ANTIBODY PRODUCTION IN *RANA PIPIENS* AS INFLUENCED BY AMBIENT TEMPERATURES

A Thesis

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INTRODUCTION

One of the major features of acquired immunity is the formation of specific antibody in response to an antigen. The specific antibody may be a precipitin if the antigen is soluble or an agglutinin if the antigen is particulate.

In response to an invading antigen, the phagocytic cells of the host attempt to engulf the foreign material and, if possible, degrade it. If the antigen enters the host by a subcutaneous or intramuscular route, it will enter the lymphatic system first. Miller and Nossal (1964), using radioactive iodine and fluorescent antibody techniques, found the antigen was localized in the primary follicles and germinal centers within the lymph nodes.

If the antigen is not completely taken up by the phagocytic cells of the lymph nodes it will enter the circulatory system. The foreign material will then either be taken up by the blood leukocytes or by the reticuloendothelial cells in the various organs through which the blood passes.

Aschoff (1924) found that when he injected carbon particles or various dyes into a normal mammalian host he could delineate the reticuloendothelial system by observing which cells took up the injected substances. Baillif (1960) confirmed Aschoff's (1924) work by using essentially the same techniques. He found the cells in the liver, spleen, bone marrow, lymph nodes, adrenals and lungs took up the injected dyes and retained the substances for weeks. Loutit (1960) found the reticuloendothelial system was composed of a network of reticular cells of the spleen, thymus and other lymphoid tissues along

with cells lining the sinuses of the spleen, bone marrow and lymph nodes and capillary endothelium of the liver, the adrenal glands and pituitary gland.

To show the effectiveness of phagocytosis by the reticulo-endothelial system, Bull (1915) injected dogs intravenously with typhoid bacilli. He found the concentration of typhoid bacilli in the blood dropped from 10,000,000 per ml at one minute to 40 organisms per ml after 40 minutes.

It has been found there are marked differences in the rates of clearance of different types of bacterial antigens. Benacerraf and Miescher (1960) injected "normal" mice intravenously with heat killed Staphylococcus aureus and Escherichia coli labeled with P^{32} and noted S. aureus was removed from the blood in 7 minutes in contrast to 90% clearance of E. coli in 61 minutes. Phagocytosis of E. coli was found to be accelerated in the presence of antibody and complement. These results agreed with the observations of Wardlaw and Howard (1959) that specific antibody and complement increase the rate of phagocytosis. Because antibody plays such an important role in a host's defense mechanism, it would be desirable to elucidate some of the characteristics of antibody synthesis.

Bjornebae and Gorman (1943) immunized rabbits with pneumococcus and noted a pronounced hyperplasia of the plasma cells in nearly all organs studied. They noticed the increase in the number of plasma cells paralleled the concentration of circulating antibody.

Coons, Leduc and Connoly (1955) demonstrated the cellular localization of antibody in animal tissues in response to a protein antigen utilizing fluorescent antibody techniques. Leduc, Coons and Connoly (1955) demonstrated antibody in cells within the medulla of popliteal lymph nodes draining the site of injection. These cells were later seen to proliferate and differentiate into nodules of mature plasma cells.

Schoenberg, Stavitsky, Moore and Freeman (1965) determined by radioactive labeled amino acids and fluorescent antibody techniques that 19S antibody appeared first after antigenic stimulation and originated from large mononuclear cells present in the wall of the sinusoids of the red pulp of the spleen. They believed smaller 7S antibody appeared after the 19S macroglobulin in plasma cells found in the white pulp of the spleen.

To further understand the mechanism of acquired immunity, lower animals have also been studied extensively. Finstad and Good (1964) could detect no immune response in invertebrates. They also detected no circulating antibody or specific acquired response in the lowest known vertebrate, the California hagfish. In contrast, the sea lamprey was found to be the lowest known vertebrate tested to produce antibody. Papermaster et al. (1963) and Evans et al. (1965) substantiated Finstad and Good's (1964) findings that the sea lamprey is the lowest known chordate capable of producing antibody.

Papermaster (1963) found that the ontogeny of the immune response in mammals is thymus dependent. This was substantiated by Hildeman and Cooper (1963) who thymectomized newborn mammals and found there was

a depletion in the number of circulating lymphocytes along with a definite impairment of the host's immunological capabilities. In contrast, thymectomized bullfrog larva demonstrated no impairment of immunological competence but there was inhibition of their growth rate. Papermaster (1964) found the California hagfish has no evidence of thymus tissue in contrast to the sea lamprey which does exhibit a primitive thymus.

Hildeman and Cooper (1963) also found lower vertebrates have a less differentiated lympho-myeloid system than mammals. Kent, Evans and Attleberger (1964) confirmed the previous work by demonstrating a lymphatic system in the marine toad, Bufo marinus, with India ink. The lymph nodes were fewer in number than those found in a mammal but demonstrated the characteristic morphology of a lymphoid reticulum. Antibody forming cells were detected in the lymph nodes of the marine toad using the immunofluorescent technique and a bovine serum albumin antigen (BSA).

There seems to be a relationship between the ability of an animal to clear an antigen from its circulation and its ability to synthesize antibody. Condie, Pih and Monson (1964) found the hagfish cannot remove an injected antigen from its circulation to any measurable degree. This compares favorably with the fact no antibody has been detected in the hagfish. Condie et al. (1964) found Rana pipiens would not clear BSA from its blood but would remove and degrade a particulate antigen. Also, the frog would not produce any detectable antibody against the BSA antigen. Im comparison, rabbits injected with either a soluble or a particulate antigen quickly removed the

foreign substances from the blood and formed antibody against the specific antigen. Alcock (1965) did demonstrate slow clearance of BSA in Rana temporaria but could not detect the formation of antibody.

Clem and Sigel (1963) compared titers in rabbits, margates and sharks that were injected with BSA. The rabbit demonstrated a titer of 1:800,000, the margate 1:50,000, and the shark 1:3,000. Antibody was detected by gel diffusion and passive hemagglutination.

Maung (1963) studied immunity in the tortoise and detected small amounts of antibody in response to the soluble antigen BSA. This compares favorably with the studies of Alcock (1965) that a low molecular weight soluble antigen does not appear to be as antigenic in some of the lower vertebrates and is cleared from the blood with difficulty.

An additional difference between the antibody response of a mammal and a cold blooded vertebrate was demonstrated by Everhart and Shefner (1966). They found by immunodiffusion techniques that fish antibody was less specific than mammalian antibody since it gave a greater number of cross reactions with antigens present in other serums.

An important humoral component of serum that combines with antigen-antibody complexes to cause lysis is complement. Legler and Evans (1966) found that three amphibians Bufo marinus, Rana pipiens, and Necturus maculosus have levels of complement comparable to concentrations found in mammalian serum.

Grey (1963) summed up the major difference between the adaptive mechanisms of mammals and lower vertebrates. He stated that teleosts, amphibia and reptiles give a prolonged heavy antibody response (19S) followed by a smaller size (7S) 2-mercaptoethanol sensitive antibody.

In contrast, mammals have a short heavy antibody response followed by a 7S 2-mercaptoethanol resistant antibody phase.

In addition to the differences between the adaptive mechanisms of mammals and lower vertebrates just described, a major influence on antibody synthesis in poikilotherms is temperature. Since poikilothermic animals have no temperature regulating mechanism, the temperature is a variable that can be manipulated by the investigator to study various aspects of antibody formation.

Emerson and Norris (1905) were one of the first groups of investigators to observe the effect of temperature upon the course of an infection in cold blooded animals. They found the disease known as "red leg" could be controlled in frogs by maintaining the animals at a temperature just above freezing.

