracts. The typical pattern of 2 antigens was observed with celomic fluid and 1 antigen common to eggs was noted in celomocyte extracts.

Egg extracts reacting with embryo antiserum showed 5 antigens while embryo vs anti-egg serum demonstrated 7 antigens. The significance of this finding would seem to be that, after fertilization, egg antibodies would demonstrate more antigens in the zygote than were present in unfertilized eggs. Therefore, the egg extracts must have contained material which induced the formation of multiple antibody populations containing at least several antibodies to 1 or more individual antigens. The egg extracts might not have evidenced all antigen types present for several reasons: (1) the concentration of the antigen was too small, (2) the antigen was bound or at least was

insoluble, or (3) 1 or more antigens were bound together to form a conjugated molecule which would show a single antigen-antibody precipitate. The author favors the latter possibility because nearly all existing evidence suggests that there is a decrease in solubility after fertilization (see physicochemical discussion below). Furthermore, if the concentration of antigen in the egg was low enough not to allow detection, it seems unlikely (though not impossible) that a strong antibody could have been elicited.

It is becoming more apparent as research progresses that there are catabolic as well as anabolic cycles in the egg which are unleashed at fertilization. Proteolytic enzymes become activated to degrade yolk proteins and intensive anabolic processes are begun to build new embryonic proteins. Kavanau, for example, has presented detailed analyses of sea urchin amino acid metabolism and solubility changes in yolk and non-yolk proteins (26, 27) which confirm the latter statement. Therefore, it seems likely that the results of this paper suggest the breakdown of antigenic (yolk?) proteins, thus giving rise to additional precipitates in gel studies on early postfertilization stages vs anti-egg serum. Lapresle (33) showed that human albumin induced the formation of an antiserum which gave a single reaction with purified albumin. However, when the albumin was enzymatically degraded, the same antiserum gave 2 or 3 precipitates depending on the method of hydrolysis. It is certain, therefore, that antibodies formed against an "antigen" may be multiple and that fractionation of the antigen will lead to multiple antigen-antibody precipitates.

Went (69) showed an illustration demonstrating a possible 7 antigens in egg with anti-egg serum, and Perlmann (51) has showed possibly 7 antigens in his work with P. lividus. Both workers, therefore, obtained more antigens from egg extracts than are described in this paper. It is quite interesting in view of this difference of results, that Went and Perlmann have not been able to show the close antigenic correspondence between eggs and the several adult urchin tissues they have investigated with anti-egg serum. In my experience, it was quite simple and very reproducible to show fairly close tissue antigenic similarity in most cases.

Changes in egg antigens after fertilization will be discussed in more detail in the following section.

Embryos. A majority of the results with embryo extracts and antiserum have been discussed above, and little more than correlation need be presented here. It should be pointed out again that the term embryo, in reference to homogenates, has been used to describe the major larval stage investigated -- late prisms. Blastulae and gastrulae were also studied and antisera were prepared to these stages. Since much more material (3 grams wet weight of packed embryos) was obtained from prism cultures, and since they represent later stages of differentiation, it was decided to confine most embryo studies to prism stages. Now that characteristic findings have been described for prisms it will be desirable to work "backwards" and determine the exact time of appearance of these changes.

The specificity of anti-embryo serum with regards to the anodic g and h antigens (Fig. 5, area C_{12}) has already been discussed. And it

was noted that antiserum to egg, ovary, and testis revealed one of the anodic embryo antigens (either g or h) in embryo extracts. However, the anti-embryo serum which showed 2 anodic antigens with embryo homogenates failed to show any anodic antigens with the 3 mentioned extracts. It did demonstrate 1 anodic gut antigen (area C_{11} of Fig. 5), thus establishing a correspondence between gut and embryo not extant in egg, ovary, and testis.

Ovary and egg antisera demonstrated a cathodic antigen with each other and also with embryo. The anti-embryo serum also demonstrated this cathodic (a) antigen in the 3 extracts.

Since both egg and sperm shared 2 antigens in common with celomic fluid, it was not surprising that the embryo also shared these antigens. However, egg and sperm shared only 1 antigen with celomocyte extracts while there were 2 in embryo homogenates. It is not known whether the single antigen in egg vs celomocytes is identical to the antigen observed in sperm vs celomocytes, and no positive conclusions can be drawn until antisera are obtained to celomic fluid and celomocytes.

Further discussion of embryonic antigens is presented after the section dealing with physicochemical studies.

Physicochemical Analysis of Urchin Proteins

The mitotic apparatus (MA) of first cleavage stages of urchin embryos was initially isolated and studied by Mazia and Dan (38). Various modifications of their original procedures have been presented

(36, 37, 70). It is now established that there are 2 main components of the isolated MA which are designated as the major and minor components. The major component which migrates slowly during free boundary electrophoresis (pH=7.4), has a molecular weight of about 315,000, a ribonucleotide content of 5-6% and an isoelectric pH of about 4.6. The minor component migrates rapidly during electrophoresis and appears to be a ribonucleoprotein. Very recently (69), it was demonstrated that MA extracts possessed at least 2 antigens which have been named the "precursor-1" and "precursor-2" components. Went (68, 69, 70) has also shown that the MA precursors-1 and 2 are soluble in the presence of calcium ion at concentrations of o.05M. This observation is discussed below. All of the above results have been obtained on MA material from S. purpuratus.

