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A METHOD FOR STUDYING THE PRODUCTION OF IMMUNOGLOBULINS BY SINGLE CELLS

By

Frank S. Gold, B.Sc.

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Faculty of Medicine,
The University of Ottawa,
Ottawa, Canada.

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Supervisor

Thesis Examiner

Approved for the Faculty of Graduate Studies

Chairman of Examining Board

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List Of Abbreviations

Abbreviation	Description
ml	milliliter
g	gram
mg	milligram
µg	microgram
G	unit of gravity
SRBC	sheep red blood cells
EMEM	Eagle's minimal essential medium
DEAE	diethylaminoethyl
RS	rat sera
RG	rat globulin (ammonium sulphate precipitated rat sera)
IgG	Immunoglobulin type G
IgA	Immunoglobulin type A
IgM	Immunoglobulin type M
C	plaque containing single cell
CARG	commercial rabbit anti-rat gamma globulin serum
ARG	rabbit anti-rat globulin serum
CARS	commercial anti-rat serum
ARIgG	rabbit anti-rat IgG

ABSTRACT

It is generally accepted that there are two types of cells which produce antibody: the plasma cell and a large lymphoid cell. Also it has recently been demonstrated that a single cell can produce antibody to more than one antigen (Attardi et al. 1964 a, b, c, d, e, Trentin et al. 1966). However, little work has been done on determining whether a single cell can produce different types of immunoglobulin. Nossal (1964) did a limited study in this field and he concluded that cells can produce both 19S and 7S antibody at the same time during the animal's "switchover" from 19S to 7S antibody production. The present author has tried to develop a method of detecting immunoglobulin production in single cells using Jerne's plaque technique (Jerne et al. 1963) as a basis for isolating single antibody producing cells. The plaques were removed and placed into the central well of an Ouchterlony type gel diffusion plate, on the agar surface of an Oudin type gel diffusion tube, or in a well of a slide prepared for agar electrophoresis. After a suitable incubation period, the plaques were removed, dried, and later stained. The type of protein(s) released by the cell was then detected by precipitation in the agar gel using specially prepared antisera. The results indicate that a single cell can produce more than one type of immunoglobulin at the same time.

A Review Of The Literature

Types Of Cells Involved In The Production Of Antibody

In reviewing the literature it has been found that there are two types of cells associated with the production of antibody: lymphocytes and plasma cells. Ehrich and Harris (1942) injected antigens subcutaneously into rabbit's feet; they then studied the formation of antibodies in the popliteal lymph node in order to compare them with the output of lymphocytes from the lymph node and the changes in the lymph node. They found that antibodies appeared in the efferent lymph two to four days after inoculation and reached its highest titer after six days. This rise in antibody titer was preceded by a sharp rise in output of lymphocytes through the efferent lymph while in the lymph node itself there was lymphatic hyperplasia preceded by an infiltration of granulocytes and monocytes. They felt that since the tissue response was chiefly lymphocytic in nature, it indicated that lymphocytes were concerned with antibody formation.

Dougherty et al. (1944) removed the lymph nodes and thymi from mice which had been previously injected with sheep erythrocytes. A comparison of the titer of antibody within these tissues to the titer of the sera from the animals revealed that the lymphocyte tissue extracts had higher titers than that of the sera. Although they did not show that the lymphocytes actually produced antibody they did demonstrate that antibody activity was greatest in lymphoid tissue.

Harris et al. (1945) repeated Dougherty's work and they found that

the lymphocyte cell extract had a higher titer than the lymph plasma from which the lymphocytes had been initially separated. The difference in titer between the lymphocytes and the lymph plasma was most significant at the time of the greatest rate of increase of antibody titer in the whole lymph node. They concluded from these experiments that either the lymphocytes produce antibody or that they take it up from the surrounding lymph plasma. To demonstrate whether the lymphocytes actually absorb or adsorb antibody from the lymph plasma they incubated lymphocytes containing antibody in lymph plasma containing a different antibody. Antibody passed from the cells to the surrounding plasma until an approximate equilibrium existed between the lymphocytes and the plasma; however, no antibody passed into the cells from the plasma. On the basis of these experiments they felt that lymphocytes were instrumental in the formation of antibody.

Kass (1945) used rabbit antiserum to purified human gamma globulin to show that extracts of lymphocytes of human origin react specifically with the specific rabbit antiserum. He suggested on the basis of these serological experiments that the formation of gamma globulin occurred in lymphocytes.

Harris et al. (1948) used a histochemical technique, pyronine stain for identification of ribonucleic acid, to determine the type of cell actively engaged in protein synthesis. His results showed that in lymph nodes which were actively producing antibody a wide range of lymphocytes, although primarily the immature forms, contained cytoplasmic granules and nucleoli which were stained with pyronine.

Similar stained granules and nucleoli were found in transitional forms between reticulum cells and lymphocytes, but in no other cell types. He concluded that lymphocytes were concerned with antibody production because they were actively synthesizing protein.

Other investigators felt that the plasma cell was chiefly responsible for antibody production. Bjorneboe and Gormsen (1943, 1947) immunized rabbits with a series of different antigens to ensure an intense antibody response. They found that the spleen enlarged greatly after immunization and that splenomegally paralleled a plasma cell proliferation. Furthermore, immunization produced massive plasma cell infiltration (90%) and slight lymphocyte infiltration (10%) in the adipose tissue of the renal sinus. Extracts of this tissue revealed that it contained high antibody titer. They hypothesized that antibodies were produced by plasma cells.

Fagreus (1947, 1948a, 1948b) attempted to demonstrate that plasma cells produce antibody by employing tissue culture techniques. After injecting rabbits with horse serum intravenously she found that from five to eight days after injection, at the height of the sera titer, there was an increase in the number of plasma cells in the red pulp of the spleen. The plasma cells originated apparently from reticulum cells going through a series of developmental changes: "transitional cell ⟶ immature plasma cell ⟶ mature plasma cell". From her <u>in vitro</u> studies of excised splenic tissue she found that the red pulp, rich in plasma cells, produced considerably more antibody than did the white pulp of the spleen which was rich in lymphocytes,

but devoid of plasma cells. Also, immature plasma cells seemed to produce more antibody than the transitional cell or the mature plasma cell. Furthermore, injected antigen accumulated in those areas in which a proliferation of plasma cells subsequently occured. She concluded on the basis of her experiments that antibody was "formed by cells of the R.E.S., passing through a chain of development the final link of which is the mature plasma cell".

Fagreus' work stimulated a number of other people to try and substantiate or disprove her findings. Reiss et al. (1950) demonstrated that certain lymphoid cells, found in lymph nodes producing antibody to a bacterial antigen had the ability to agglutinate the specific bacteria in vitro. These lymphoid cells were identified as belonging to the plasma cell series, whereas small lymphocytes did not exhibit this phenomenon. From these observations they concluded that the plasma cell rather than the lymphocyte was responsible for producing antibody.

Keuning et al. (1950) retested the role of the lymphocyte and the plasma cell in antibody production. Using a technique similar to Fagreus' in vitro synthesis of antibody from splenic tissue, they found that the red pulp of the spleen produced the greatest amount of antibody, and that a large immature lymphoid cell was responsible for the production of antibody. They concluded that the reticular and immature plasma cells are instrumental in antibody production and that large lymphoid cells were also capable of synthesizing antibody. However, immature lymphoid cells and small lymphocytes were not capable of antibody production. From the results of their experiments Keuning and co-workers

hypothesized that plasmacytogenesis and lymphocytogenesis were in some manner closely related to each other.

Ringertz et al. (1950) studied the response in regional lymph nodes and the spleen of animals which had received subcutaneous injections of various antigens. He found that the general response was a combination of reactions in several cell systems co-operating in a characteristic uniform fashion. They felt that the immature plasma cell was also involved with antibody production; and like others (Keunig et al. 1950) they thought that the immature plasma cell was related in some manner to the immature lymphocyte in the lymph node.

Marshal et al. (1950) found that following intravenous injection of an antigen two types of cellular proliferation took place in the spleen. The initial change was a multiplication of reticular cells leading to plasma cells in the red pulp of the spleen and to lymphocytes in the germinal centers. They deliberately destroyed the lymphocytes in the white pulp of the spleen by nitrogen mustard and by X-radiation; but they did not find a release of antibody in the tissue culture cells as would be expected if the lymphocytes stored or produced antibody. The plasma cells were highly resistant to both of these destructive agents. On the basis of their experiments they assumed that plasma cells were responsible for antibody production.

In 1955 Coons et al. developed a new technique which proved conclusively that plasma cells did in fact produce antibody. This method consisted of a two stage immunological reaction using specific fluorescein-labelled antibody. Frozen sections of spleen tissue from

hyperimmune animals were allowed to react in vitro with the dilute antigen used for immunization. Those areas where the antigen was specifically absorbed were detected by means of a precipitation reaction carried out with fluorescein-labelled antibody. Under a fluorescent (UV) microscope these areas, where an antigen-antibody reaction had taken place, fluoresced yellow-green in colour. They found that antibody was present in groups of plasma cells in the red pulp of the spleen, the medullary areas of the lymph nodes, the submucosa of the ileum and the portal connective tissue of the liver. Small amounts of antibody were present in the lymphoid follicles of the spleen and lymph nodes. Although they thought that the immature plasma cell was primarily responsible for antibody production they did not rule out the fact that the lymphocytes could play a minor role in antibody production.

Askonas et al. (1955) substantiated the claim that the plasma cells were involved in antibody synthesis. In their experiments they found that the capacity of various lymph nodes, based on a unit weight measurement, varied greatly; but that the antibody production of individual lymph nodes varied according to the number of plasma cells found within these nodes.

Ortega et al. (1957) studied the cellular sites of formation of gamma globulin in man by a fluorescent antibody technique. They showed that gamma globulin was formed in the germinal centers of lymphatic nodules and in the cytoplasm of mature and immature plasma cells. They concluded that there were three morphologically distinct categories

of cells which had the ability to synthesize antibody: plasma cells without Russel bodies, plasma cells with Russel bodies, and lymphocytes.

Harris et al. (1958) attempted to prove that the lymphocyte was also responsible for antibody production. After a series of injections of antigens into rabbits' hind feet they removed the popliteal lymph nodes and teased them apart to obtain lymphocytes. They injected these cells intravenously into normal rabbits. Antibody to these antigens appeared in the recipient's serum on the first day of transfer and rose in titer on the second and third days. By the fifth to seventh days, the titer diminished. When damaged cells from non-immunized rabbits were transferred into the normal rabbits, in a different series of experiments, no antibody formed in the recipients. They concluded on the basis of these experiments that lymphocytes which were transferred might possibly contain a mechanism for antibody formation.

Smith (1960) found 19S antibody in neonatal infants, yet there were no plasma cells present. This indicated that another type of cell, perhaps the large lymphocyte mentioned by Harris et al. (1945, 1948, 1958), might be responsible for synthesis of this type of antibody.

Kritzman et al. (1961) found 19S antibody, macroglobulin, in a patient who had lymphocytoid cells.