Allen and McDaniel (1937) discovered that frogs injected with human erythrocytes produced hemolysins at room temperature but failed to produce detectable antibody at refrigerator temperatures. Thus, it became evident synthesis of antibody could be studied more closely in cold blooded vertebrates by varying the ambient temperature.

Recognizing the importance of temperature on the immune response, Bisset (1946) found in studying infections of fish there seemed to be a balance between the action of the invading bacteria and the defensive powers of the host when the temperature is lowered. Bisset (1946) postulated that when the temperature was raised the delicate balance was upset and either the host was killed by the multiplying bacteria or the host eliminated the organisms and survived. Bisset (1947a) found that when frogs were injected at temperatures of 20 C and 8C

against lethal organisms, protection was afforded only at the higher temperature. Also, he found that adult and larval frogs rid themselves of a bacterial infection more rapidly at 20 C than at 8 C. He postulated there may be a condition of asymptomatic parasitism at the lower temperatures (1947b).

Hildeman and Haas (1959) demonstrated a vigorous isoimmune response to skin homografts in bullfrog larva. They found the mean survival time varied inversely with the temperature i.e., as the ambient temperature was lowered the homografts were rejected at a much slower rate.

From the previous studies, it becomes evident that lowering the ambient temperature of a poikilothermic vertebrate has an inhibitory effect on the concentration of circulating antibody. Bisset (1948) concluded from his experiments that it is possible for cold blooded animals to acquire specific immunity at cold temperatures if the temperature is subsequently raised.

In later experiments, Bisset (1949-50) injected immunized frogs held at 25 C and at 5 C with adrenocorticotropic hormone (ACTH) and periodically tested them for the appearance of circulating antibody. Bisset (1949-50) detected antibody and concluded antibody was probably being synthesized by the antibody producing cells, but not released into the circulation until triggered somehow by the injection of ACTH even though he immunized the animals at room temperature before transferring them to 8 C.

Krueger and Twedt (1963) extended Bisset's (1949-50) observations by studying antigenically stimulated splenic cells of Rana pipiens in vitro. The cells were first incubated at 4 C and subsequently

raised to 26 C. They noted production of antibody at the higher temperature. Antibody was detected by observing immobilization of a motile bacterial antigen (Salmonella typhosa), and by titering the supernatant fluid with agglutination tests. Their conclusions were similar to those of Bisset (1949-50) in that antibody may be produced at refrigerator temperatures and released when the temperature is raised.

Evans (1963) immunized marine toads and detected antibody only when the animals were raised from refrigerator temperature to room temperature. He concluded the potential for synthesizing antibody is probably acquired in the cold.

Rees, Perkins and Elek (1963) found that lowering the temperature of the toad, Xenopus laevis, reduced the rate of protein metabolism and immunized toads failed to produce detectable antibody until removed to a temperature of 27 C. They also found that circulating antibody appeared as soon as 60 hours after warming the animals.

Maung (1963) immunized the tortoise at refrigerator temperatures and subsequently injected cortisone. He failed to detect circulating antibody even after several months. It should be noted there is a major difference in Maung's (1963) work as compared to that of Bisset et al. (1948, 1949-50). In Maung's (1963) work the injected animals were never allowed to come to room temperature in studying the antibody response at refrigerator temperatures. This casts some doubt on Bisset's (1949-50) conclusions that antibody is actually formed in the cold but not released by the antibody forming cells.

Alcock (1965) attempted to confirm Bisset's (1949-50) hypothesis concerning the release of antibody from immunized poikilotherms maintained in the cold. She immunized Rana temporaria at refrigerator temperatures and later administered cortisone. As a result, she could not detect any circulating antibody in the cold adapted frogs.

The primary purpose of this study was to investigate the antibody synthesizing mechanism at the cellular level in Rana pipiens in relation to the ambient temperature using a fluorescent conjugated bacterial antigen.

As a preliminary study, it was planned to demonstrate the distribution of injected material via the dorsal lymph sac in order to observe later the disappearance of a bacterial antigen. Kent, Evans and Attleberger (1964) sucessfully studied the lymphatic system of Bufo marinus by injecting carbon particles (India ink) and by histological studies. Similar techniques were used in this study.

Also, an additional objective of this study was to determine if a bacterial antigen (Francisella tularensis) was removed from the blood and broken down by the phagocytic cells of the reticuloendothelial system at refrigerator temperatures. For this work, a fluorescent antibody conjugate specific for F. tularensis was used.

As a supplement to the previous work, Rana pipiens serum from immunized animals maintained at 5 C and 25 C was analyzed by agar gel electrophoresis, immunoelectrophoresis, and indirect immunofluorescence to characterize the immunoglobulins as compared with human serum.

MATERIALS AND METHODS

Experimental animals

The frogs, Rana pipiens, were obtained from a commercial supply house, The Lemberger Company, in Oshkosh, Wisconsin. Young adult rabbits were purchased from local merchants.

Bacterial cultures

F. tularensis, strain Jap, was obtained from the Department of Microbiology of the University of Kansas at Lawrence, Kansas. The cultures were maintained on glucose cysteine blood agar (GCBA).

Preparation of a bacterial antigen

F. tularensis, strain Jap, was cultured on GCBA slants for 24 hrs and harvested with a 1% formol-saline solution. The organisms were allowed to stand in the formol-saline for 48 hrs and then washed three times in 0.85% saline solution by centrifuging at 4000 rpm for 30 minutes. The organisms were adjusted to a final concentration of 1×10^9 organisms per ml by spectrophotometric determination. As a final check for viability, a culture plate was routinely inoculated (Burrel and Mascoli, 1962).

The bacterial agglutination test

Two-fold dilutions of the serum were made in 10 x 75 mm test tubes using normal saline as the diluent yielding a final volume of 0.3 ml per tube. An equal volume of antigen was added to each tube (0.3 ml) including a saline control. After three hours incubation at 37 C, the

tubes were placed in the refrigerator overnight. The following morning the tubes were read for agglutination using a microscope mirror and a strong light by the method of Burrel and Mascoli (1962).

Phlebotomy procedure

The frog's heart was exposed by a midventral incision and the pericardium removed. A 24 guage needle and a 5 ml syringe were used to draw blood slowly from the right branch of the truncus arteriosus. The blood was allowed to clot for 10-15 minutes at room temperature, centrifuged at 3000 rpm for 10 minutes, and the serum removed.

Preparation of fluorescent conjugates

F. tularensis antiglobulin solution was tagged with fluorescein isothiocyanate to detect F. tularensis in tissues according to the procedure of Cherry, Goldman, Carski and Moody (1961).

A young, adult rabbit was immunized with F. tularensis by the procedure described by Burrel and Mascoli (1962) After a titer of 1:640 was reached, the rabbit was exsanguinated and the serum separated from the clot as previously described. The globulins were fractionated by slowly adding an equal volume of saturated ammonium sulfate with constant shaking.

Following overnight precipitation, the globulins were centrifuged at 3000 rpm for 30 minutes and the supernatant fluid discarded. After resuspending the precipitate in a small amount of distilled water, the solution was dialyzed against normal saline until all of the ammonium and sulfate ions were removed from the saline. The complete removal of ions was indicated when no precipitate was formed after combining equal amounts of saline and saturated barium chloride.

The protein solution was quantitated by the biuret method and adjusted to 1% with normal saline. Carbonate-bicarbonate buffer (0.5 M, pH 9.0) was added until pH 9.0 was obtained.