Kane and Hersh (25) separated 3 groups of proteins from unfertilized eggs of S. purpuratus by use of the ultracentrifuge. They defined the 3 particles as $S_{w,20}$=3.5, $S_{w,20}$=7, and $S_{w,20}$=20. They reported from their investigation that the 7S particle was a long fibrous protein (axis ratio of 15:1 at pH=7), with a molecular weight of about 350,000, and that it formed an insoluble gel in 0.05M Ca^{++}. The 20S particle was noted to be a nucleoprotein which did not precipitate out of solution in 0.05M Ca^{++} unless left overnight after which it did form a gel. The 3.5S particle would not precipitate in the presence of 0.05M Ca^{++} under any conditions studied. It was suggested by these authors that the 7S particle might correspond to Mazia's "major protein" of the MA. However, this now seems very unlikely in view of the recent studies of Went and Mazia (68, 69, 70) which demonstrated the Ca^{++}

solubility of MA proteins. The results presented in Figs. 3e and 5F demonstrated that the author's ultracentrifugal separations were not single proteins but rather that the 7S fraction contained a non-migrating and a cathodic migrating antigen.

Electrophoretic studies (free boundary) of unfertilized S. purpuratus eggs by Connors and Scheer (3) revealed 4 peaks (in Weber's solution: 0.6M KCl in 0.04M $NaHCO_3$ and 0.01M Na_2CO_3, pH=8.5-8.8). In the author's experience with paper electrophoresis of the egg homogenates at least 2 main peaks with possible subdivisions were observed. It is likely that Weber's solution extracts different materials than the 0.1M NaCl.

Monroy and Monroy-Oddo (40, 41, 42) have shown 4 electrophoretic peaks in unfertilized eggs of Arbacia lixula and 5 or 6 peaks with the eggs of Paracentrotus lividus using distilled water for the extractions. After removing all water-soluble proteins from A. lixula eggs, they then extracted the remaining egg material with 1.0M KCl and obtained 3 additional proteins. From their studies with the KCl extracts these workers observed that there was a quantitative change represented by a precipitate of protein material. The insoluble protein appeared within 5 minutes after fertilization or upon freezing unfertilized eggs and thawing them again. In the latter case, the fraction which became insoluble after freezing contained much nucleic acid and migrated electrophoretically faster than any other fraction. Monroy et al. (40, 41, 42) has gathered evidence that following fertilization, there is (1) a decrease in solubility of at least one or more proteins, (2) the appearance of a new protein, and (3) a suggestion that

this new protein arises from unfertilized egg proteins which are fractionated during the process of fertilization.

S. purpuratus and A. punctulata eggs were studied before and after fertilization by Mirsky (39). He observed that between 10 minutes and 2 hours after fertilization there was a loss of 1M KCl solubility of a long fibrous protein (at a pH=7) which accounted for 12% of the total egg proteins. It was also noticed that 12% of the 1M KCl soluble unfertilized egg proteins were insoluble if the eggs were first frozen and then thawed. Monroy and associates confirmed Mirsky's original work concerning a loss of 1M KCl protein solubility upon fertilization of unfertilized eggs. Both Monroy and Mirsky have pointed out that the main loss of solubility occurred with a protein fraction that was salted out by low concentration of $(NH_4)_2SO_4$ (under 50%).

The Mirsky and Monroy studies were similar except for the fact that Monroy studied a different species, A. lixula, and employed water as a solvent in the freezing-thawing experiments. Therefore, it is not totally surprising that there might be a difference between the freeze-coagulated proteins of these 2 workers. Such a difference did, in fact, exist. It has been pointed out above that Monroy and Monroy-Oddo (42) observed that the freeze-coagulated protein contained large amounts of nucleotides. However, Connors and Scheer investigated purified extracts of the 1M KCl "Mirsky protein" and found that there was no appreciable nucleotide content in the freeze-coagulated protein.

Gross (17, 18, 19) has investigated the nature of protein precipitation in unfertilized A. punctulata eggs by the addition of Ca^{++}.

His work is much more extensive than that of Kane and Hersh. Gross speculated that Ca^{++} increases the acidity of egg extracts and serves to bring about the aggregation of particles which contain a high concentration of nucleoprotein. Ultraviolet absorption analysis and electron microscopy have led Gross to conclude that the Ca^{++}-aggregating particles are simply monomers of ribonucleoprotein which upon aggregation constitute the Palade granules of the egg. He also observed that eggs before Ca^{++} treatment showed 4 electrophoretic peaks while after Ca^{++} they evidenced 5 peaks. It is not yet possible to equate the work of Gross, Mirsky, Monroy and Monroy-Oddo, Connors and Scheer, Kane and Hersh, and Went and Mazia, but much might be gained by such a correlation.*

It would be of interest to isolate the g antigen (area A_1 of Fig. 5) which is a rapidly migrating particle to determine whether it corresponds to the water soluble RNP of Monroy and Monroy-Oddo. Also, one would desire to know whether the "20S particle" of Kane and Hersh might correspond to the RNP fractions of Gross, and possibly to the g antigen from unfertilized eggs which has been described in this paper. The ultracentrifugal work presented here was inadequate as shown in Figs. 5F and 3e because the preparations were not immunologically pure, and better separation is desired.