Zucker-Franklin et al. (1962) used C^{14} - lysine to try and determine whether cells of the lymph node could produce protein. In conjuction with this they used fluorescent antibody to macroglobulin to determine whether the protein(s) produced by the lymphocytes was 19S

type (macroglobulin) antibody. They found that in the lymph nodes of three patients with macroglobulinemia of the Waldenstrom type the lymph tissue was able to synthesize 19S gamma globulin in vitro. Furthermore the cells associated with the 19S antibody were large and medium sized lymphocytes rather than mature lymphocytes or plasma cells. They hypothesized that 19S antibody might also be synthesized by cells belonging to lymphoid cells under normal circumstances, but in the case of macroglobulinemia the medium and large lymphocytes produce the 19S gamma globulin.

Cruchard et al. (1962) found that in some children there was an absence of plasma cells in the lymph nodes, spleen and bone marrow. However, by a fluorescent antibody technique they detected cells resembling transitional cells in splenic tissue which produced 19S gamma globulin.

De Petris et al. (1963) studied the distribution of specific antibody in the cytoplasm of plasma cells from immunized animals. Using an electron microscope and employing ferritin as a marker they found that in the lymph node preparations incubated with crystalline horse ferritin a great number of plasma cells contained ferritin; and that most of it was located in the cisternae of the endoplasmic reticulum. Since they did not study large lymphocytes they did not report finding any ferritin in these cells.

Mellors et al. (1963) carried out a study to verify the types of cells which produce antibody, and also to determine the kind of immunoglobulin produced by each cell. The results of their experiments

are in close agreement with the others previously mentioned (Ortega 1957, Zucker-Franklin 1962, Coons et al. 1955, Coons 1958). They found two closely related families of cells forming immunoglobulin in human tissue: 1) cells in the germinal reticular center and 2) plasma cells. They also showed that Y_A (IgA) type antibodies were formed in the same family of cells as Y_2 (IgG) globulin. They even reported a few instances where YM (IgM) and Y_2 (IgG) globulin were present in the same cell, but the greater majority of immunoglobulin-producing cells contained only one type of globulin.

Bauer et al. (1963) did a study of the primary and secondary antibody response with respect to the sequence of synthesis of 19S (IgM) and 7S (IgG) antibody. They employed a phage neutralization assay combined with ion exchange chromatography to detect the molecular species of antibody. They found that 19S (IgM) antibody was synthesized first and then diminished in titer as 7S (IgG) antibody rose in titer. They postulated from their findings that 19S and 7S antibody were formed in different types of cells. The 7S antibody being formed in plasma cells while the 19S antibody being formed "by short-lived cells, by cells of limited synthetic capacity".

Wellensiek et al. (1964) tried to detect the presence of ferritin, used as an antigen, in antibody-forming cells. Using a fluorescent antibody technique they found intact ferritin molecules in the cytoplasm of various reticular and phagocytic cells in the sinuses of lymph nodes, in hemacytoblasts which, according to their definition, correspond to the transitional cell described by Fagreus (1948a),

and in immature and mature plasmocytes. However, ferritin molecules were not found in lymphocytes which is in agreement with De Petris et al. (1963).

Scheonberg et al. (1964, 1965) found similar results as Bauer et al. (1963). Furthermore, they found 19S antibody present in the serum when there was a preponderance of large mononuclear cells in the spleen; plasma cells were also associated with 7S antibody production. However, they did not rule out the possibility that one cell might produce both 19S and 7S antibody or that a 19S antibody producing cell might be converted to a 7S producing cell. (These two points are discussed in greater detail in the next section, Antibody Production By Single Cells).

Svehag et al. (1964) found that the formation of 19S and 7S antibody differed in a number of respects: antigen dose requirements necessary for induction; kinetics; retention of memory; and sensitivity to X-irradiation. They explained these differences on the basis that different cells produce 19S and 7S antibody. A number of workers have also found that plasma cells and other cells (large lymphocytes) are involved in the production of antibody. (Harris et al. 1966, Peterson et al. 1966, Cunningham et al. 1966, Hannoun et al. 1966, Porter et al. 1968).

Antibody Synthesis By Single Cells

The previous section has attempted to demonstrate that there are two types of cells concerned with antibody production: 1) the plasma cell which produces primarily 7S (IgG) antibody but is also capable of producing 19S (IgM) and 7S (IgA) antibodies (Mellors et al. 1963); 2) A large lymphoid cell producing 19S (IgM) antibody.

There are now two questions which arise from the study of the cellular origins of antibody. First, can a single antibody-producing cell synthesize specific antibodies with distinct activity to two or more different antigens at the same time? Second, can a single antibody-producing cell synthesize both normal (possessing activity) and atypical immunoglobulins at the same time?

A number of investigators have devised quite elaborate techniques to solve the first question, but the second question has not been examined to any great depth. Coons (1958) used his fluorescent antibody technique (Coons et al. 1955) to determine whether single cells produce antibodies with specific activity to two or more different antigens. He injected egg albumin and diphtheria toxoid into the footpad of a rabbit; a month later he reinjected the rabbit with a mixture of the same antigens. He then stained alternate sections of the rabbit's spleen for anti-albumin or anti-diphtheria toxoid, or both. He found that the response to egg albumin was about the same as the response to the diphtheria toxoid. Also the two slides stained for one or the other contained about the same number of cells with antibody in them. When he stained for both antibodies at the same

time he found that the number of visible cells which fluoresced was about double that of either slide. From these observations he concluded that a single cell can generally synthesize only one type of antibody at any one time. However, he did not rule out the possibility that there were a few cells making both antibodies.

Nossal (1958, 1959, 1960) and co-workers (Nossal et al. 1958) carried out a series of experiments to try and solve both questions concerning single cell antibody production. The experiments are based on the specific production of antibody to flagella from strains of Salmonellae. The antibodies which are directed against flagellar antigens has the specific ability to immobilize the Salmonella strain used in the injection. Rats were immunized with flagellar antigen from Salmonella adelaide H^{fg}, and Salmonella typhi H^{a}, by injecting a mixture of equal parts of both antigens into each hind foot. (In later experiments sometimes three or four different strains were used together as antigens instead of just two strains). The animals were sacrificed three days after their tertiary set of injections and their popliteal lymph nodes were removed. The nodes were gently teased apart and the released cells washed to remove free soluble antibody. A slight modification of de Fonbrune's oil chamber method was used to isolate single cells in microdroplets. Basically, the method consisted of putting tiny droplets (10^{-7} - 10^{-6} ml in volume) on a coverslip's surface, and then immersing the droplets in paraffin oil. The coverslip was then inverted on an oil filled chamber. Droplets containing from one to six cells were prepared, but the one cell droplets were

most difficult to obtain as the cells tended to adhere to the sides of the micropipettes. After incubation at 37°C for four hours, the chamber was examined at 100 X magnification with a dark ground field. Then the drops were inoculated with about seven to ten bacteria per drop of either Salmonella adelaide or Salmonella typhi. After 20 minutes the bacteria were examined for motility. A total loss in motility was recorded as inhibition. Any cells which were found to be producing antibody against one type of bacteria were subsequently inoculated with the second strain of bacteria to determine whether the cell was also producing antibody against the second type of Salmonella. However, in later experiments they realized that just testing those cells which completely immobilized the first strain applied gave incomplete results. Therefore, in subsequent tests they examined all cells for production of antibody to each strain of Salmonella used. If motility did not occur with the first strain applied a specific antiserum was used to immobilize the first strain, and then the second strain of Salmonella was tested. Antisera against each serotype showed little cross-reaction with the other. In 1958 Nossal tested 456 single cells for antibody production; of these 33 were active against Salmonella adelaide and 29 against Salmonella typhi. None of these 62 cells immobilized both strains. In 1960 Nossal found 347 single producers but no double producers; and of 351 cells studied from animals immunized with 3 antigens 104 formed antibody against one or the other. No double or triple producers were detected. From these results he concluded that a single cell can produce antibody to

only one antigen.

Makela et al. (1961) immunized rats with a variety of motile bacteria. Five days after their second injections the animals were sacrificed and their lymph node cells were removed. These cells were studied to determine whether bacteria would either adhere to the surface or become immobilized. Their results showed that antibody-producing cells could be positive for immobilization and adherence or positive for adherence and negative for immobilization. This indicated that the cells were producing either anti-H (flagellar) or anti-O (somatic) antibody. However, by this technique they were unable to determine whether the cells were producing both anti-H and anti-O at the same time.

Attardi et al. (1959, 1964a, 1964 b, 1964c, 1964d) used bacteriophages as antigens and assayed antibodies to these bacteriophages in single cell studies. They injected three serologically distinct bacteriophages T_2, T_5 and C into the footpads of rabbits. When the animals were in a hyperimmune state they were bled out and their popliteal lymph nodes removed. A suspension of cells was prepared from these nodes. Four bacteriophages were added to the suspension; T_1 the fourth bacteriophage was added as an internal standard. A number of microdrops were dispensed from the suspension and incubated at 37°C for 48 hours. The number of cells and the number of nuclei per cell were noted per microdrop incubated. Although they dispensed about 2,500 drops only 472 were chosen; drops were chosen which were free of debris and which contained cells resembling plasma cells.

After incubation, the drops were recovered in nutrient broth. Dilutions were made from the broth and added to the three bacteriophages. The degree of bacteriophage inactivation was measured. Drop volume studies using the T_1 bacteriophage as an internal standard were carried out; if the T_1 bacteriophage could not be detected then it was assumed that an insufficient volume was used to inoculate the bacterial plates. From these experiments it was found that 3.6% of the drops containing one cell inactivated more than one bacteriophage. This meant that a single cell, according to their findings, could synthesize antibody to more than one antigen. To check their results on single cells they did a series of experiments using microdrops which contained two cells. They calculated the expected double inactivating drops using their data on single cell drop studies. Their results showed a ten-fold higher incidence of drops producing antibodies to two bacteriophages than the "accidental overlap frequency". They were unable to detect single cells which produced antibody to all three bacteriophages. A series of controls were done to rule out the possibility of artifacts in their findings. They concluded from their experiments that a single antibody-producing cell can synthesize antibodies to at least two different antigens at one time. Later (Attardi et al. 1964e) they found that the T_5 bacteriophage itself had two antigenic determinants. This finding added further proof to their results and increased the amount of single cells producing two different antibodies to 65%.

Ingraham et al. (1964) used suspensions of lymphoid cells

prepared from rabbits that had been immunized with sheep erythrocytes to study single cell antibody production. The lymphoid cells were mixed with sheep erythrocytes and complement in a suitable culture medium which had been thickened by adding gum. The mixture was then spread on microscope slides under sealed cover-slips and incubated at 37° C. (This method is very similar to Jerne's plaque technique except that Jerne used agar instead of gum to gel the mixture (Jerne et al. 1963)). Small zones of hemolysis indicated the presence of single cells producing antibody to sheep r.b.c. (hemolysin). They used metabolic inhibitors to determine whether or not the cells were actively synthesizing antibody or merely releasing stored antibody. They found that the cells incorporated amino acids into protein (antibody) and required messenger RNA to actively synthesize antibody. Furthermore, an active energy-requiring process seemed to be involved in the release of antibody indicating that the cells were actively engaged in synthesizing antibody. Using this technique they tried to determine whether single cells could produce antibodies to two or more antigens. They coupled sulfanilazo groups (SA-rbc) or bovine globulin (BGG-rbc) to the erythrocytes and used these as antigens instead of sheep erythrocytes. They only reported preliminary findings using lymph nodes from animals immunized with SA-rbc and BGG-rbc, and none of these cell preparations gave lysis which could be attributed to the attached SA or BGG. However, they do not attach considerable importance to their initial results using coupled antigens, as on occasion, they even failed to observe lysis of sheep erythrocytes by cells from

rabbits immunized with only sheep erythrocytes.