The buffered globulins were placed on a magnetic stirrer in the refrigerator and cooled to 5 C. The fluorescein isothiocyanate powder was dissolved in 2 ml reagent grade acetone and added slowly with stirring to the chilled globulins in a ratio of 0.5 mg powder per mg protein.

After overnight conjugation, the globulins were dialyzed against normal saline until the overnight dialysate showed no fluorescence under a Wood's ultra violet lamp. The conjugate was then dialyzed against buffered saline for 24 hrs, aliquoted into tubes and frozen at -10 C.

For the cellular localization of antibody in tissues, a fluorescent conjugated bacterial antigen was prepared according to the procedure used by Eveland (1964).

F. tularensis was grown in large quantities on GCBA for 48-72 hrs to obtain maximum growth. The organisms were harvested by washing the slants with a minimum amount of saline solution. The organisms were washed three times with normal saline and centrifuged at 4000 rpm for 30 minutes. The bacteria were disrupted by intermittent sonification for 30 minutes at a power setting of 7-8 amperes using the Bronson sonifier. The solution was centrifuged at 5000 rpm for 30 minutes at 5 C.

The supernatant solution was quantitated for protein as previously described and the solution concentrated to 1% by pervaporation. Dialysis

and tagging the protein with the fluorescein isothiocyanate was accomplished as previously described.

A third fluorescent conjugate was prepared for detecting F. tularensis antibody in serum. A young, adult rabbit was immunized with frog serum according to the procedure of Burrel and Mascoli (1962). The rabbit anti-frog serum was fractionated and tagged as described.

Before the tularensis antibody conjugate could be tested on tissue, it was adsorbed with tissue powder to eliminate non-specific fluorescence. For the adsorption process, rat liver tissue was prepared according to the procedure of Cherry, Goldman, Carski and Moody (1961). A quantity of liver was taken from several rats and was homogenized with a mortar and pestle. The mixture was centrifuged and washed three times with distilled water to remove the hemoglobin. The sediment from the final washing was suspended in saline, filtered through cheese cloth, centrifuged and the saline decanted.

The mixture was transferred to a Buchner funnel by washing with acetone. Most of the water was removed from the sediment by pulling through generous quantities of acetone. Before all of the water could be removed, the filter cake was broken up into a fine powder. Finally, the powder was dried overnight at 37 C and stored in the refrigerator with a dessicant.

For adsorption of the labeled globulins, the powder was stirred into an aliquot of conjugate in a ratio of 100 mg of powder per ml of conjugate and allowed to stand for one hour at room temperature with occasional shaking. The supernatant fluid was harvested by centrifuging at 18,000 rpm for 30 minutes at 5 C. The adsorption procedure was usually repeated a second time with a similar amount of liver powder.

Precipitin test

Ten-fold dilutions of antigen were made and layered over an equal amount of undiluted antisera in 10 x 75 mm test tubes forming an interface where the two components met. For a negative control, saline was substituted for serum. Tests were examined after 3 hrs and 24 hrs for a precipitin ring at the interface. The procedure was a modification of one by Burrel and Mascoli (1962).

Tissue embedding techniques

Tissue to be tested by the immunofluorescent technique was fixed in cold 95% ethanol and embedded in paraffin by the method of Sainte-Marie (1962).

After fixation for one hour in cold 95% ethanol, the tissue was trimmed to slices 2-4 mm thick, placed in cloth bags and returned to the cold ethanol for 15-24 hrs.

The tissue was dehydrated in cold absolute alcohol, cleared in cold xylene and brought to room temperature in the final xylene. The tissue was embedded in paraffin and the blocks refrigerated. The tissue was sectioned at 10 microns and floated onto acid cleaned microscope slides with distilled water. The slides were dried for two hours at 37 C and the paraffin removed in cold xylene. Before testing with the fluorescent conjugate, the slides were dehydrated in cold absolute alcohol, hydrated in 95% alcohol and rinsed in cold buffered saline.

For morphological studies, tissues were fixed in formol-Zenker's solution and embedded essentially as described.

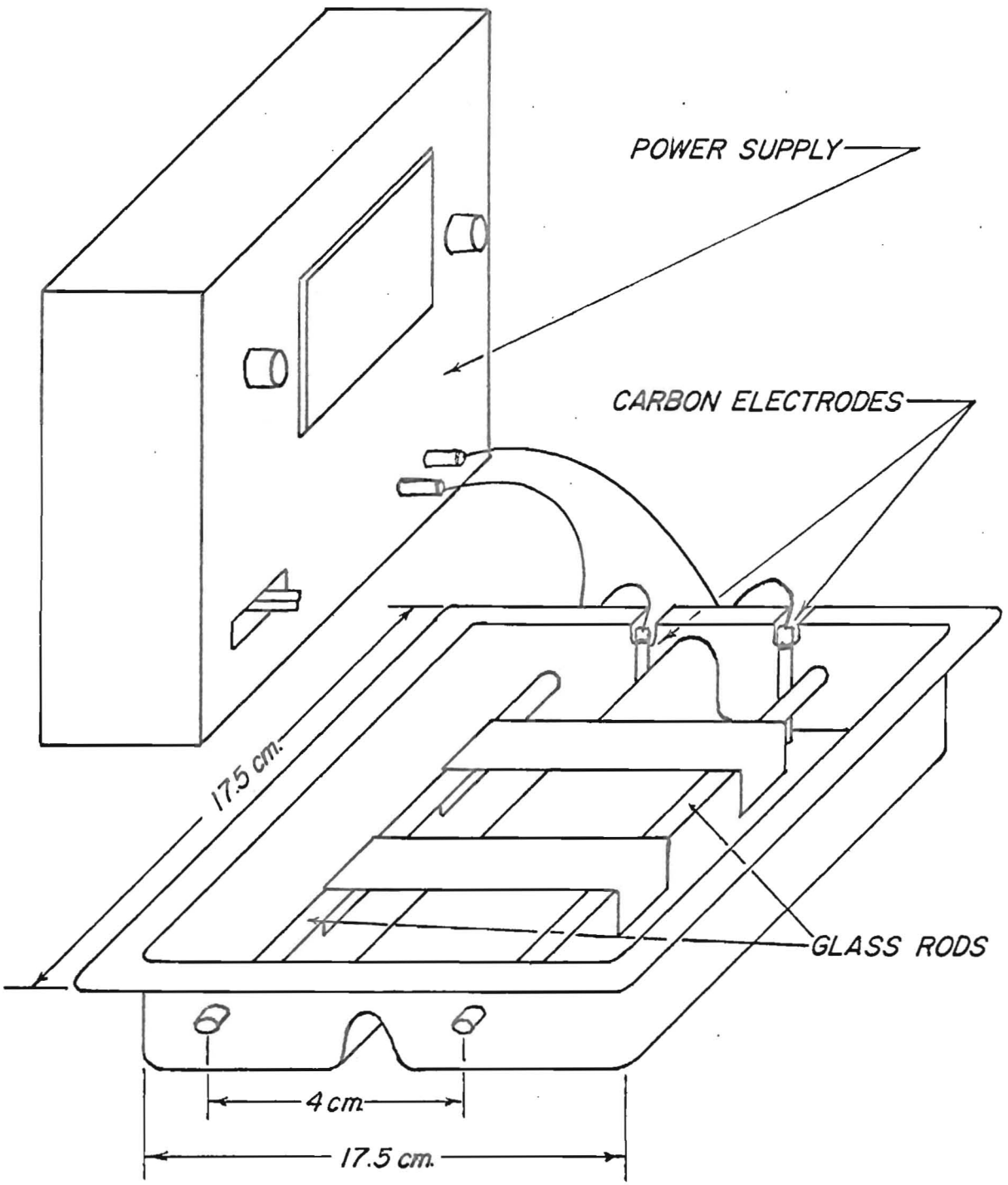
Electrophoresis procedures

Before electrophoresis could be done, an electrophoresis cell had to be constructed (Fig. 1). For the construction, a styrofoam container (17.5 cm x 17.5 cm) that was partitioned in the center was used. Two glass rods were inserted into one end 2 cm on either side of the middle partition and extended through the opposite end. The glass rods served as supports for the agar film strips during electrophoresis. For the electrodes, carbon cells were removed from two pen light batteries and coupled to the wire and to the cell with modeling clay. An equal volume of veronal electrophoresis buffer (0.05 ionic strength, pH 8.6) was poured into each side of the cell and the unit was covered with a glass plate before use.