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*Since different species of urchins, different experimental conditions and techniques, and different investigators have done the separate experiments, it is probably impossible to correlate information and the various procedures should be repeated on a single species by one laboratory.

Changes in Embryos During Development

No attempt will be made to discuss the results of this investigation with reference to the extensive general literature on embryonic differentiation. The general knowledge of tissue differentiation prior to 1956 with proposed methods of investigation was presented by Trinkaus (62). He emphasized the importance of environment and reversibility on the differentiation of tissue cells. Other general reviews on differentiation and immunoembryology have been mentioned in the introduction.

Changes in urchin gonads during the year were not investigated in this paper. Much work has been done on various species of urchins which revealed that the main seasonal changes are quantitative rather than qualitative. Giese and associates (14, 15) studied S. purpuratus and found that nucleic acid content in the gonads increases strikingly in preparation for spawning. They showed that DNA increased by 31 times in testes and 9 times in ovaries, while RNA increased by 7 times in testes and 27 times in ovaries. Protein increased by 17 times in testes and 27 times in ovaries. These results suggested that the testis was devoted toward increasing the DNA; sperm is largely DNA. Apparently the ovary was largely synthesizing RNA and protein for storage in the eggs. It is believed that the urchins spawn several times each season. No obvious seasonal antigenic differences appeared in the gonads during the present investigation, but adequate analyses have not been performed.

Now with a full complement of constituents, the gametes are shed

into the water where fertilization occurs. Much mystery centers about the exact nature of fertilization which has been reviewed in detail by Perlmann (50, 51) and Tyler (65). Figure 5 demonstrated no sperm antigens that were not also seen in eggs, but recent results not included in Fig. 5 may express unique antigens in sperm extracts. It must be recalled, however, that sperm is largely DNA and that DNA is insoluble under the procedures of this investigation.

Fertilization initiates a breakdown of yolk proteins into amino acids or peptides that will then be used for construction of new proteins. Not all of the new proteins formed in early embryonic stages will be present in the adult. The appearance and disappearance of embryonic proteins is probably best understood in the case of the optic lens of the chick which has recently been reviewed by Langman (31, 32).

It has been shown in the present work that prism embryos are, antigenically, far more complicated than any of the other extracts investigated here. A change in certain egg (and/or sperm) antigens was also detected with anti-embryo serum by the discrepancy of antigen-antiserum reactions discussed above. Several anodic antigens in prisms were not observed in egg, ovary, and testis; however, these latter extracts did contain an antigen that is quite closely related to 1 of the anodic embryo antigens. A cathodic gut antigen (g antigen) apparently not present in egg extracts was observed in the prism homogenates suggesting the differentiation of an adult gut protein as early as the prism stage. Many other relationships undoubtedly are

concealed by the lack of resolution obtained with the present method of investigation.

Perlmann and associates (49, 52, 54) studied the soluble antigens of P. lividus during development. They showed by gel diffusion that egg antigens persist at least to the pluteus stage and that 3 new antigens appear between the gastrula and pluteus stages. With the use of Oudin gel diffusion analysis, they discovered (54) that 2 egg antigens were quantitatively disappearing during embryonic development and nearly gone by the pluteus stage.

Amino acid metabolism of urchins studied by Kavanau (26, 27) with S. purpuratus was discussed above. The changes of DNA and RNA during larval development to the pluteus stage in Arbacia were reported by Schmidt et al. (60). They found that DNA increased by 10 times and that RNA did not significantly increase during development to the pluteus stage. Therefore, it is possible that the embryo synthesizes large quantities of genetic material at least during early stages. This might be expected because of the increase in nuclei relative to cytoplasm during cleavage and early development.

Echinochrome pigment has not been found in eggs of S. purpuratus (10, 11) but has been noted to appear after initiation of gastrulation. During the course of this work, the activity of echinophores and development of pigment in prisms was observed. The pigment has been extracted and tentatively identified as echinochrome by Dr. Jack Fellman of the Biochemistry Department, following a modification of the methods described by Nishibori (46). The appearance of echinochrome in embryonic stages of Lytechinus variegatus has

been studied in great detail by Young (78) and corresponds remarkably with what has been discussed above for S. purpuratus. Young also noted that echinochrome did not appear in completely animalized (ectodermalized) embryos and that it would develop in exogastrulae. This suggests that the pigment may be of importance to metabolic events occurring in mes-endoderm structures. In fact, collection of echinochrome in embryos is always greatest in regions where metabolic activity is greatest. The function of echinochrome and its synthesis in the embryo is of concern to many investigators and may be of basic importance to sea urchin development.

Maternal and paternal contributions to development of urchin-sand dollar crosses have been investigated by Moore (43) and Flickinger (9). This application promises to be of much value in assessing the metabolic influence of sperm vs egg in enzyme systems, pigment formation, etc. Studies will soon be initiated in our laboratory to investigate these influences in cooperation with Dr. A. R. Moore.