Trentin et al. (1966) gave intravenous injections of 10^5 bone marrow cells from adult normal (CBA X T_6) donor mice to lethally irradiated CBA mice. Ten days after the injections the spleen colonies of these irradiated mice were removed and used to repopulate secondary irradiated CBA mice. The myeloid and lymphoid tissues of some of these hosts were used to repopulate tertiary hosts of lethally irradiated CBA mice. Those mice which survived after thirty days were injected with a number of different antigens. Since the hemopoietic and lymphoid systems of the repopulated mice were all of the original donor origin, indicated by the T_6 marker chromosome, any antibodies which were formed must have been produced from the original 4 to 13 discrete colonies which developed in the spleens of the secondary hosts after injection of the normal bone marrow cells. The mice were found to produce antibodies to these antigens just as well as normal mice. They concluded from these experiments that single cells had the potential of synthesizing antibody with more than one specificity.

Nossal (1964) investigated the question of whether single cells produce more than one type of immunoglobulin. He used his microdrop technique to isolate single antibody-producing cells. If the isolated cells were immobilized by mercaptoethanol then he assumed that they were 19S producers; 7S producers were similarly immobilized by using specific anti 7S serum. Four days after a secondary injection he found cells containing either 7S or 19S antibody. Cells which appeared to contain both 7S and 19S antibody were found only at times

when a switchover from 19S to 7S antibody was in progress; this could be detected by a rising 7S and a declining 19S titer in the serum. Of 144 cells studied 123 of them contained detectable antibody. And of these 123 antibody-producing cells 64 showed 7S only, 42 showed 19S only and 17 showed 19S and 7S antibody. He concluded on the basis of his experiments that antibody-producing cells go through a sequential stage---19S being produced first followed by 7S antibody. Mellors et al. (1963) also reported finding some cells containing 7S and 19S antibody.

Fahey et al. (1967) studied 29 human lymphoid cultures and found that "many, but not all, produce immunoglobulin, (b) some lines produce several classes of immunoglobulin, (c) the immunoglobulins are restricted to a relatively few molecular forms, (d) the newly formed molecules appear normal in terms of size, antigenicity, and gross polypeptide chain composition". They used immunofluorescent staining techniques to determine whether individual cells could synthesize more than one type of immunoglobulin. Their results show that single cells produced γ and μ heavy immunoglobulin chains.

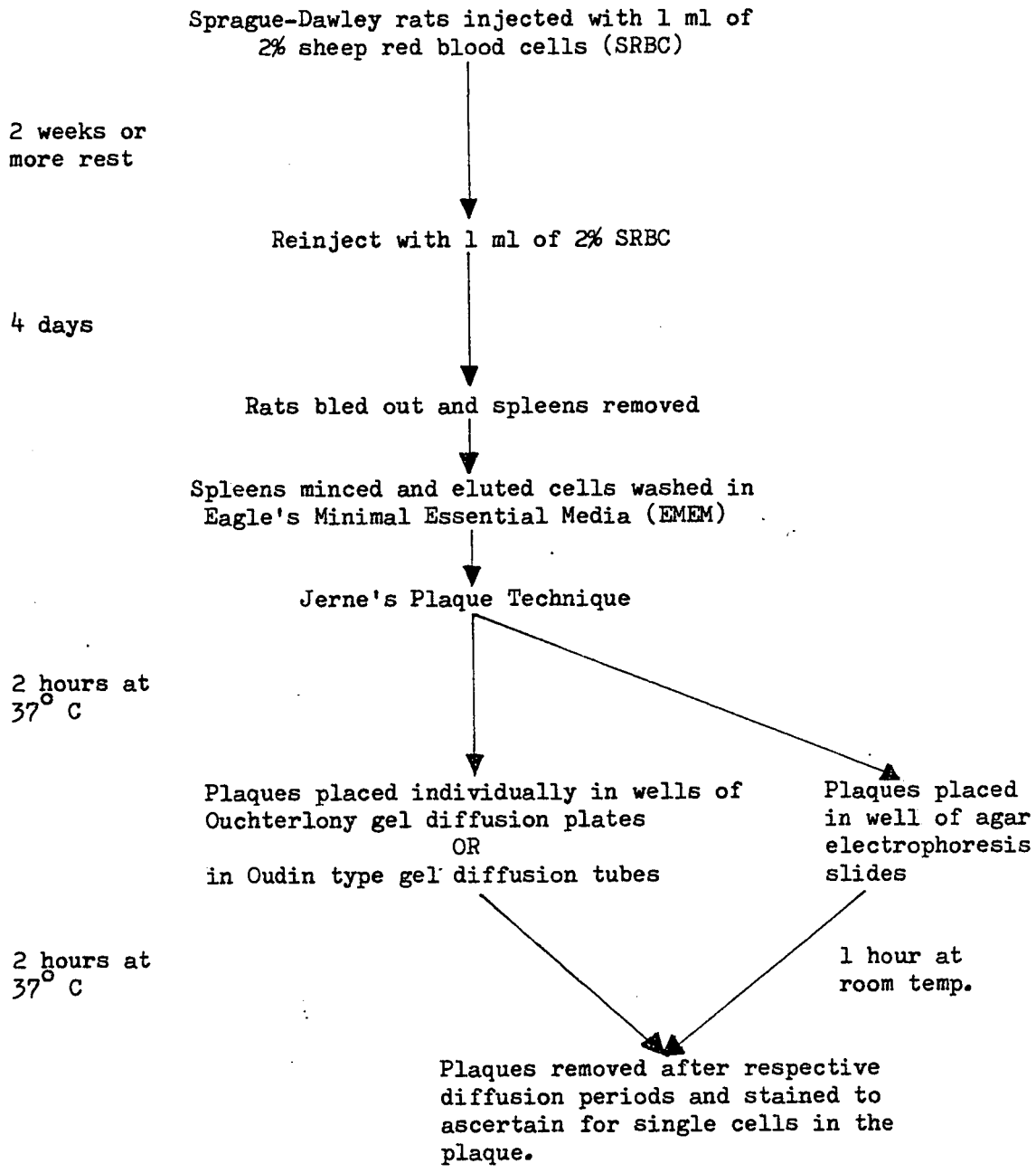
Yagi (1967) reported finding similar results. He stained cells of LKID cell line with a mixture of fluorescein (FI)-, and tetramethylrhodamine (TMR)- conjugated antibodies of two different specificities (for example FI-anti-γ and TMR-anti-α antibodies). The specially purified antibodies used in the conjugations were each monospecific to γ-, α- or μ- heavy chains. He found that both γ and α

heavy chains were present in single cells.

It is now recognized that about 2-5% of single antibody-producing cells can produce antibody with more than one specificity. It is important to determine two additional features of antibody production: 1) can a single antibody producing cell synthesize both 19S and 7S antibody with the same specificity and 2) can a single antibody producing cell synthesize both 19S and 7S antibody with more than one specificity. A great deal of the research concerned with the type of immunoglobulin produced by single cells has been carried out using fluorescent antibody techniques. In these studies the cell is killed in the staining procedure; therefore, only that protein in greatest concentration at the time of cell death can be detected in the cell. For this reason a new method is needed which can detect immunoglobulins in single cells while the cells are still viable and actively synthesizing protein (antibody). Moreover, the protein (antibody) should be given an opportunity to diffuse out of the cell to enable the cell to synthesize more protein (antibody).

METHODS

Experimental Design



Preparation of Sheep Red Blood Cells for use in Injections and Jerne's Plaque Technique

Sheep blood in Alsever's solution was obtained through the courtesy of the Department of Agriculture, Animal Diseases Research Institute, Hull, Quebec. These cells were stored for a minimum of one week after bleeding; and after a period of five weeks the blood was discarded.

The blood was poured into a centrifuge tube and centrifuged at 615 G for fifteen minutes. The plasma and the top layers of cells (buffy coat) from the pellet were discarded. The erythrocytes were resuspended in 0.9% NaCl solution and then washed by pouring them from one centrifuge tube into another and then back into the first tube. The cells were then centrifuged at 615 G for fifteen minutes. The cells were washed in this manner four times. A 2% (vol/vol) red blood cell suspension was prepared in 0.9% NaCl solution for use in the injections of the rats.

For Jerne's plaque technique the cells were washed three times in 0.9% NaCl solution; the fourth washing was done in veronal buffer pH 7.4 (See Appendix) and a 4% red blood cell suspension was prepared in the veronal buffer.

Inoculation of Rats for Spleen Cell Preparations

Forty male Sprague-Dawley rats between the ages of three months to one year were injected interperitoneally with one ml of a 2% suspension of sheep red blood cells in 0.9% NaCl solution. They were allowed to rest for three weeks or more; then twenty of them were

reinoculated with one ml of 2% sheep red blood cell suspension in 0.9% NaCl solution. Four days later they were anaesthetized and bled out by either heart puncture of by cutting the jugular vein. The blood from these rats was used to prepare rat globulin.

The remaining twenty rats were used for studying single cell production of rat immunoglobulins. Four days prior to their sacrifice they were reinoculated interperitoneally with one ml of a 2% sheep red blood cell suspension in 0.9% NaCl solution. Usually two rats per experiment were used for the preparation of the spleen cells for Jerne's plaque technique.

Purification and Isolation of Rat Immunoglobulins

Ammonium Sulphate Precipitation

All twenty rats were first anaesthetized with ether. They were then bled out by either heart puncture or by cutting the jugular vein. The blood was allowed to clot and the serum pooled. This pooled serum was centrifuged at 760 G for thirty minutes to sediment the cells. A total of 75 ml of serum was obtained in this manner. The globulin fraction of the pooled serum was fractionated by precipitating the globulins with ammonium sulphate at a 33 1/3% of concentration, at a pH of 7.8 adjusted with a N sodium hydroxide. (One volume of serum, one volume of 0.9% NaCl solution, one volume of saturated ammonium sulphate). The solution containing the precipitated globulins was centrifuged at 1710 G for thirty minutes. The supernatant was stored at 4° C overnight and recentrifuged the next day at the same speed

and for the same period of time. The pellets from the first and second centrifugations were dissolved in 25 ml of 0.9% NaCl solution. The entire fractionation process was repeated twice more.

The 25 ml containing the dissolved globulins were dialysed against 0.9% NaCl solution for two days at 4° C; the 0.9% NaCl solution was changed three times daily. The contents of the dialysis sacs were diluted with 0.9% NaCl solution to a final volume of 75 ml; the same as the original volume of serum. This was then frozen in sterile vials and stored until needed for further separation techniques.

A small volume (1.5 ml) of this fractionated serum was further dialysed against veronal buffer pH 8.2 (μ= 0.05); the buffer was changed three times within a period of two days. An immunoelectrophoresis of this dialysed serum was carried out to ascertain the purity of the ammonium sulphate fractionation. Figure 3 shows the immunoelectrophoresis slide of the fractionated serum.

Sephadex G-200 Chromatography

Sephadex G-200 (Pharmacia, Upsala, Sweden) was swollen in 0.01 M phosphate buffer 0.9% NaCl pH 7.4 for a period of three days or longer. After swelling, the slurry was poured into large cylinders and the "fines" removed. The column was straightened before packing. It was filled with 0.01 M phosphate buffer 0.9% NaCl pH 7.4; and then a funnel was attached to the top of the column. The funnel was filled with the slurry of Sephadex. After 2-3 cm of Sephadex formed at the bottom of the column the stopcock was opened to allow the buffer to drain out and be replaced by the sephadex. After the column was

packed, the Sephadex was washed with two liters of the buffer.