Agar gel electrophoresis was performed on Dupont photographic film base according to the procedure of Cawley (1965). The agar solution was dissolved in the electrophoresis buffer and pipetted in a thin layer onto the film strips that were cut into desired lengths. The surface of the film strips facing the inside of the roll was always placed upward. After the agar hardened, the strips were placed in position in the electrophoresis cell.

Strips of Whatman #1 filter paper were cut (2 mm x 15 mm) and saturated with the test serum. After shaking off the excess serum, the filter paper was placed on the agar strips at right angles to the length of the strip. To observe the progress of electrophoresis, a drop of brom phenol blue was placed on the agar over the glass rod nearest the negative electrode. The current was passed through the strips and adjusted to 5-8 milliamperes per strip. Current was applied until the marker dye reached

Figure 1: Illustration of electrophoretic cell used in study.


POWER SUPPLY
CARBON ELECTRODES
17.5 cm.
GLASS RODS
4 cm
17.5 cm.

the opposite glass rod (4 cm). The number of volts and the time of migration was not considered important unless the voltage exceeded 250 volts. After completion of electrophoresis, the current was turned off and the strips fixed in 5% methanol-acetic acid for 20 minutes. After fixation, the strips were dried in a drying oven at 60 C.

The strips were dried, followed by staining in Ponceau S solution for 5 minutes and decolorizing in 3 changes of 5% acetic acid. The strips were dried at room temperature and scanned on a Beckman Spinco analytrol. The slit width of the analytrol was set at 0.5 mm. Scanning was performed with a B-2 cam and 500 millimicron interference filters. The slit length was 1 cm.

Immunelectrophoresis was performed on agar film strips essentially as described by Cawley (1965) with the following modifications. For the electrophoresis, serum was applied on discs of Whatman #1 filter paper punched out with a paper punch. Electrophoresis took place as previously described, the current turned off, and the strips removed from the electrophoresis cell. Excess length was cut from each end of the strips and troughs were cut into the agar parallel to the separated antigen. The strips were placed in moisture chambers and the troughs filled with homologous antiserum. The strips were incubated at room temperature until maximum precipitation occurred (usually 24-48 hrs).

Excess antigen and antibody were removed from the strips by soaking for several days in 2% saline solution. The salts were removed by soaking the strips in distilled water for several hours. The strips were dried at 60 C in a drying oven and stained with Ponceau S solution as previously described.

The film used for a base was clear 35 mm motion picture film base that was obtained from the E. I. Du Pont de Nemours and Company, Photo Products Division, Wilmington 98, Delaware.

RESULTS

Distribution of carbon particles injected into the dorsal lymph sac

In order to study the distribution of material when injected into the dorsal lymph sac of Rana pipiens, the following procedure was carried out.

Duplicate frogs were injected with 0.5 ml of a 1:2 dilution of carbon particles (India ink) via the dorsal lymph sac and maintained at room temperature. After 2 hrs, the animals were killed by pithing and the internal organs exposed by a midventral incision. The lymph nodes in the upper thoracic region were exposed by removing the sternum.

The lymph nodes, spleen, liver and other organs containing phagocytic cells were black as a result of the injected particles. Two lymph nodes, seen in the upper thoracic region, were kidney bean shaped, flattened and approximately 2-3 mm in length.

Sections taken from normal uninjected frogs were stained with hematoxylin and eosin solutions to observe the histology of frog lymphoid tissue and were found to have a structure similar to that found in mammals.

Effect of temperature on the uptake of carbon particles

As a preliminary study, the effect of a cold temperature on the uptake of carbon particles was investigated to determine the feasibility of a later investigation of the fate of a phagocytized bacterial antigen as affected by temperature. Six frogs were injected with 0.5 ml of a 1:2 dilution of carbon particles and maintained at 5 C along with six uninjected animals. At 2 hrs, 24 hrs, and one week after the injection, frogs from the injected and uninjected groups were sacrificed, their livers and spleens excised, and

placed in 95% alcohol for fixation. The tissues were embedded, sectioned and stained with eosin solution.

Carbon particles were found to have been taken up by the phagocytic cells of the spleens as soon as 2 hrs after the injection as indicated by the presence of black India ink particles within the cells. After one week, there was a significant increase in the number of cells containing carbon particles. These results indicated that although distribution of colloidal material through the lymphatic system of the frog with subsequent phagocytic uptake occurred more slowly in the cold, it did, however, occur. Therefore, it could be assumed that a bacterial antigen would be similarly handled by frogs maintained at reduced temperatures.

Effect of temperature on circulating antibody after antigenic stimulation

To study the effect of ambient temperature on the immune response, frogs were divided into two groups and adapted to 5 C and 25 C. The two groups were immunized via the dorsal lymph sac with F. tularensis, strain Jap, (3×10^{10} organisms per ml). Six 0.6 ml injections were given with the first three occurring on alternate days and the last three at seven day intervals. Beginning on the tenth day after the initial injection, two frogs were sacrificed from each group once a week for three weeks. At each period, the serum was assayed for circulating antibody. The group at room temperature had no detectable antibody at one week, 1:40 at 2 weeks, and 1:80 at 3 weeks. The animals at 5 C produced no detectable antibody during the three week period. The results demonstrated the inhibitory effect of temperature upon the antibody response.

Immunofluorescent studies

To study the mechanism of inhibition of the immune response at

refrigerator temperatures more definitively, the cellular localization of antibody was investigated with a fluorescent conjugated bacterial antigen (F. tularensis). Before the fluorescent conjugate was used on tissue sections, its specificity was determined as follows.

Smears of E. coli and F. tularensis were fixed in 95% ethanol and rinsed briefly in buffered saline. The organisms were reacted with untagged F. tularensis anti serum (1:320) for 30 minutes at room temperature and then rinsed in three changes of buffered saline for 10 minutes. In the second stage of the reaction, the organisms were reacted with fluorescent conjugated antigen for 30 minutes and rinsed in three changes of buffered saline for 10 minutes. The slides were mounted in buffered glycerol and examined under ultra violet microscopy. The slide of F. tularensis fluoresced a bright apple green color in contrast to the slide of E. coli which showed only slight autofluorescence. The results indicated a specific immune reaction for F. tularensis and its homologous antibody.

The following experiment was carried out to determine if antibodies were actually being synthesized at refrigerator temperatures. Frogs adapted to 5 C and 25 C respectively were immunized with formalin killed F. tularensis (1×10^9 organisms per ml) via the dorsal lymph sac. The vaccine was mixed with equal parts of Freund's adjuvant and injected in 1 ml amounts at seven day intervals for four weeks. Beginning at one week after the initial injection, duplicate frogs were sacrificed before each injection and also one week after the final injection. Uninjected animals were sacrificed at the same periods. Sera from each pair were pooled and assayed for agglutinating antibody.