Disadvantages of the Immunological Approach to this Problem

Brief mention must be made about the recognized difficulties encountered during this investigation.

The antigens must be obtained in sufficient concentration without denaturation and be injected into an animal which will respond actively to each antigen injected. These qualifications are seldom, if ever, realized. Seasonal and individual differences must be also anticipated in the urchin materials.

Of great importance, of course, is the choice of a suitable technique for analyzing the results of the antigen-antibody reaction. The analysis is inherently limited by antigen or antibody concentration, specificity of reactions, and reproducibility of results.

In short, many extracts must be obtained by various methods of extraction. Then these must be injected with several techniques into many different rabbits. Finally, appropriate analytic techniques must be carefully selected by repeated trials.

Possibilities for Future Related Investigations

The various tissue antigen homologies suggested above must be established by appropriate experiments and characterized where possible. These characterizations should include physicochemical procedures, specific staining, biological activity, etc.

Mitochondria, microsomes, and specifically, the nucleoproteins should be studied; possibly with the aid of fluorescent and/or radiolabelled antibodies.

Culture methods employing animalizing and vegetalizing substances should be investigated. Dihybridization and blastomere isolation studies would be of great significance. The treatment of cultures with high concentrations of specific antigens might reveal interesting effects and suggest an investigation of self-regulation of growth.

Normally occurring specific differentiating substances will, in time, be implicated in the development of many forms. These specific substances will control and possibly even cause morphogenetic change. The importance of such agents is of paramount interest to man in the

study, not only of embryonic differentiation and growth; but also, and, perhaps of more importance to the general population, the study and eventual curtailment of neoplastic growth.

SUMMARY

Preliminary antigenic analysis of urchin gonads, gametes, gut, embryonic stages (to prisms), celomic fluid, and celomocytes was presented. Every homogenate investigated was found to possess antigens also shared by several other extracts.

The greatest number of antigens was demonstrated in prism extracts and all adult tissues studied contained antigens in common with the prisms. It was also noted that a cathodic adult gut antigen not present in eggs (or sperm) had appeared by the prism stage. A suggestion was advanced that the prism possesses 2 anodic antigens which are not found in egg, ovary, or testis extracts; one of these antigens, however, was noted in gut homogenates, thus suggesting the presence of another adult gut antigen in prism embryos.

Results of the antigen analysis were discussed and compared with the work of other investigators.

The relative efficacy of various methods of gel analysis was investigated with simultaneous studies on the use of agar preservatives and precipitate stains. It was concluded that IEA is the superior technique, that preservatives are usually detrimental to precipitate formation, and that staining was not necessary.

APPENDIX

TABLES

TABLE I

PREPARATION OF HOMOGENATES

Antigen	Homogenization	Solvent	Lyoph.	Dialysis	Conc.	Inj.
Egg	VT, TG, S	N	±	±	±	a,I.V.
Ovary	VT, TG, S	N	±	±	±	a,I.V.
Sperm	VT, TG, S	N	±	±	±	a
Testis	VT, TG, S	N	±	±	±	a,I.V.
Blastula	TG	N	-	-	-	a
Gastrula	TG	N	-	-	-	a
Prisms	VT, TG	N	-	-	±	a
Whole gut (♀)	VT, TG, S	N	±	±	±	a
Whole gut (♂)	VT, TG, S	N	±	±	±	a
Esophagus (♀+♂)	VT	N	-	-	+	-
Small intestine(♀+♂)	VT	N	-	-	+	-
Large intestine(♀+♂)	VT	N	-	-	+	-
Celomocytes	VT	N	-	-	±	-
Celomic fluid	none	none	-	-	±	-
3.5S particle	from VT eggs	N.KCl	-	+	±	-
7S particle	from VT eggs	N.KCl	-	+	±	-
20S particle	from VT eggs	N.KCl	-	+	±	-

Lyoph., Dialysis, and Conc. = whether or not the homogenate was lyophilized, dialyzed, or concentrated, respectively; Inj. = route of injection; VT = Vir-Tis blender; TG = tube grinder; S = sand; N = 0.1 M NaCl; KCl = 0.1 M KCl; a = antigen-adjuvant mixture; I.V. = intravenous.

Legend to Table II

A, B, C, and D correspond to horizontal rows in Fig. 5. Card #=reaction card number; μ=ionic strength (a=0.075, b=0.0375); V=voltage for cell; t=time in hours.