The column was 2.5 x 100 cm; and the flow rate was between 20 and 30 ml per hour. A total of 4 ml of the ammonium sulphate-precipitated globulin was applied each run. Two ml samples were collected and read at 277 mμ using a Coleman spectrophotometer. Figure 5 shows a typical run of the fractionated globulin.

The tubes (17-25) from the first peak were pooled and concentrated by lyophilizing. The lyophilized material was dissolved in 4 ml of distilled water. The tubes (32-45) from the second peak were also pooled and concentrated to 4 ml in the same fashion.

A small quantity (0.2 ml) of both peaks was dialysed separately against veronal buffer pH 8.2 ($\mu = 0.025$). An immunoelectrophoresis analysis of these peaks was performed to determine the type of protein present in each peak. Figure 6 shows the immunoelectrophoresis of peak 1 and 2.

Diethylaminoethyl (DEAE) Cellulose Chromatography

DEAE cellulose (Mann Research Laboratories) was prepared with 0.5 N HCl and 0.5 N NaOH (See Appendix 7). After preparation it was poured into large cylinders to remove the "fines". Once the "fines" were removed the DEAE was degassed by suction. A large funnel was attached to the top of the column; and then the column was filled with 0.01 M phosphate buffer pH 7.8. The DEAE cellulose was poured into the funnel. After 2-3 cm of DEAE formed at the bottom of the column the stopcock was opened to allow the buffer to drain out and be replaced by DEAE. The packed DEAE was washed with two liters of the phosphate buffer.

The column was 2.5 x 45 cm long and the flow rate was between 50 to 100 ml/hr. Five ml of protein from either Peak 1 or Peak 2 from the Sephadex G-200 separation of the ammonium sulphate precipitated rat globulin were applied per run. The DEAE had a wet weight of 0.91 mg/ml. The column was repacked after each run. Five ml samples were collected and read at 277 mμ using a Coleman spectrophotometer. Figures 9 and 8 show typical runs of peak 1 and peak 2 respectively. Tubes 10-22, 250-256, 260-340 from Figure 9 were pooled and concentrated by lyophilizing. Similarly tubes 48-57, 58-80, 81-121, 143-180 from Figure 8 were pooled and concentrated.

A small quantity (0.2 ml) of the various concentrated peaks from Figures 8 and 9 was dialysed separately against veronal buffer pH 8.2 (μ= 0.025). An immunoelectrophoresis analysis of these peaks was performed to check the proteins present in each peak. Figure 4 shows the immunoelectrophoresis of tubes 48-57, 143-180 from Figure 8.

Inoculation of Rabbits

The ammonium sulphate precipitated globulin was used as an antigen to obtain specific rabbit anti-rat globulin serum. New Zealand albino rabbits were used between the ages of three months to six months. The inoculations were given using either the ammonium sulphate fractionate globulin in alum or in complete Freund's adjuvant (See appendix).

When the alum precipitated protein was employed the injections were given intravenously in the marginal ear vein. On the first day of injections 0.5 ml of the alum protein solution was given intravenously.

The second and third injections, given on two consecutive alternate days, consisted of 1 ml of the alum-protein solution each day. After a week's rest, the animals were test bled to determine the strength of the serum against ammonium sulphate precipitated rat globulin.

In most cases the response was poor and a booster injection was given in Freund's complete adjuvant. A total of 1.7 ml of the rat globulin in adjuvant was injected into the following regions of the rabbit's body: 0.2 ml subcutaneously in either side of the dorsal section of the neck; 0.2 ml subcutaneously in either side of the dorsal posterior region of the back; 0.2 ml into each of the hind footpads; and 0.5 ml intramuscularly into the right thigh of the hind leg.

The rabbits were test bled two, three, and four weeks after the adjuvant injections. When the rabbit serum at a dilution factor of 1/32 (gel diffusion test) could detect the ammonium sulphate precipitated rat globulin the rabbit was anaesthetized with ether and bled out by either heart puncture or by cutting the jugular vein. The serum from the rabbits was pooled and then fractionated with 33 1/3 % ammonium sulphate precipitation of the globulins. The globulins were dissolved in 0.9% NaCl solution and dialysed against 0.9% NaCl solution for three days. It was then stored frozen in small aliquots. Inoculations of rabbits were also done using rat immunoglobulins IgG, IgA, IgM as antigens. The method for obtaining specific antiserum against these immunoglobulins was exactly the same as used for the ammonium sulphate precipitated rat globulin.

Spleen Cell Preparation

All rats sacrificed in these experiments were first anaesthetized with ether. All operations involving the removal of the spleen were done as aseptically as possible. Just prior to usage the dissecting instruments were immersed in 70% methyl alcohol, removed, flamed, and cooled. The rat was bled out by cutting the jugular vein. The spleen was removed and placed into a sterile plastic, 100 x 15 mm non-toxic Petri dish (Standard Hospital Supply Ltd., Toronto) containing ice cold Eagle's minimal essential medium (Eagle 1959). Care was taken in handling the spleen by grasping the surrounding fat. The fat and the connective tissue were then trimmed from the spleen. It was transferred to another sterile plastic Petri dish containing EMEM. The spleen was kept cold by using crushed ice in a bucket. It was then cut into small pieces and a nylon mesh screening was placed over the spleen tissue. A forceps was gently but firmly drawn across the nylon mesh repeatedly, causing the spleen cells to be released into the EMEM.

The EMEM containing the spleen cells, was then pipetted into a sterile 15 ml centrifuge tube. Larger pieces of tissue settled to the bottom of the tube. After five minutes the supernatant was pipetted into another sterile centrifuge tube to allow larger pieces of tissue to settle out. The supernatant from this tube was transferred to a third sterile centrifuge tube and centrifuged at 615 G for fifteen minutes. The cells were resuspended and washed twice with EMEM centrifuging each time at 615 G for ten minutes. At the end

of the second washing most of the EMEM was removed. The spleen cells were resuspended in the remaining medium (3-4 ml). Two-fold dilutions of the spleen cells were made in EMEM. The dilutions ranged from undiluted to a dilution factor of 1/64. These various dilutions were later used in Jerne's plaque technique.

Jerne's Plaque Technique

A volume of 0.1 ml of the various spleen cell suspensions was added to separate test tubes containing 2 ml of 4% sheep red blood cells prepared in veronal buffer (See text) and 2 ml of liquid agarose prepared in Eagle's minimum essential medium. The agarose was kept at 45° C to prevent solidification and the sheep cells were brought to that temperature just before the addition of the spleen cell suspensions. After adding the spleen cells to the sheep cells agarose mixture, the tube was inverted a number of times to allow mixing. The contents of the tube were then poured into small Petri dishes (sterile, non-toxic, 60 x 15 mm, plastic, Falcon Plastics, Division of B-D Laboratories Inc., Los Angeles, California, U.S.A.). After gelation of the agarose the Petri dishes were placed into a hooded water-bath shaker incubator at 37° C (Warner Chilcott Laboratories, Instrument Division). A 90% oxygen - 10% carbon dioxide mixture was passed through the incubator. Two hours later the dishes were removed and sufficient guinea pig complement at a dilution of 1:5 was added to flood the entire surface of the Petri dish (approximately 1.5 ml). The Petri dishes were then placed into the refrigerator, 4° C, for

thirty minutes to allow diffusion of the complement, followed by a two hour incubation period at 37° C to allow the reaction to take place.

Upon removal from the 37° C incubator the dishes were examined with a Leitz inverted microscope at a magnification of 35X, 90X and 100X. Single plaques were removed with a specially designed instrument. (Figure 1 shows a picture of the instrument); the end of it was placed directly onto the agarose containing the plaque. The surrounding agarose was cut with the tip of the instrument and the plaque was sucked up into the tube by drawing the plunger of the attached syringe. The plaque was removed by pushing on the plunger. The single plaques were placed into either the central well of an Ouchterlony type gel diffusion plate, on the agar surface of an Oudin type gel diffusion tube, or in a well on a slide prepared for agar electrophoresis. A drop of EMEM was placed on top of the plaque to allow the protein(s) present in the plaque to diffuse into the surrounding agar. The plaques on the gel diffusion plates and tubes were removed after a two hour incubation period at 37° C in the water-bath incubator with the 90% oxygen - 10% carbon dioxide atmosphere. The plaques on the electrophoresis slides were removed after a one hour incubation at room temperature. When the plaques were removed they were placed on microscope slides to dry. These were later stained. A drop of liquid 0.5% agar was added to the well of the gel diffusion plates, the agar surface of the Oudin gel diffusion tubes, and the well of the electrophoresis slides which had contained the plaques.

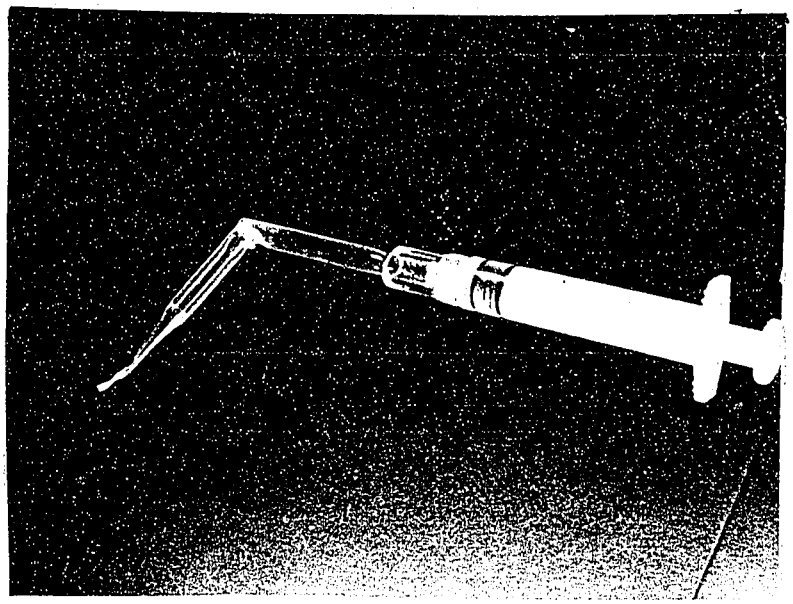


Figure 1

Instrument designed for removal of plaques. A 5 ml syringe was attached to a Pasteur pipette. The Pasteur pipette was bent at a 90° angle; and the tip was drawn out to a fine bore. The bore was constricted 1 mm (approx.) from the tip to prevent the plaque from entering too far up the pipette.

Gel Diffusion Studies

Ouchterlony Type Gel Diffusion Plates

The slides used for gel diffusion were 75 x 50 mm in size. They were cleaned by washing in mild detergent and then rinsing thoroughly in distilled H_2O. The slides were then immersed in methyl alcohol and flamed after removal from the alcohol. All of the slides used in gel diffusion studies were pre-coated with hot liquid 0.5% agar (approximately 4 ml). After gelation the agar was dried down by placing a wet strip of filter paper over the agar surface and then leaving them at room temperature until dry. When dry they were rinsed with distilled water to remove any of the fibre which had come off of the filter paper. The pre-coated slides were then placed in a special plastic tray (each section of the tray measured 54 x 80 x 2.5 mm). Six ml of liquid 1% purified agar (Mann Research Laboratories) prepared in 0.01 M phosphate buffer 0.85% NaCl pH 7.4 was pipetted on each slide. Sodium azide at a concentration of 0.01% had been previously added to the agar to prevent microbial contamination. Wells were punched in the agar using a specially designed cutter. The wells were 4 mm in diameter and the central well was 6 mm distant from each of the six peripheral wells. Figure 2 shows a picture of the cutter. The agar in the wells was removed by suction.