For the detection of cellular antibody, splenic sections were subjected to a 1:2 dilution of fluorescent conjugated antigen for 30 minutes, washed in three changes of buffered saline for 10 minutes and mounted in buffered glycerol. The sections were examined under ultra violet microscopy and compared with identically treated sections from normal uninjected frogs.

One week after the initial injection, frogs at 25 C demonstrated a circulating titer of 1:20. In contrast, frogs at 5 C showed no detectable antibody at this period. At the cellular level, no antibody containing cells were seen in sections from either group.

At two weeks, a titer of 1:40 was detected in the group at 25 C in contrast to the animals at 5 C which demonstrated no detectable titer. On examination of splenic sections, the group at 25 C demonstrated an occasional cell giving the characteristic apple green fluorescence in the cytoplasm (Plates 1, 2, and 3). The cells contained a single large nucleus and usually resembled a lymphocyte or a plasma cell in their morphology. The fluorescence was homogenous and was restricted to the cytoplasm of the cells. In contrast, cells from frogs at 5 C resembled the uninjected control in that slight autofluorescence was seen.

Three weeks later, frogs at 25 C demonstrated a titer of 1:80. On the other hand, frogs at 5 C still gave no detectable antibody titer. Sections from animals at 25 C showed a significant increase in the number of antibody containing cells that correlated with the circulating titer. Sections from frogs at 5 C again resembled the normal control in that slight autofluorescence was observed. Autofluorescence was

Plate 1: Photomicrograph of splenic section from a frog immunized and kept at room temperature showing typical antibody producing cell using immunofluorescent technique (1000 x).

Plate 2: Splenic section from frog injected and maintained at 5° C showing no antibody producing cells (1000 x).

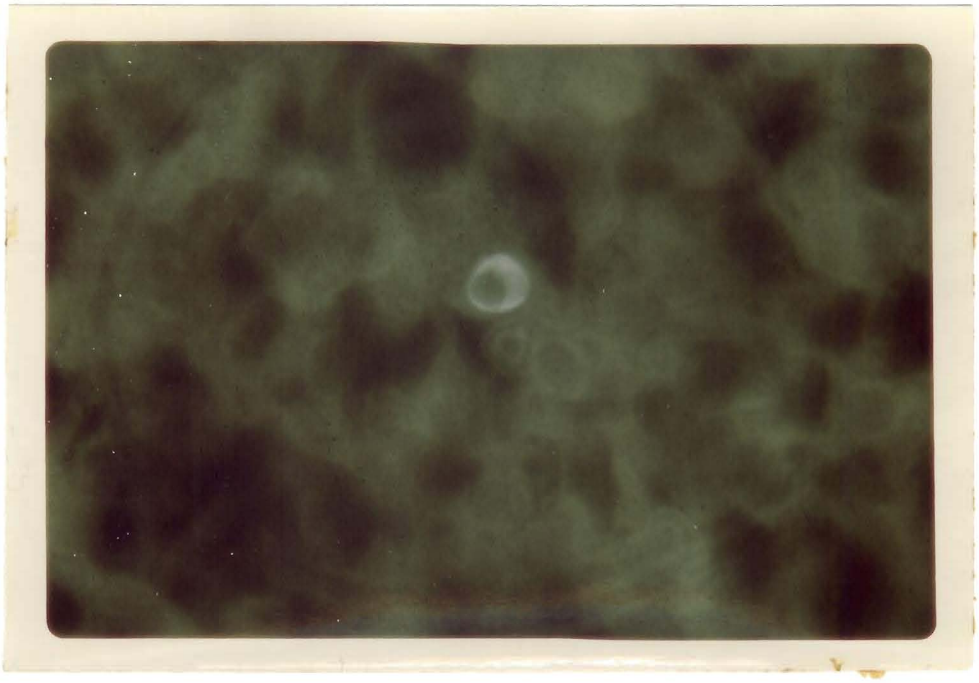
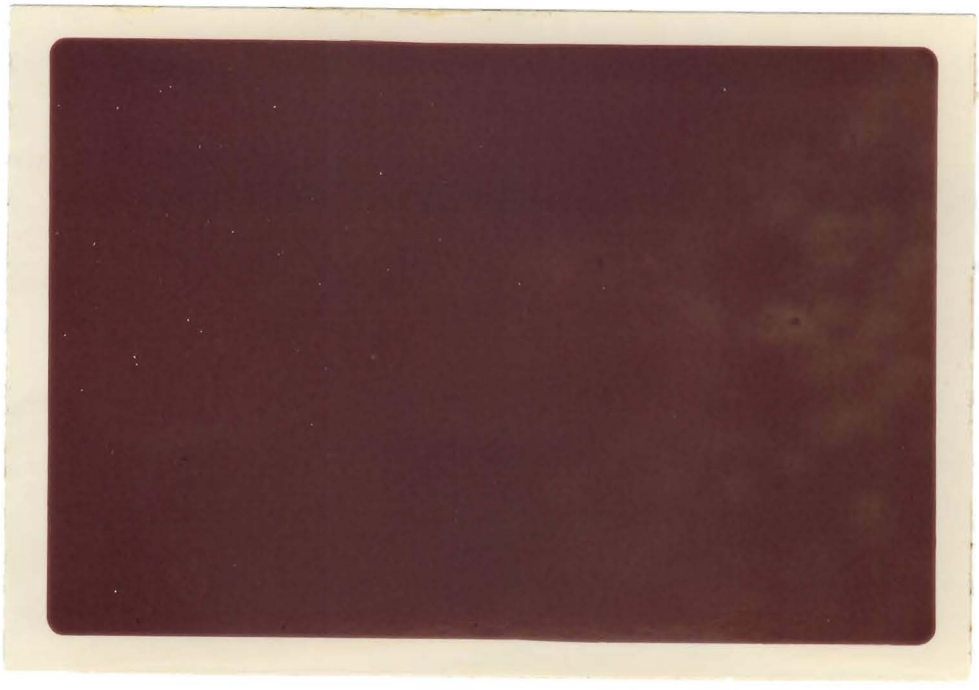


Plate 3: Splenic section of a normal uninjected control frog tested by immunofluorescence (1000 x).



occasionally a problem in the interpretations of the slides, but definite differences could consistently be seen and reproduced between the two groups.

Four weeks after the initial injection, a titer of 1:160 was seen in the frogs at 25 C and, as before, no titer was detected in the group at 5 C. The number of antibody forming cells did not appear to increase markedly in the sections from the group at 25 C in comparison to those seen from this group at three weeks. As for the sections from the animals at 5 C, again only slight autofluorescence was observed (Table 1).

Although not indicated in Table 1, a titer of 1:320 was observed in the group at 25 C one week after the final injection. Sections from the group at 25 C demonstrated several antibody producing cells correlating with the circulating titer although the number of fluorescing cells from this group did not increase to a great extent over those seen at four weeks. Sections from the group at 5 C again contrasted sharply with the frogs at 25 C in that only slight autofluorescence was observed. Thus, it seemed detectable amounts of antibody were not synthesized at 5 C as shown by the lack of antibody containing cells and negative circulating titers.

To prove the specificity of the immunofluorescent reaction, a blocking control was set up. Splenic sections demonstrating fluorescing cells were first reacted with untagged F. tularensis antigen. After washing the sections in buffered saline, the sections were subjected to the tagged antigen. The slides were washed and mounted as before. The cells showed a definite decrease in the intensity of the green fluorescence as compared with the actual test for antibody containing cells.

Table 1: Antibody producing cells in relation to circulating titer.

++++ 10-12 antibody producing cells per splenic section.