TABLE II DATA ON ELECTROPHORESIS

Reaction Area of Figure 5	A Card #	μ	V	t	B Card #	μ	V	t	C Card #	μ	V	t	D Card #	μ	V	t
	60L4-5	b	100	2.5	59BB12-1	a	68	2.0	60B1-9	a	66	2.5	60B2-8	a	70	2.5
1	59L8-3	a	50	2.0	59BB12-5	a	65	2.0	60B3-4	b	70	2.5				
2	60L4-14	b	100	2.2	60L4-14	b	100	2.2	60L4-16	b	100	2.2	60L4-16	b	100	2.2
	60B1-5	a	64	2.5	60L4-20	b	100	2.5	60B3-2	b	70	6.0	60L4-36	b	100	2.5
3	60L4-18	b	100	2.5	60B1-16	a	68	3.0	60L4-19	b	100	2.5				
	60B1-16	a	68	3.0					60B2-8	a	70	2.5	60B2-7	a	70	2.5
4	60L4-21	b	100	2.5									60B2-8	a	70	2.5
	60F3-15	b	100	3.0	59BB12-7	a	68	2.0	60B1-14	a	66	3.0	60L4-37	b	100	2.5
5	59BB12-8	a	68	2.0	60F3-9	b	100	3.0	60L4-33	b	100	2.5				
	60L4-32	b	100	2.5	60F3-15	b	100	3.0	60F3-10	b	100	3.0	60L4-37	b	100	2.5
6									59BB12-7	a	60	2.0				
	60L4-19	b	100	2.5	60F3-16	b	100	3.0	40L4-18	b	100	2.5	60L4-36	b	100	2.5
7					60B1-17	a	68	3.0	59BB12-6	a	60	2.0	60L4-37	b	100	2.5
	60F3-16	b	100	3.0	59BB12-1	a	68	2.0	60L4-18	b	100	2.5	60L4-37	b	100	2.5
8	60F3-8	b	100	2.5	60F3-15	b	100	3.0								
	60B3-2	b	70	6.0	59BB12-8	a	60	2.0	60L4-11	b	100	2.5	60B1-11	a	66	3.0
9	60B3-3	b	70	6.0									60L4-38	b	100	2.5
	60B3-2	b	70	6.0	60F3-8	b	100	2.5	60B3-1	b	70	2.5	60L4-38	b	100	2.5
10									60F3-14	b	100	3.0				
	60L4-31	b	100	2.5	60L4-32	b	100	2.5	60F3-9	b	100	3.0	60L3-12	b	100	2.0
11									60B3-4	b	70	6.0	60L4-38	b	100	2.5
	60F3-12	b	100	3.0	60L4-32	b	100	2.5	60F3-11	b	100	3.0	60L4-38	b	100	2.5
12									60F3-9	b	100	3.0				

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ILLUSTRATIONS

Figure 1

Dimensions (in millimeters) of micro (a), IEA (b), and macro (c) plates. An IEA trough form is shown in (d). A glass-slide box for macro double diffusion is shown in (e). Two IEA plates (f) are shown in place in a Durrum cell (cover not included). An aluminum-foil lined photographic box is shown in (g).

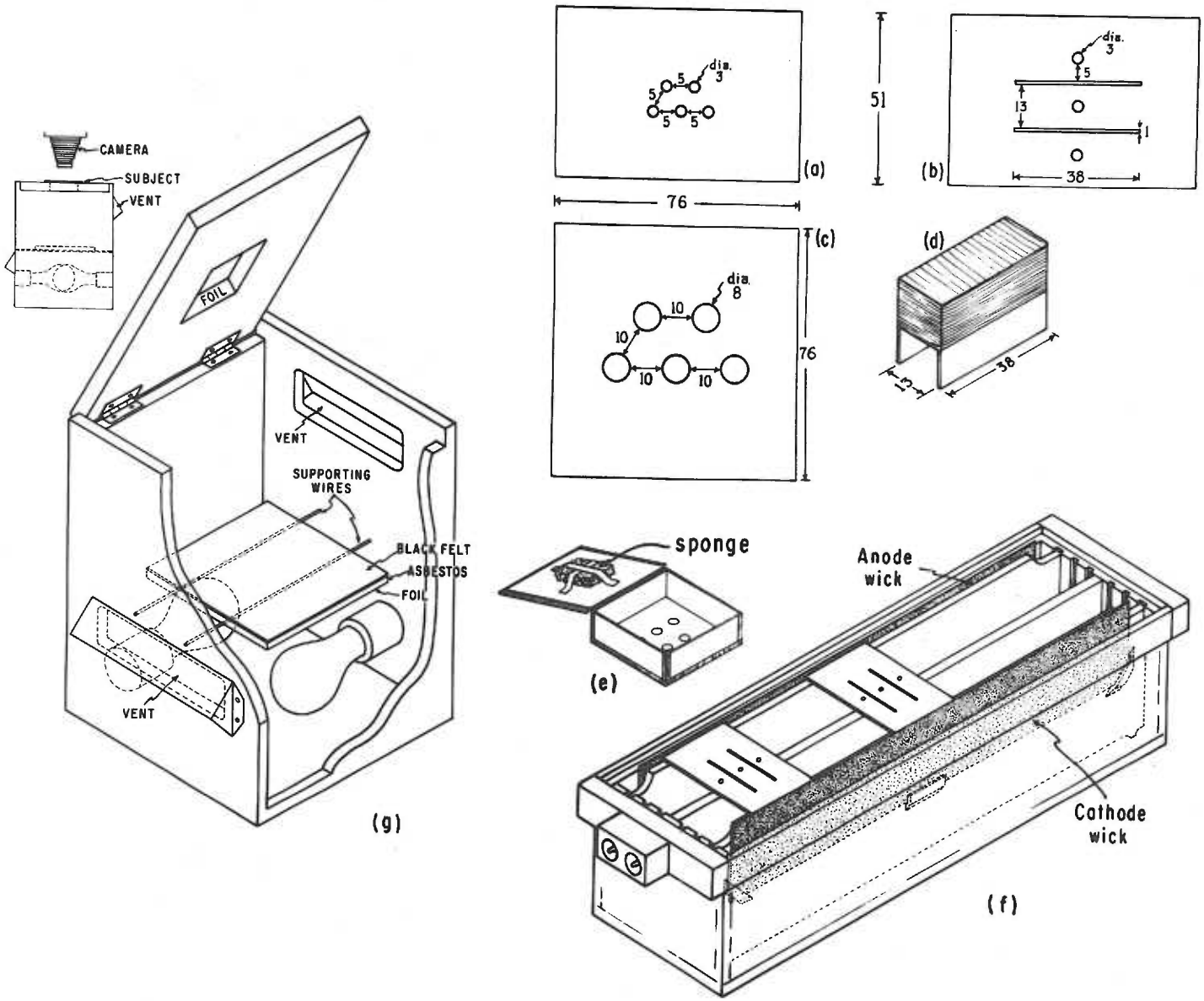
CAMERA
SUBJECT
VENT
FOIL
VENT
SUPPORTING WIRES
BLACK FELT
ASBESTOS
FOIL
VENT
(g)
dia. 3
5
5
5
5
(a)
76
51
(b)
dia. 3
5
13
1
38
(c)
dia. 8
10
10
10
10
76
(d)
38
sponge
(e)
Anode wick
Cathode wick
(f)