When working with plaques, the plaques were placed in the central well and the rabbit anti-rat globulin serum and purified immunoglobulins (to act as internal standards) were placed in the peripheral wells.

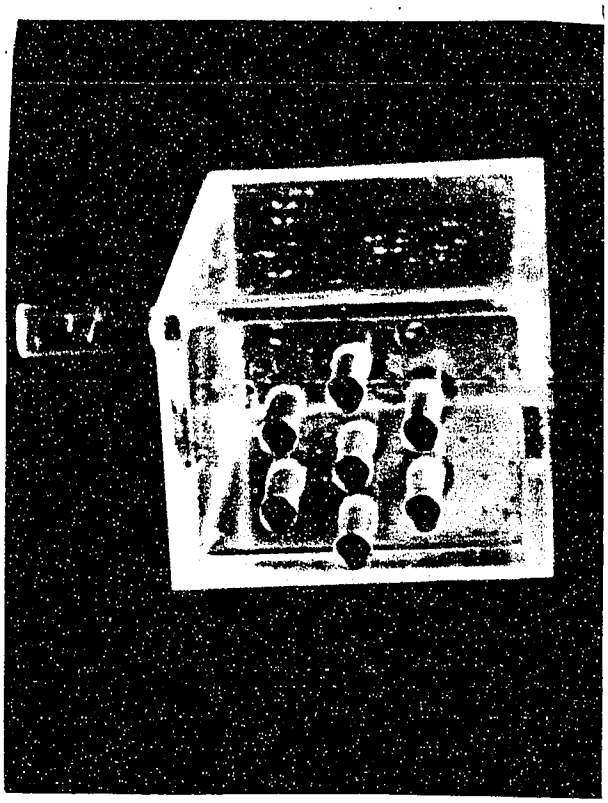


Figure 2

Specially designed cutter for use in Ouchterlony type gel diffusion plates. The tubing used for the cutter was 4 mm in diameter and the central tube was 6 mm distant from each of the peripheral six tubes.

Gel diffusion precipitation tests were also performed to determine the dilution to which the specific rabbit anti-rat globulin serum could be diluted and still detect the ammonium sulphate precipitated rat globulin. Ouchterlony type gel diffusion plates were used as well to check the purity of the isolated immunoglobulins.

After the application of the reagents to the wells of the gel diffusion plates they were incubated in humid Petri dishes. The dishes were kept on a flat surface at room temperature until a visible precipitin line(s) could be detected. They were then placed in the cold, 4^{o} C, for a minimum of 24 hours to increase the sharpness of the lines. After this, the slides were washed in 0.9% NaCl solution at a slightly basic pH for three days. The 0.9% NaCl solution was changed at least three times daily. On the fourth day the slides were washed twice with distilled water to remove any NaCl from the agar. They were then dried in a manner similar to the first coating of agar by placing a wet strip of filter paper over the slides and leaving them at room temperature until dry. The dried slides were stained with 0.1% thiazine red or 1.0% coomassie blue (See Appendix). Although coomassie blue stained the lines from the plaques more intensely than the thiazine red it was found upon photographing that the thiazine red stain was more sensitive. For this reason the plates which were photographed were stained with thiazine red.

Oudin Type Gel Diffusion Tubes

Glass tubing (5 mm in diameter) was cut in 2¼ inch lengths.

One end was blocked by stuffing it with plasticine. Agarose (1.5%) was prepared in 0.01 M phosphate buffer 0.85% NaCl solution and kept liquid at 45° C. Specific rabbit anti-rat globulin serum was brought to 45° C and 0.5 ml of this antiserum was mixed with 1.0 ml of the agarose. The agarose antiserum mixture was carefully pipetted into the glass tubing. The mixture was allowed to gel and then a plaque was placed on top of the surface of the agarose. A drop of EMEM was placed on top of the plaque to allow the protein(s) present in the plaque to diffuse into the agarose. After the two hour incubation period the plaque was removed and placed on a microscope slide to dry. These were later stained. A drop of 0.5% liquid agar was then placed on top of the surface of agarose. The tubes were allowed to incubate at room temperature for several days until visible line(s) could be seen.

Wright-Giemsa Stain

Once the plaques had air dried on the slides they were washed with 0.9% NaCl solution to elute the hemoglobin from the zone of lysed sheep erythrocytes. After drying, the slides were stained with Wright-Giemsa stain for a period of ten minutes. The sheen was flushed from the top of the solution with neutral water and the slides were placed in neutral distilled water for 1 minute. The slides were then dried. Using the Wright-Giemsa stain, a differential stain, the nuclei of lymphoid cells stained a reddish purple and the cytoplasm blue.

A series of spleen cell smears were also prepared and stained with Wright-Giemsa stain to determine the presence of lymphocyte-like and plasma-like cells. Figures 14 and 15 show plaques and spleen cell smears stained with Wright-Giemsa stain.

Immunofluorescent Staining

A total of 20 ml of rabbit anti-rat globulin serum containing 1160 mg of protein was labelled using 23.2 mg of fluorescein isothiocyanate (FITC) (Mann Research). The serum was diluted with 11.6 ml of carbonate bicarbonate buffer pH 9.0 (See Appendix) and 84.4 ml of 0.15 M sodium chloride solution. The dilution gave an end result of 10 mg/ml of protein and a ratio of 0.02 mg FITC/mg protein. The ratio was approximately two molecules FITC : one molecule protein.

The antiserum was chilled in an ice bath. The dry FITC powder was added in small aliquots, and mixed with a magnetic stirrer. Frothing of the protein was avoided by slow gentle stirring. The mixing was continued for nine hours at 4° C. The antiserum was then dialysed, 4° C, against 0.01 M phosphate buffer 0.85% NaCl pH 7.0 for two days. The buffer was changed at least three times daily. The dialysis was done to remove any excess dye which might have caused non-specific background staining. The antiserum was then removed from the dialysis sacs and stored in small aliquots.

The FITC labelled antiserum was used to stain spleen cell smears and plaques. The slides of the smears and the plaques were flooded with the labelled antiserum and were incubated for 30 minutes at 37° C

in a humid chamber. After this time the excess antiserum was washed off with 0.01 M phosphate buffer 0.85% NaCl pH 7.8; the slides were washed three more times with this buffer. The slides were then flooded with a buffered-glycerine solution containing one part 0.01 M phosphate buffer 0.85% NaCl pH 7.8 and nine parts glycerine. After covering the slides with cover slips they were examined with a fluorescent microscope (Reichert, Austria, No. 259938) under a magnification of 400 X. Figure 13 shows a spleen cell smear and Figure 12a plaque stained with the labelled antiserum.

RESULTS

Purification and Isolation of Rat Immunoglobulins

Ammonium sulphate precipitation

Figures 3 and 4 show an immunoelectrophoretic pattern of the ammonium sulphate precipitated rat sera. It can be seen from these two figures that the 33 1/3% of ammonium sulphate precipitation separated the Y and B_2 globulins from the albumin and the a_1 globulin. The immunoglobulins IgG and IgA are easily visible, but IgM immunoglobulin does not seem to show a precipitation line. Ammonium sulphate precipitation is the first step in a series of procedures designed to obtain purified immunoglobulins. The ammonium sulphate precipitation removes the albumin, which is the main component of the serum, from the globulins. The fractionated serum is then further separated according to molecular size by Sephadex G-200 chromatography.

Sephadex G-200

The Sephadex G-200 chromatography of the ammonium sulphate precipitated rat serum (rat globulin) resulted in the separation of the globulins into two distinct peaks according to molecular size. It has been demonstrated that the proteins which are eluted first in a Sephadex G-200 chromatography of (rat) globulins (Peak 1, Fig. 5) are 19S proteins while the second peak (Peak 2, Fig. 5) contain 7S proteins. The results of the agar gel immunoelectrophoresis of the proteins from these two peaks (Peaks 1 and 2, Fig. 5) show the Peak 1 contains a protein different from that in Peak 2; this is illustrated in Figure 6.

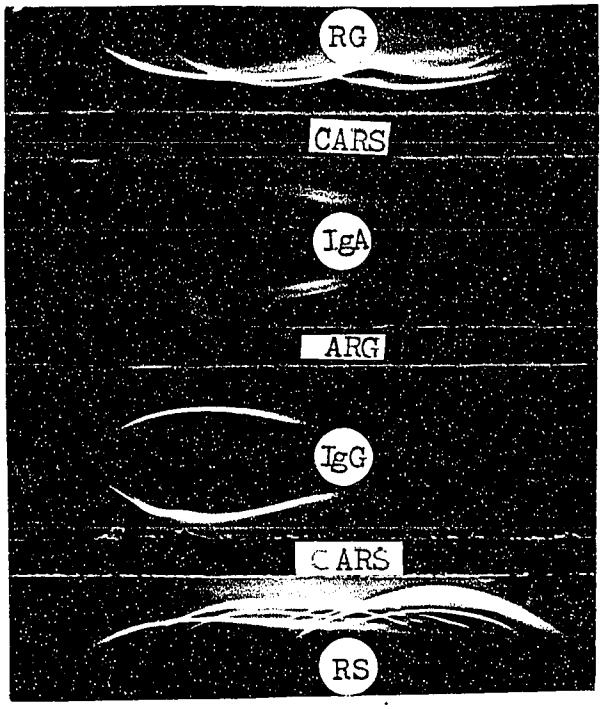


Figure 3

Agar gel immunoelectrophoresis of: rat sera, ammonium sulphate fractionated rat sera, rat IgG (Peak A from DEAE cellulose separation (Fig. 8) of Peak 2 from Sephadex G-200 separation of ammonium sulphate precipitated rat serum (Fig. 5), rat IgA (Peak D from DEAE cellulose separation (Fig. 8) of Peak 2 from Sephadex G-200 separation of ammonium sulphate fractionated rat serum (Fig. 5). Anti-rat serum used for identification of rat proteins.