+++ 8-10

++ 3-5

- 0

TIME WEEKS	TITER		antibody producing cells	
	warm injected	cold injected	warm injected	cold injected
1	1:20	—	—	—
2	1:40	—	+	—
3	1:80	—	+++	—
4	1:160	—	+++/++++	—

This decrease in the intensity of the green fluorescence was interpreted as a true blocking reaction indicating the suspected cells were antibody producing cells.

Because antibody does not appear to be synthesized in large amounts at refrigerator temperatures, an experiment was carried out to determine if a bacterial antigen is actually degraded by the phagocytic cells in order to relate this to the lack of detectable antibody at the colder temperature.

To study the breakdown of bacterial antigen within cells, a fluorescent conjugate was prepared against F. tularensis antisera. The specificity of the conjugate was first determined by incubating smears of E. coli and F. tularensis with the conjugated globulin, washing with buffered saline, and mounting with buffered glycerol as previously described. The slides of F. tularensis fluoresced a bright apple green color in contrast to the smears of E. coli which demonstrated slight autofluorescence. The green fluorescence observed on the slide of F. tularensis and the lack of it on the smear of E. coli was interpreted as a specific immune reaction. For the detection of F. tularensis in tissues, the conjugate was adsorbed with rat liver tissue powder as previously described.

For the test, eighteen frogs were adapted to 5 C and ten frogs were adapted to 25 C. Fifteen of the cold adapted frogs and all of the frogs at room temperature were given a single injection of 1 ml of live F. tularensis (5×10^6 organisms per ml) via the dorsal lymph sac. The three remaining cold adapted frogs were injected with 1 ml of sterile saline. Two antigenically stimulated frogs from each group were sacrificed

at 2 hrs, 24 hrs, one week, two weeks, and three weeks after the injection. For a normal control, one saline injected animal was sacrificed at 24 hrs, two weeks and three weeks. The spleens were excised, fixed and embedded as previously described. Additional tests for the cellular localization of antibody and for the presence of circulating antibody were also done at the latter time periods.

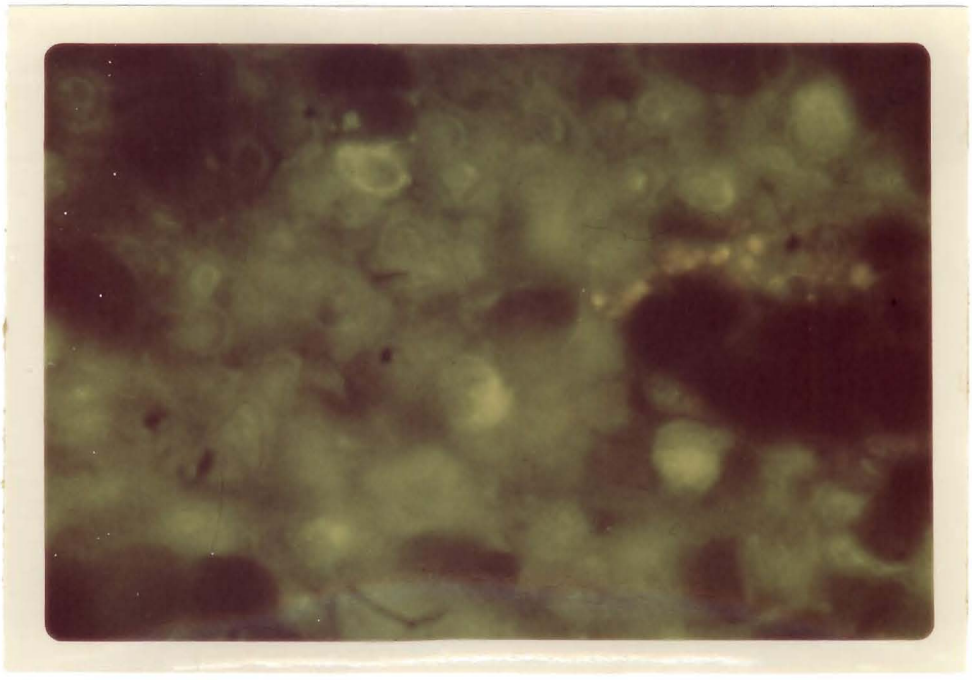
Two hours after the injection, sections from frogs at 5 C demonstrated numerous cells containing particulate bacterial cells that fluoresced a bright apple green color (Plate 4). Sections from frogs at 25 C were seen to have a comparable number of phagocytic cells containing particulate organisms (Plate 4). The two groups of frogs appeared to have taken up the organisms at approximately the same rate and neither group seemed to have degraded the organisms to any great extent as indicated by their particulate form and the apparent lack of lysis.

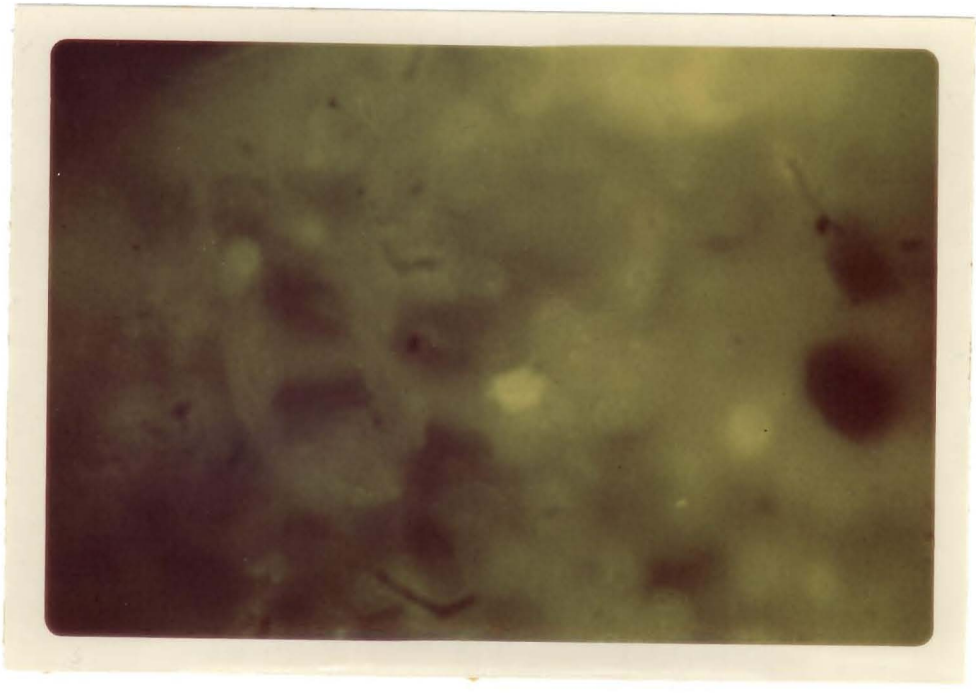
Twenty-four hours after the injection there were marked differences between the two groups. The phagocytized bacteria in cells from the group at 25 C were undergoing the first stages of degradation as evidenced by their more swollen appearance and diffuse fluorescence (Plate 5). The ingested organisms in the cells from the frogs at 5 C did not exhibit similar changes in morphology or intensity of fluorescence (Plate 6).

One week after the injection, sections from frogs at 25 C showed no detectable organisms within the phagocytic cells indicating their degradation was complete. In contrast, sections from frogs at 5 C still showed numerous cells containing intact organisms. The ingested organisms were slightly swollen in comparison to earlier observations but their cell

Plate 4: Section treated with fluorescent antibody conjugate showing particulate bacterial antigen in both cold and warm injected frogs 2 hrs after injection (1000 x).