Figure 1

Figure 2

Partially dissected female urchin to demonstrate gut, 3 lobes of ovary, gonoduct and genital (aboral) sinus. The gonoduct empties through test on upper surface of animal. Approximately 2x.

Figure 2

Figure 3

Comparisons between IEA (a and c), macro (b and d), and micro (f) diffusion corresponding to Fig. 4. Ultracentrifugal analysis in (e) corresponds to Fig. 5F. Scanned paper electrophoregrams of egg and ovary are shown in (g); arrow indicates application point (veronal buffer pH=8.6, μ=0.075). Importance of taking photographs at least once every day is demonstrated in (b)and (d) which represent a difference of 24 hours development. In all cases where electrophoresis was performed, anode is to left.

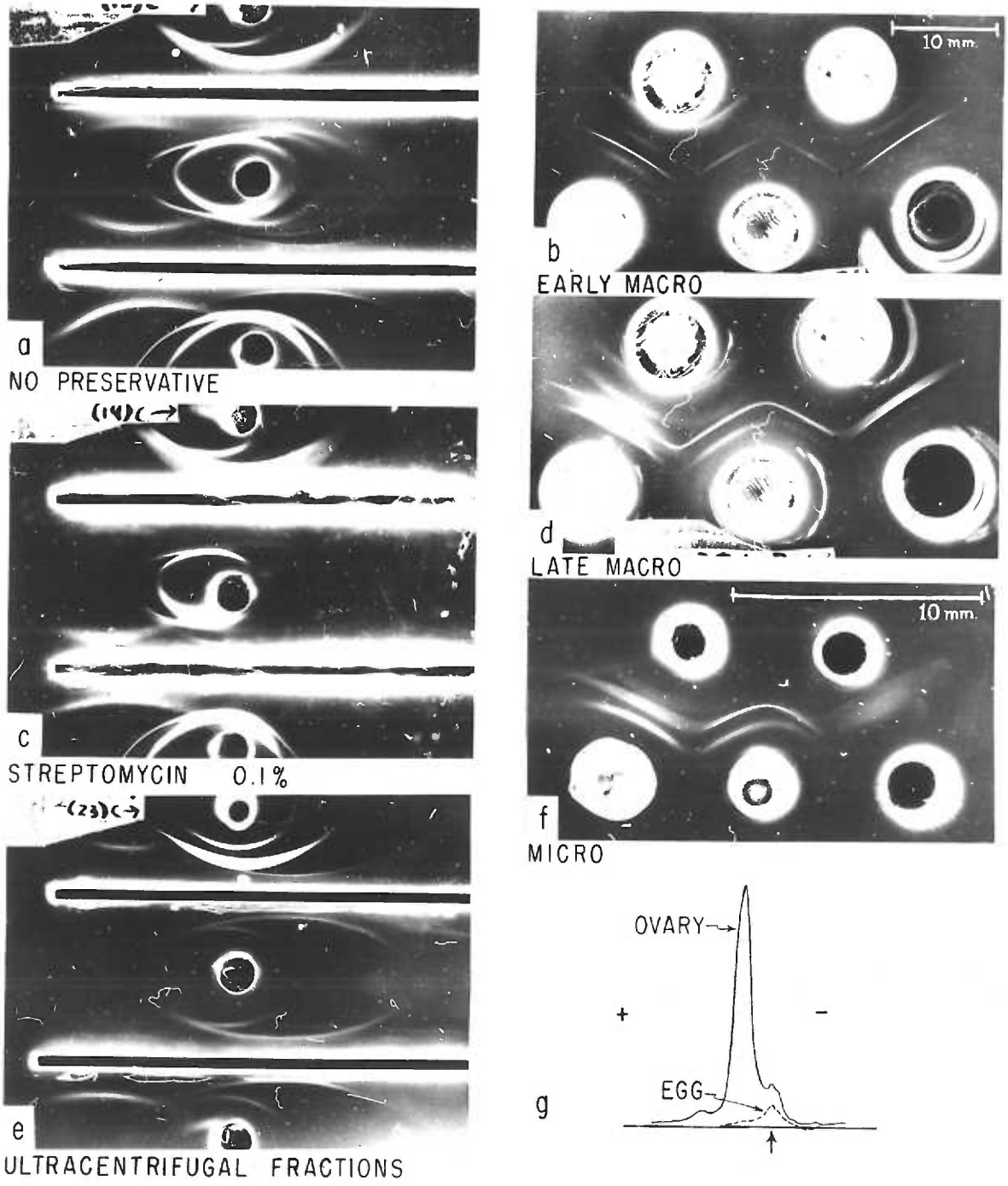
a
NO PRESERVATIVE
c
STREPTOMYCIN 0.1%
e
ULTRACENTRIFUGAL FRACTIONS
10 mm.
b
EARLY MACRO
d
LATE MACRO
10 mm.
f
MICRO
OVARY
+
−
EGG
g