RS : Rat serum
IgG : Immunoglobulin type G
IgA : Immunoglobulin type A
RG : Rat globulin (ammonium sulphate precipitated rat serum)
ARG : Rabbit anti-rat globulin
CARS: Commercial anti-rat serum

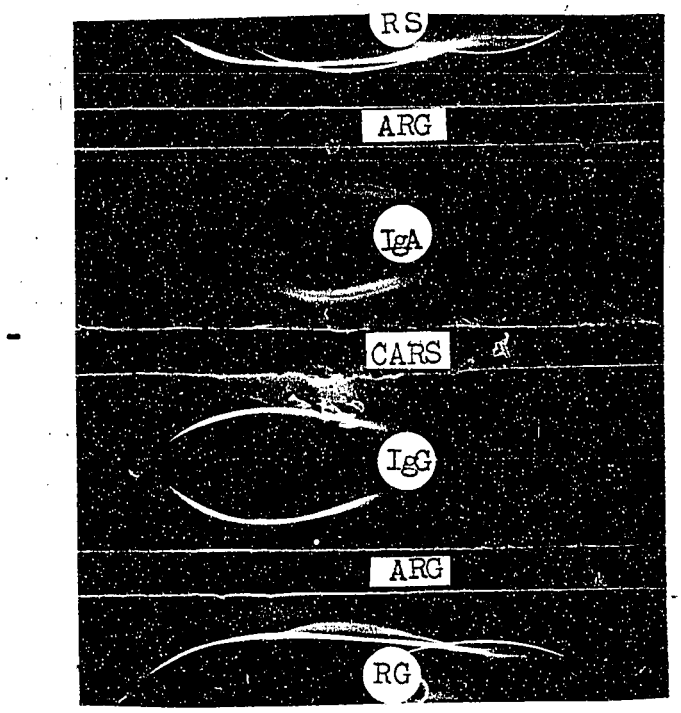


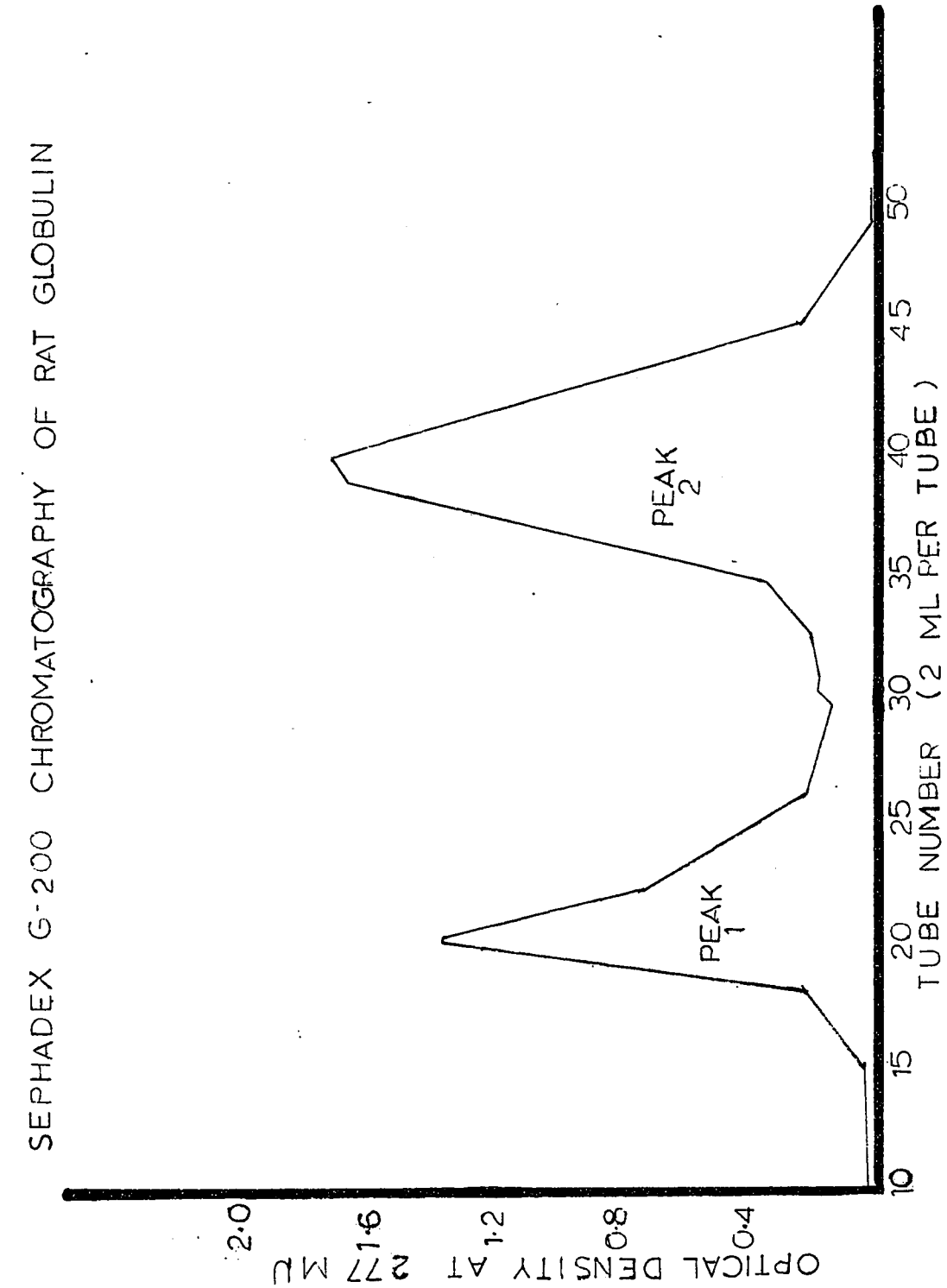
Figure 4

Agar gel immunoelectrophoresis of: rat sera, ammonium sulphate fractionated rat sera, rat IgG (Peak A from DEAE cellulose separation (Fig. 8) of Peak 2 from Sephadex G-200 separation of ammonium sulphate fractionated rat serum), rat IgA (Peak D from DEAE cellulose separation (Fig. 8) of Peak 2 from Sephadex G-200 separation of ammonium sulphate fractionated rat serum). Anti-rat globulin used for identification of rat proteins.

RS : Rat serum
RG : Rat globulin
IgG : Immunoglobulin type G
IgA : Immunoglobulin type A
CARS: Commercial anti-rat serum
ARG : Rabbit anti-rat globulin

Figure 5

Sephadex G-200 chromatography of ammonium sulphate fractionated rat sera.



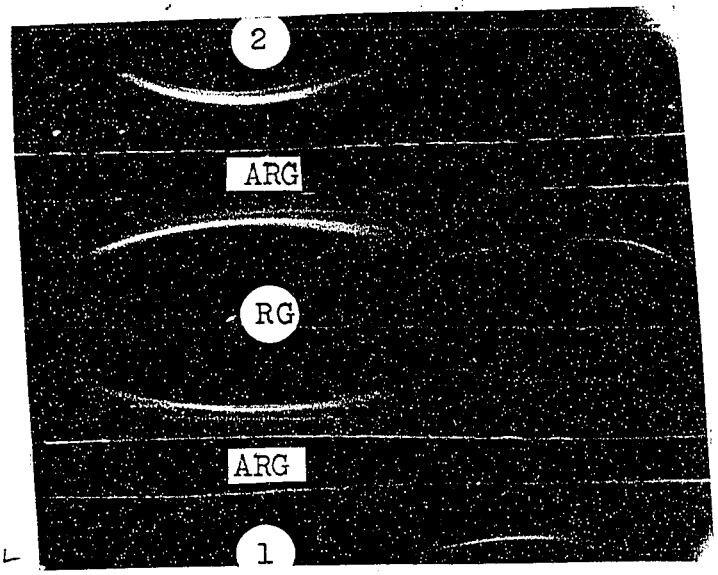


Figure 6

Agar gel immunoelectrophoresis of Peak 1 and Peak 2 from Sephadex G-200 chromatography of ammonium sulphate precipitated rat sera.

ARG: Anti-rat globulin
RG : Rat globulin

Whether this protein is, in fact, IgM (19S) immunoglobulin is questionable; from its position in immunoelectrophoresis (Fig. 6) it does not appear to be IgM. Moreover, if this protein is not IgM then the concentration of IgM present in Peak 1, Figure 5, was relatively low. Furthermore, Figures 6 and 7 show that this protein is different than either IgG or IgA immunoglobulin; this can be seen from the fact that (Fig. 6) the IgG and the IgA have migrated to the negative electrode during electrophoresis while the protein from Peak 1 (Fig. 5) seems to have migrated slightly towards the positive electrode.

The results of the immunoelectrophoresis of Peak 2 (Fig. 5) showed only two major proteins present (Fig. 6). From their position in the immunoelectrophoresis they were identified as IgG and IgA immunoglobulins.

Sephadex G-200 chromatography is the second step in the purification of immunoglobulins. It separates proteins according to molecular size; IgM immunoglobulin has a molecular weight of approximately 1,000,000 compared to IgG and IgA's molecular weights of about 160,000 each. Thus, IgM is located in the first peak of Sephadex G-200 separation and is usually relatively free from the other immunoglobulins provided that there is no polymerization of these 7S immunoglobulins. However, since IgG and IgA have similar molecular weights the second peak in Sephadex G-200 chromatography of (rat) globulins contains a mixture of IgG, IgA, and the other immunoglobulins. For this reason a different type of chromatography based on ionic charge is necessary to separate IgG from IgA and the other immunoglobulins (IgD, IgE).

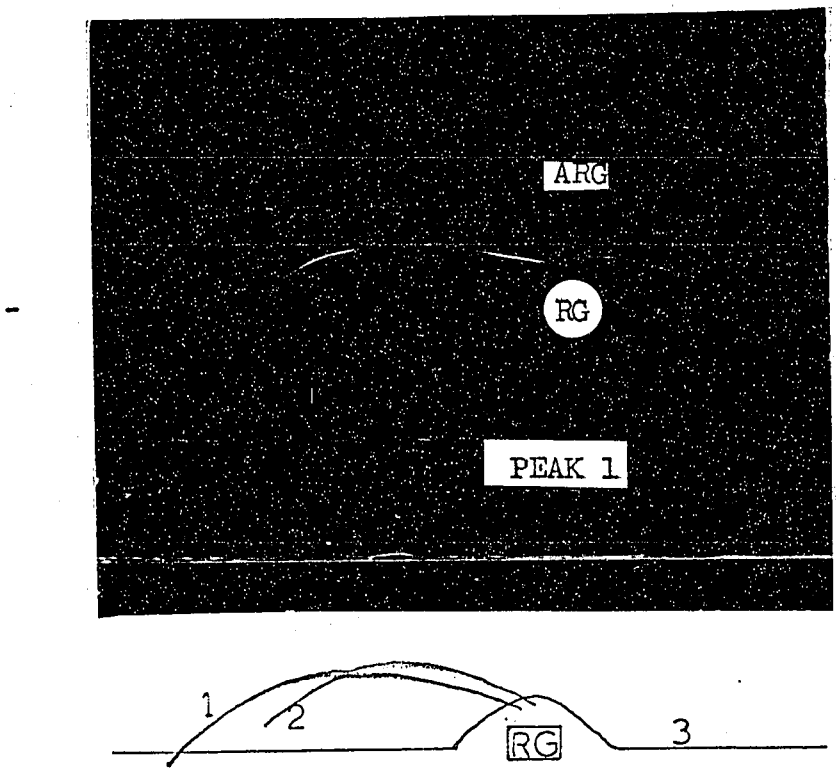


Figure 7

Identification of protein producing precipitation line No. 3 in Peak 1 from Sephadex G-200 separation of ammonium sulphate-precipitated rat sera (Fig. 5). An immunoelectrophoresis was done on rat globulin (ammonium sulphate precipitated rat sera); rabbit anti-rat globulin was placed in one of the troughs (top of the photograph) and protein from Peak 1 was placed in the other trough (bottom of the photograph).

ARG: Anti-rat globulin
RG : Rat globulin

DEAE cellulose chromatography

DEAE cellulose chromatography is the last in the series of steps designed to isolate immunoglobulins. This type of chromatography separates protein according to ionic charge. This means that proteins with similar molecular weights can be separated providing that they have different ionic charges.