Plate 5: Splenic section from a frog maintained at room temperature 24 hrs after injection. Organisms appear to be swollen which is perhaps indicative of onset of lysis (1000 x).





walls still appeared to be intact (Plate 7). This was the first indication that degradation was beginning in the cells at 5 C.

After two weeks, no organisms were detected within the cells of either group indicating degradation of the antigen was not only complete at 25 C, but also at 5 C. At this time, sections from the two groups were tested for the presence of cellular antibody. No antibody containing cells were observed in either group as indicated by the lack of specific fluorescence. These results compared favorably with the lack of a detectable antibody titer in either group (Table 2).

Although not presented in Table 2, again no organisms were seen within the cells of either group three weeks after the injection as shown by the lack of specific fluorescence within the cells. Also, no circulating antibody was detected in either group. The absence of a circulating titer and of antibody producing cells was probably due to the lack of sufficient antigenic stimulus from the single injection of organisms.

To substantiate the previous studies that specific antibody was not detected in the blood of frogs injected and maintained at 5 C, the blood samples were pooled and tested by indirect immunofluorescence. For this purpose, a fluorescent conjugate was prepared by injecting a normal young adult rabbit with frog serum and tagging the anti-frog globulin with fluorescein isothiocyanate. Before the conjugate could be used for the test, the correct dilution of serum was determined. Serial dilutions of normal serum and F. tularensis anti serum were made and incubated with smears of E. coli and F. tularensis, washed in buffered saline and then incubated with the conjugated anti-frog serum. The slides were washed in

Table 2: Rate of antigen disappearance from frog lymphoid tissue as influenced by temperature.

++++ maximum specific fluorescence
+++ reduced
++ slight
- no

TIME	WARM INJECTED	COLD INJECTED	SALINE INJECTED
2 hr.	++++	++++	—
24 hr.	++	+++	—
1 week	—	++	—
2 week	—	—	—

buffered saline as before and mounted in buffered glycerol.

The preliminary test showed that a dilution of 1:20 gave a bright apple green fluorescence with F. tularensis while giving a weak autofluorescence with the smear of E. coli. This dilution was considered to be optimum for the actual test due to the specificity of the reaction described.

Pooled sera from frogs immunized at 5 C were diluted 1:20 as were F. tularensis immune sera and normal sera. The sera was reacted with smears of F. tularensis as before. The sera from frogs at 5 C exhibited fluorescence of about the same intensity as that of normal sera. The organisms gave a weak autofluorescence indicating the absence of specific antibody in the sera from animals at 5 C. The specificity of the immune reaction was also demonstrated by the appearance of bright apple green fluorescence of the organisms incubated with immune sera. Thus, by using a more specific technique, it was again substantiated that circulating antibody was not present in frogs injected and maintained at 5 C. These results, along with the tube agglutination tests performed before, reinforce the cumulative evidence that circulating antibody was not produced in measurable amounts at refrigerator temperatures.

Electrophoresis

To study the serum immunoglobulins in frog serum, agar gel electrophoresis was performed on frog serum and human serum. Frog serum was found to have six protein components compared with five to six types normally found in human serum. There were globulins in frog serum that migrated at a rate similar to that of human gamma globulin which is

Figure 2: Electrophoretic scans of human and frog serum.
—o—o—frog serum
————human serum

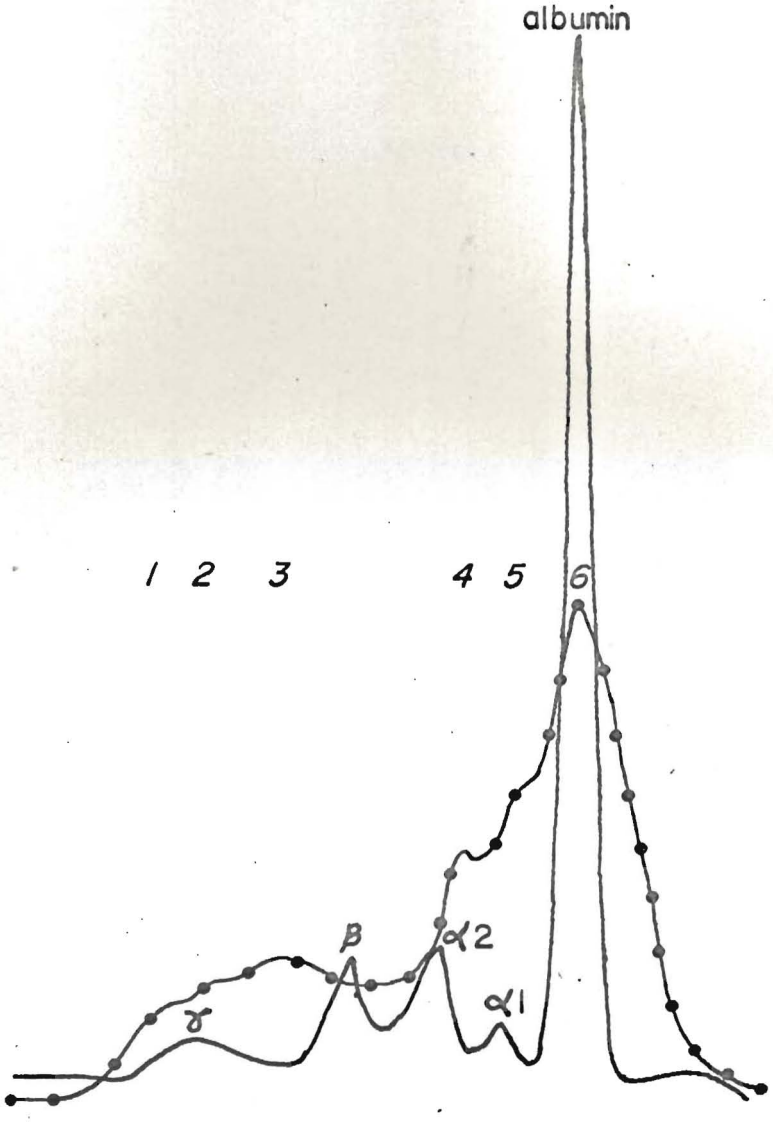
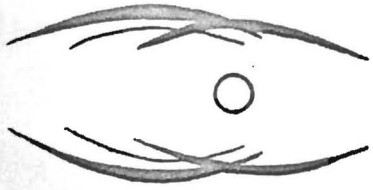
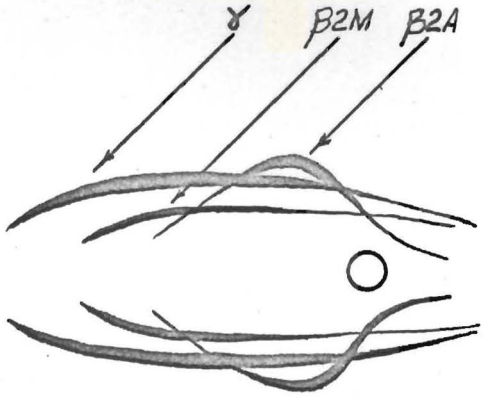

albumin
1 2 3
4 5 6
γ
β
α2
α1

Figure 3: Comparative immunoelectrophoretic studies of the gamma globulins of human and frog serum.

Figure 4: Three major immunoglobulins in human serum.




γ
β2M
β2A

where the immunoglobulins are found (Fig. 2). From these results, it appears that a gamma globulin fraction exists in frog serum that may be comparable to human gamma globulin. To clarify this, immunoelectrophoresis was employed.

Precipitin bands were found in the gamma region in frog serum that were similar to those normally found in human serum (Fig. 3 and 4). The number of precipitin bands in the gamma region, their approximate rates of immunodiffusion, and their antigenic characteristics were comparable to those found in human serum. From these results, it appears that frog serum contained globulins as complex as the immunoglobulins in human serum, at least on a qualitative basis.