Figure 3

Figure 4

Diagrams of IEA (a) and macro (b) plates summarizing results of plate analysis. In both drawings, A=egg, B=blastula, C=ovary, D=anti-egg, and E=anti-ovary serum. Lower case letters designate corresponding precipitates. The anode is to the left in (a).

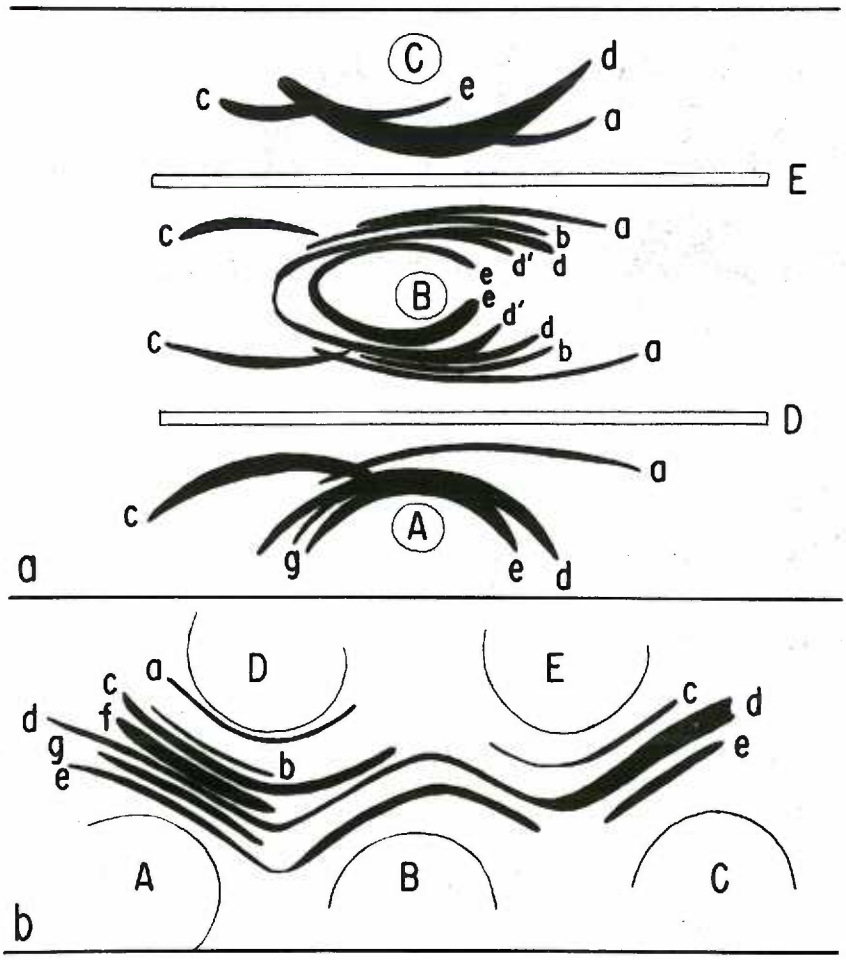

Figure 4

Figure 5

Summary of IEA reactions. Straight vertical lines represent antiserum troughs. Circles represent antigen wells. Arcs between troughs and wells indicate observed antigen-antibody precipitates. Homologous reaction precipitates are labelled with lower case letters. Antisera are labelled at top of figure over corresponding trough and each trough below the labels contains that antiserum. Numerals (together with capital letters) serve to designate each reaction area of precipitation arcs.

Gut separation analysis is presented in E with gut antiserum. Ultracentrifugal separations of egg are presented in F with egg antiserum.

See p. 22 of text for key to abbreviations.