The tube numbers from the DEAE cellulose chromatography of Peak 2 from Sephadex G-200 chromatography of rat globulin (Fig. 5) were plotted against optical density 277 mμ (Fig. 8). Figure 8 shows two major peaks, Peak A and Peak D. Two other minor peaks, Peak B and Peak C, are also visible. The four peaks were concentrated by lyophilization and were tested by immunoelectrophoresis. It was found that Peak B and Peak C contained a protein similar to that of Peak A, although Peak A had more protein than the other two peaks. The immunoelectrophoresis of Peak A and Peak D can be seen in Figures 3 and 4. From the position of the precipitation lines it appears that Peak A contains IgG immunoglobulin and Peak D contains IgA immunoglobulin. This seems logical because according to Oh et al. (1966), IgG is eluted from the column at a higher pH and lower NaCl molarity (pH 7.0 and 0.030 M NaCl) than IgA (pH 6.5 and 0.070 M NaCl). Both IgG and IgA appear to be quite pure as only one precipitation line is present in both cases (Fig. 3 and 4). However, the IgA precipitation line seems to split into two, but both lines join at one end. This may arise as a result of two different forms of IgA.

In the DEAE cellulose chromatography of Peak 1 from Sephadex

G-200 separation of ammonium sulphate precipitated rat sera (Fig. 5) there are four small peaks (Fig. 9): I, II, III and IV. All of these peaks were concentrated by lyophilizing and tested by immunoelectrophoresis. Not one of the concentrated peaks showed any precipitation line(s) with the rabbit anti-rat globulin serum. This would tend to indicate that none of the peaks contained rat globulin (IgM or other immunoglobulin) at a high enough concentration which could be detected by immunoprecipitation techniques. Each peak was then subsequently tested for its hemolysin activity: Peak IV was found to have a very slight hemolysin titer of 1:4. the others possessed no hemolysin activity. From the immunoelectrophoresis and hemolysin titer tests it is evident that there was little or no IgM type immunoglobulin present.

As mentioned previously, DEAE chromatography is the last step in the isolation of immunoglobulins. To obtain specific antiserum it is essential that a pure antigen be used in the immunization. For this reason purified immunoglobulins are necessary to obtain specific anti-immunoglobulin(s) serum. For example, the purified rat IgG (Peak A, Fig. 8) was used to obtain specific rabbit anti-rat IgG. Also pure antigens (protein) are necessary in identification of unknown proteins in immunoprecipitation techniques. These can be employed as internal standards in immunoprecipitation techniques or in immunoelectrophoresis according to the Osserman technique (Osserman, 1960).

Figure 8

Diethylaminoethyl (DEAE) cellulose chromatography of Peak 2 from Sephadex chromatography of ammonium sulphate fractionated sera (Fig. 5)

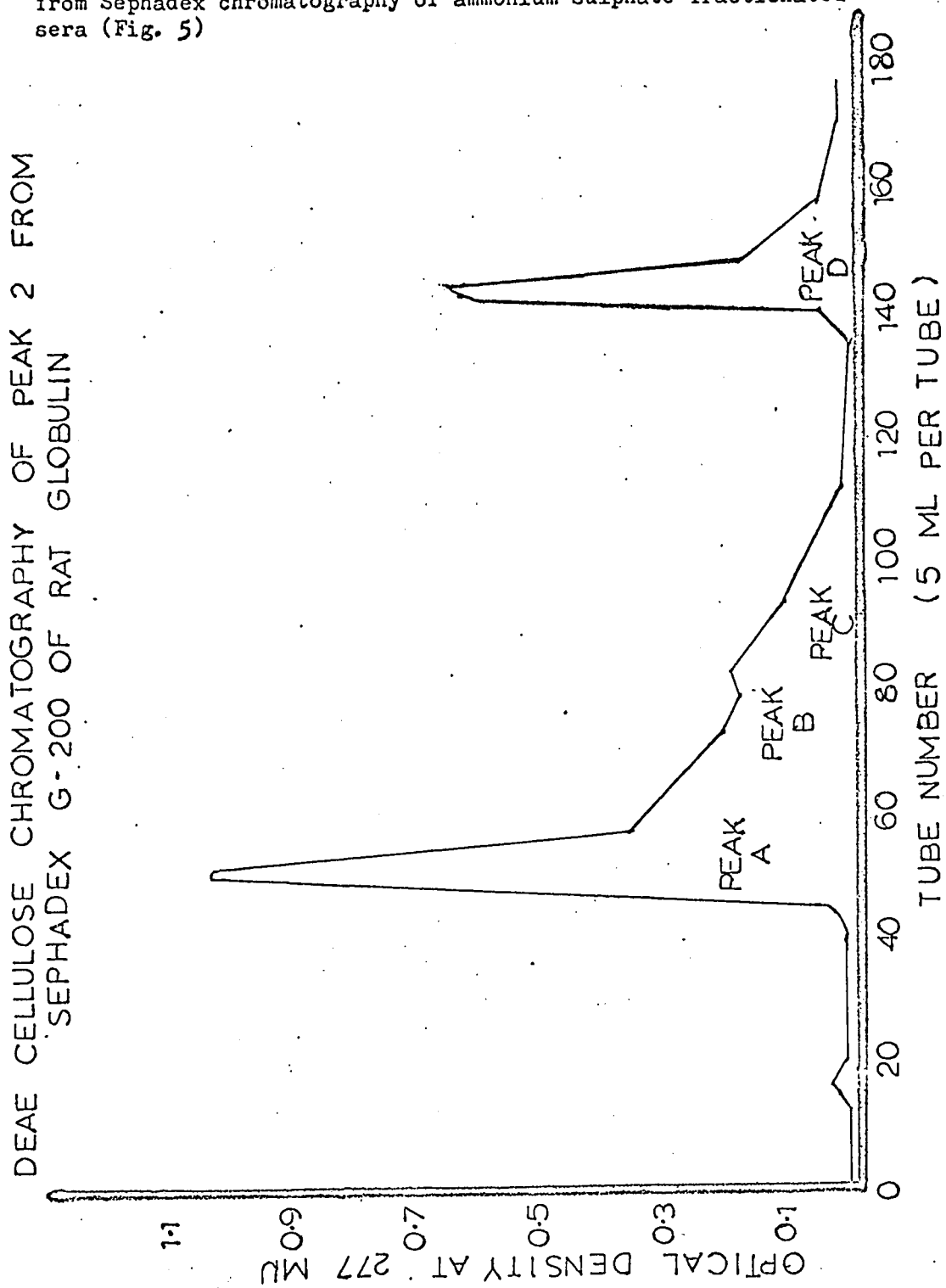
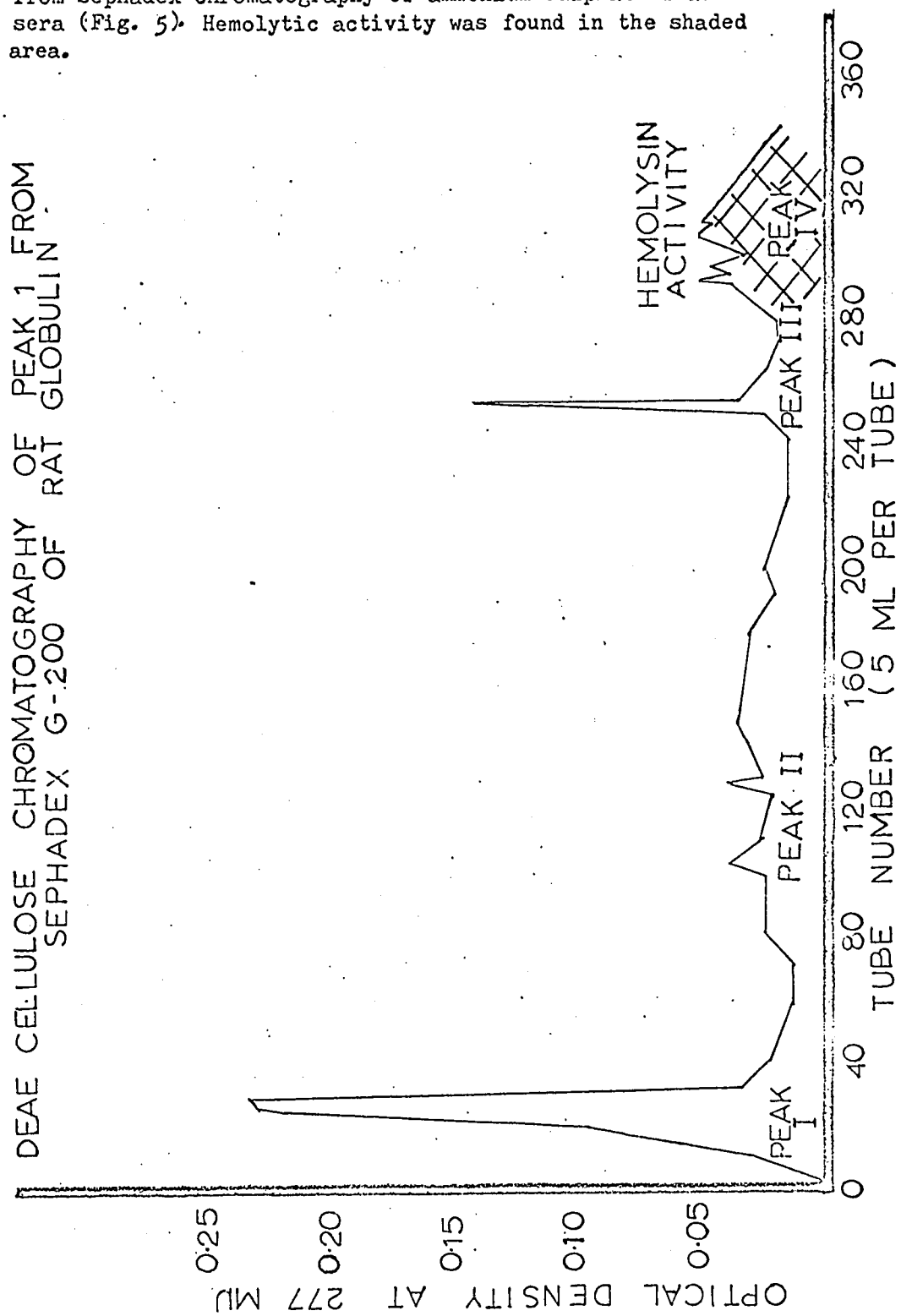


Figure 9

Diethylaminoethyl (DEAE) cellulose chromatography of Peak 1 from Sephadex chromatography of ammonium sulphate fractionated sera (Fig. 5). Hemolytic activity was found in the shaded area.



Titer Of Antiserum

The rabbits immunized with the ammonium sulphate precipitated rat sera produced a high titer of antibody against the rat globulins. Figure 10 illustrates a comparison between commercial anti-rat serum and the rabbit anti-rat globulin which was prepared by the author. The titer of this rabbit anti-rat globulin was determined by immunoprecipitation in agar gel. Various dilutions of purified rat IgG and IgA (See text) were made; these dilutions ranged from 1:5 to 1:200. Exactly 10 λ of each dilution was dispensed in the peripheral wells of an Ouchterlony type gel diffusion plate. The rabbit anti-rat globulin serum could detect at a dilution of 1:100 for rat IgG and 1:40 for rat IgA. A protein determination (Lowry et al, 1951) showed that the concentration of IgG was 2.83 mg/ml, and that the concentration of IgA was 0.810 mg/ml. This meant that the rabbit anti-rat serum could detect as little as 0.283 µg of IgG and 0.205 µg of IgA. When the antisera prepared in rabbits against IgG and IgA could detect at a dilution of 1:30 (the antiserum being diluted) they were bled out. A precise determination of the least amount of protein which these two antisera could detect was not carried out.