DISCUSSION

The results presented in this study indicate that Rana pipiens has a functioning clearance mechanism capable of removing particulate material from the blood even at refrigerator temperatures. This was demonstrated by noting the presence of carbon particles within the phagocytic cells in sections from frogs injected and maintained at 5 C.

A definite lymphatic system was observed in the frogs, particularly in the upper thoracic region. Two lymph nodes, noticably black because of their uptake of colloidal carbon, were located on either side of the esophagus just above the heart. Their histology, as studied with hematoxylin and eosin stains, was generally comparable to that found in nodes of higher forms consisting of a characteristic lymphoid reticulum. The discovery of a lymphatic system was significant in view of the fact Kent, Evans and Attleberger (1964) questioned the presence of typical lymph nodes in Bufo marinus, the marine toad, although they noted uptake of carbon in lymphatic tissues. Kent et al. (1964) found the nodes of Bufo marinus were anatomically similar to those seen in mammals confirming the results obtained here.

Hildeman and Cooper (1963), by more extensive investigations, found that lower vertebrates have a less differentiated lymphomyeloid system than do mammals. Although the extent of the lymphatic system in Rana pipiens was not investigated here, a less elaborate system could be a factor influencing the effeciency of phagocytosis and degradation of an antigen.

Even though it is apparent that the phagocytic cells of the frog function at lower temperatures in taking up foreign material, it is not known for certain whether the immunologically competent cells can synthesize antibody at these temperatures. Bisset (1947a) claimed that antibody formation in amphibia is restricted by lower ambient temperatures. In later experiments, Bisset (1949-50) injected ACTH into frogs at room temperature and transferred the animals to the refrigerator before immunizing them with a killed bacterial antigen. He found circulating antibody in the animals and concluded from these results the lack of antibody at lower temperatures was due to the inhibition of adrenocortical hormone and that this hormone was necessary for antibody release. Krueger and Twedt (1963) extended Bisset's (1949-50) findings by incubating antigenically stimulated splenic cells in vitro at 4 C and then raising the temperature to 26 C. The cells showed no antibody at 4 C, but when the incubation temperature was raised antibody was detected. Krueger and Twedt (1963) also postulated that the release of antibody must be affected by the lower temperatures rather than synthesis. On the other hand, Maung (1963) and Alcock (1965) immunized amphibia, maintained them at cold temperatures, administered cortisone but did not detect any circulating antibody. Thus, the lack of detectable antibody in immunized amphibia at lower temperatures in the presence of ACTH indicates an inhibitory mechanism other than the absence of the hormone.

The studies reported here are reinforced by Maung's (1963) and Alcock's (1965) findings that antibody does not appear to be synthesized at refrigerator temperatures in poikilothermic vertebrates. This is

illustrated by the absence of circulating antibody as well as the absence of visible splenic cells containing antibody as shown by the lack of fluorescence in sections taken from frogs at 5 C and by negative tube agglutination tests. The number of antibody containing cells from frogs at 25 C increased as the circulating antibody titer increased. The results presented here indicate the synthesis of antibody at lower temperatures in Rana pipiens is inhibited at least to the extent of not being measurable by these techniques.

It is interesting to speculate that one of the major reasons why antibody cannot be produced in detectable amounts at lower temperatures is due to the inhibition of cellular proliferation. Chute (1964) stated that cell division is inhibited in hibernating animals. It is generally believed today that cell division must take place before antibody can appear in measurable quantities (Dutton, 1967). Urso and Makinodon (1963) found by following the incorporation of tritiated thymidine in combination with immunofluorescent techniques that immunologically competent cells divide at a significantly higher rate than immunologically incompetent cells. The true mechanism for antibody synthesis is still not known but, as stated before, it is generally agreed that cellular proliferation must occur in the process of antibody formation. It is still an open question as to whether cell division occurs at lower temperatures.

In relation to the problem of inhibition of antibody synthesis at lower temperatures, it was found that a bacterial antigen (F. tularensis) was not only taken up by the phagocytic cells at 5 C but degraded within two weeks. To illustrate the possibility of a relationship between the

clearance of an antigen and the synthesis of antibody, Condie, Pih and Monson (1964) compared the hagfish, the frog and the rabbit in their ability to remove a soluble antigen from the blood and to form specific antibody. They injected serum albumin labeled with I^{131} into the respective hosts and noted the uptake of the labeled materials by the phagocytes and the appearance of circulating antibody. They found the hagfish did not remove the material from the blood and did not form antibody. The frog also failed to clear the albumin from the blood or form any detectable antibody. In contrast, the rabbit quickly removed the albumin from the blood and formed specific precipitating antibody. Alcock (1965) found that Rana temporaria did remove serum albumin from the circulation slowly but produced no precipitating antibody. In the results given here, circulating antibody did not appear at an ambient temperature of 5 C even though the bacterial antigen was taken up by the phagocytes and degraded. Therefore, it appears, at least in these studies, that the lack of degradation of the antigen was not a factor in the inhibition of the appearance of antibody at lower temperatures.

To study the immune response of Rana pipiens more closely, the serum was analyzed by electrophoresis and immunoelectrophoresis. Serum electrophoresis demonstrated six protein components which agreed closely with Alcock's (1965) studies on Rana temporaria. Alcock (1965) found that three components were elevated upon immunization, two of which demonstrated antibody activity. Her studies showed a significant increase in the component having an electrophoretic mobility similar to human gamma globulin, the region containing the immunoglobulins. A similar component

migrating like human gamma globulin was seen on electrophoretic scans performed in this study.

To study this region more closely, immunoelectrophoresis was employed. Bands were seen in the gamma region that behaved much like immunoglobulins in human serum. It appeared the precipitin bands found in the gamma region had approximately the same mobility and rates of immunodiffusion as the immunoglobulins that have been characterized in human serum. These results are supported by Marchalonis and Edelman (1966) who found by immunoelectrophoresis and other studies that bullfrog serum contained two major immunoglobulins, a macroglobulin and a 6.7S globulin that resembled human gamma M(19S) and gamma G(7S) in their electrophoretic mobility and antigenic relationships. One of the major differences they found was that bullfrog serum contained up to 40% of the gamma M fraction (Macroglobulin) in contrast to human serum which normally contained no more than 5-10% of the heavy globulin fraction. Thus, Rana pipiens appears to have globulins as antigenically complex as the immunoglobulins in human serum but may differ on a quantitative basis.

Collectively, the results illustrate the presence of an acquired immune mechanism in Rana pipiens that is intermediate to mammals and lower forms such as the sea lamprey and is inhibited from forming large amounts of antibody at lower temperatures.

SUMMARY

The influence of temperature upon the mechanism of antibody formation in Rana pipiens was studied with the following results. It was found that frogs immunized with a bacterial antigen and maintained at 5 C failed to demonstrate detectable antibody producing cells and circulating antibody in contrast to animals injected and kept at room temperature which demonstrated significant amounts of circulating antibody and several antibody producing cells.

The fate of the bacterial antigen at a colder temperature was studied by observing the phagocytosis and subsequent breakdown of the organisms in order to relate this as a possible factor in the lack of detectable antibody in the cold. It was found the organisms were taken up by the phagocytic cells in the cold and eventually broken down leading to the conclusion that inhibition of antibody formation must occur in some later step in the synthesis.

Rana pipiens serum was analyzed and compared with human serum by electrophoresis and immunoelectrophoresis. Three bands were seen in the gamma region of frog serum that were comparable to the three immunoglobulins in human serum.

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