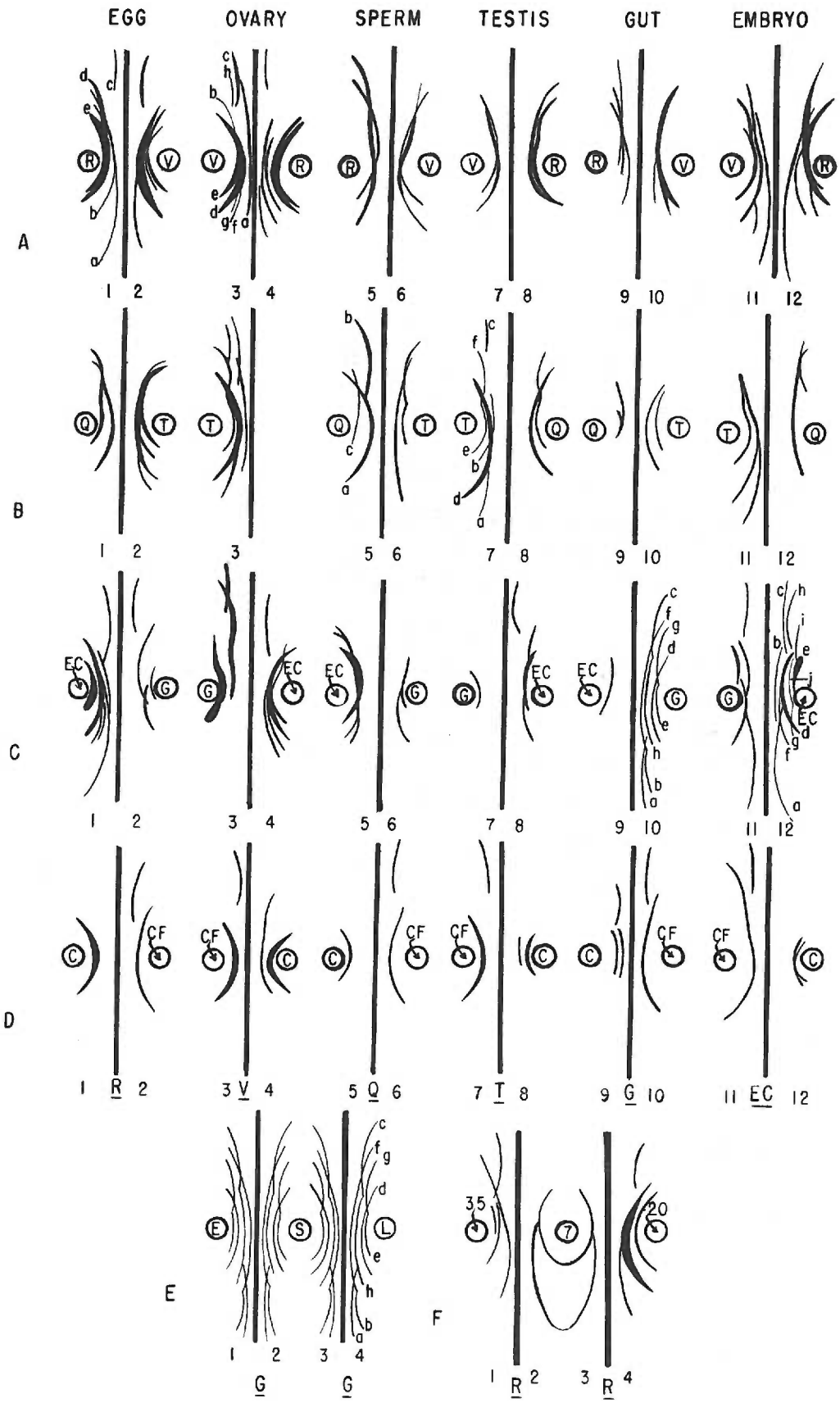
EGG
OVARY
SPERM
TESTIS
GUT
EMBRYO
A
B
C
D
E
F
EC
CF
1 2
3 4
5 6
7 8
9 10
11 12
1 R 2
3 V 4
5 Q 6
7 T 8
9 G 10
11 EC 12
1 2
3 4
G
G
1 R 2
3 R 4

Figure 5

Animal Code Number _____

IMMUNIZATION CARD

Antiserum Code Number _____

Antigen Code Number _____

Immunizations performed by _____

Injections:

Date	Antigen (code #, amount injected, note if adjuvants used etc.)	Remarks (site of injection, apparent condition of animal, etc)

TEST bleedings (dates, method of collection, reference to place of write up for results):

FINAL bleeding (date with pertinent information concerning autopsy, methods, serum, etc.):

How serum is preserved:

(OVER if this square is marked ☐)

ANTIGEN-ANTIBODY REACTION

Reaction # _____

Date set up _____

Test set up by _____ Time of Day _____

Purpose of test: _____

_____

Type of container _____

Agar _____

Thickness of Agar layer _____ mm.

Length of run _____

Voltage/plate _____

Current/plate _____

Contents of wells (code numbers, description of reagents, quantities, incidents of filling).

A: _____

B: _____

C: _____

D: _____

E: _____

F. _____

(Over if this square is marked ☐)

ANTIGEN CARD

Antigen Code Number _____

Date prepared and by whom: _____

Source of Antigen: (refer to book if necessary):

Method of Preparation:

Method of Preservation:

Strength of Antigen:

(OVER if this square is marked ☐)