An antiserum to IgM could not be produced although a number of rabbits were used in the attempt, but because there was little or no IgM present in Peak 10 (Fig. 9) which was used as a source for IgM in the immunizations, the attempts were unsuccessful.

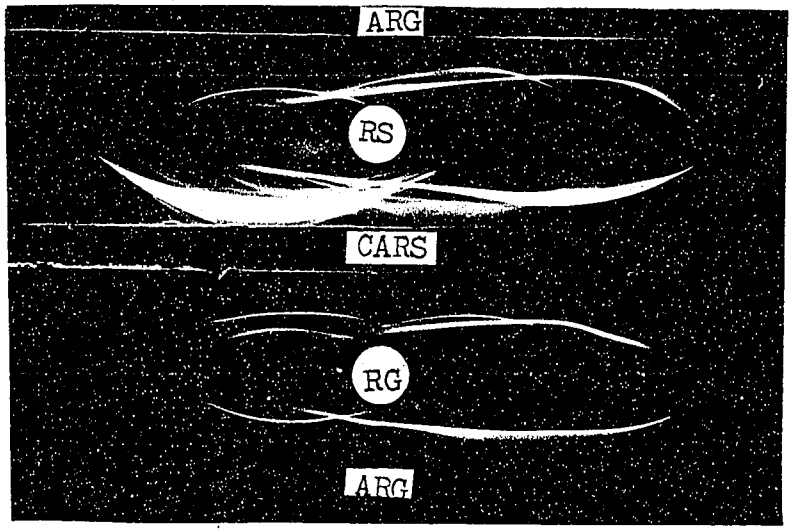


Figure 10

A comparison of commercial anti-rat serum and rabbit anti-rat globulin (produced by the author).

RS : Rat serum
RG : Rat globulin
CARS: Commercial anti-rat serum
ARG : Rabbit anti-rat globulin

Jerne's Plaque Technique

Distinct areas of hemolysis could be seen in the SRBC-agar when the Petri dishes were examined with the Leitz inverted microscope (See text). Figure 11 shows a typical plaque at 100 X. It was found that the higher spleen cell dilutions produced an insufficient number of plaques. In contrast to this the undiluted spleen cell suspension produced so many plaques that it was impossible to find isolated ones that could be removed singly. The best dilutions lay within the range of 1:2 to 1:8. A number of plaques were not circular in shape and these usually contained more than one nucleated cell type. These were not chosen in the selection of plaques.

On two separate occasions no plaques were observed. This is not that peculiar as others have found similar results (Ingraham et al. 1964). Also in two separate experiments rabbit serum was used as a source of complement instead of guinea pig serum. The rabbit serum was tried at a dilution factor of 1:2 and undiluted. No plaques were observed in both of these experiments. However, when guinea pig complement was added to these Petri dishes plaques appeared after a short incubation period at 37° C.

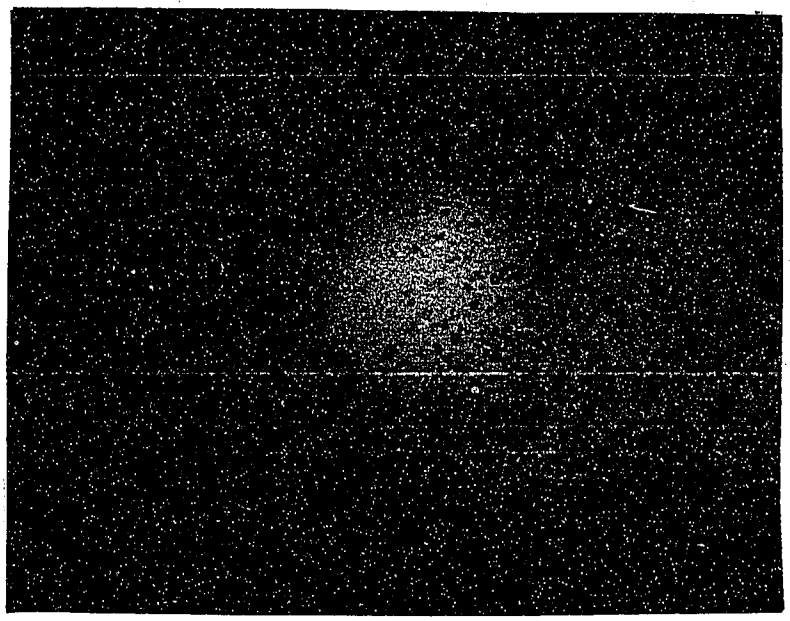


Figure 11

Plaque (showing sheep red blood cells and area of hemolysis).
100 X

Determination Of The Number Of Nucleated Cell(s) In a Plaque

The rabbit anti-rat globulin serum labelled with fluorescein isothiocyanate was quite specific in staining cells which contained rat globulin. Figure 12 shows a cell, within a plaque, stained with the fluorescent antibody. Figure 13 shows a smear of spleen cells that had been stained with the fluorescent antibody. However, staining with the fluorescent antibody was very time consuming; it took about one hour for a single plaque. Because of the numbers of plaques which were studied it was much quicker to use the Wright-Giemsa stain routinely for the recognition of nucleated cell(s) in a plaque (Figures 14 and 15).

About 85% of the plaques studied contained only one nucleated cell. However, it should also be noted that in a number of cases no nucleated cell could be found in the plaque. There are two possible explanations for this finding: 1) the plaque was an artifact and the area of hemolysis was due to some foreign particle in the blood-agar or 2) the nucleated cell in the plaque was damaged in handling the plaque.

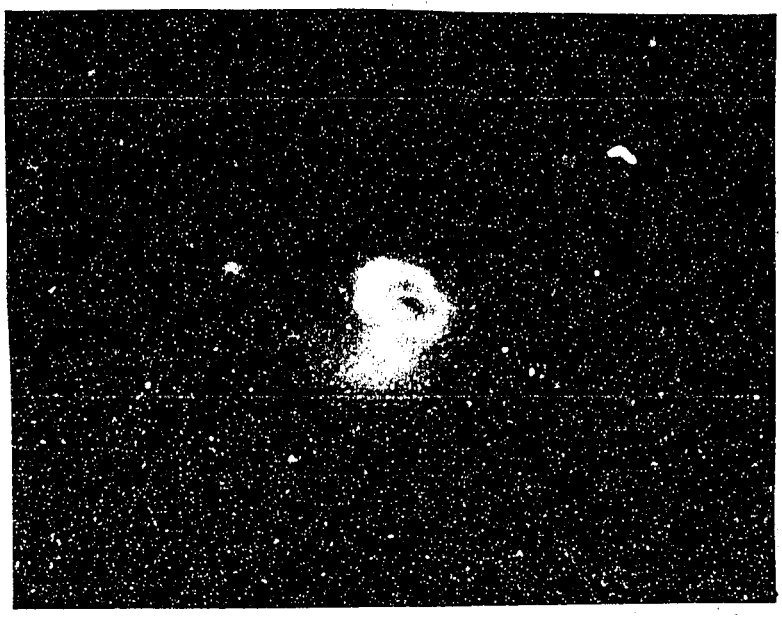


Figure 12

Cell (located in a plaque) stained by the immunofluorescent technique. The rabbit anti-rat globulin was labelled with fluorescein isothiocyanate. 400 X

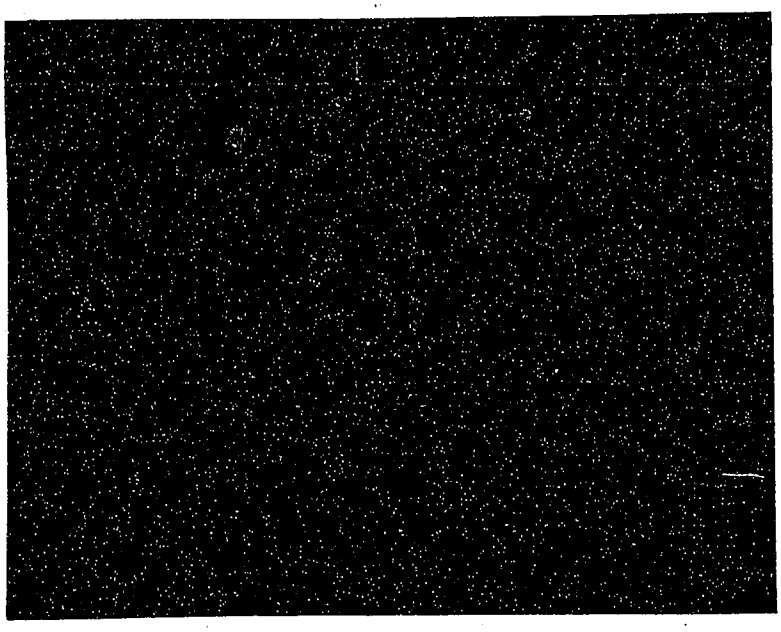


Figure 13

A smear of spleen cells stained by the immunofluorescent technique. The rabbit anti-rat globulin was labelled with fluorescein isothiocyanate. 400 X

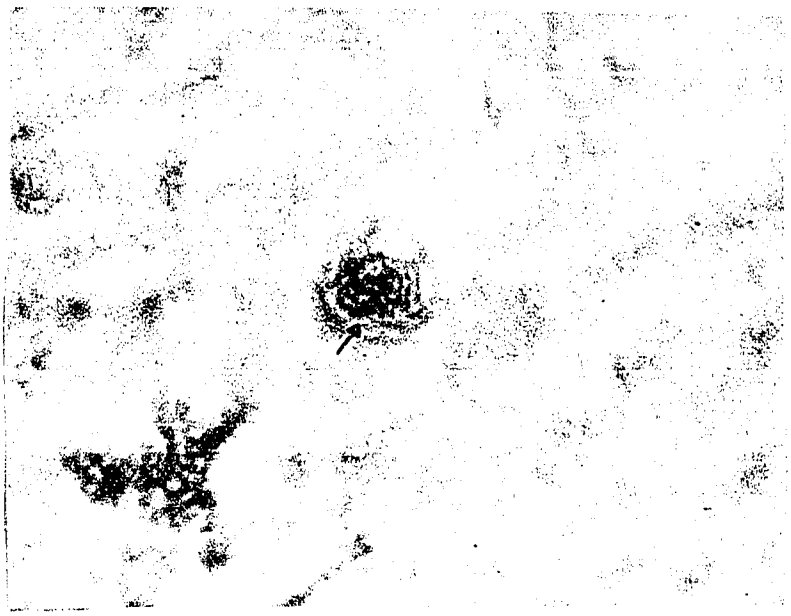


Figure 14

A cell (located in a plaque) stained with Wright-Giemsa stain.
400 X

Figure 15

A smear of spleen cells stained with Wright-Giemsa stain. 400 X

Gel Diffusion Studies Of Single Cells

Oudin gel diffusion tubes

It can be seen in Figure 16 that protein from plaques, containing antibody producing cells, formed two or more precipitation lines with the rabbit anti-rat globulin. However, with this type of gel diffusion test it is not possible to identify the type of protein(s) responsible for the reaction. These Oudin gel diffusion tests were initially used to determine whether a single antibody producing cell could remain viable in the agarose (plaque) and synthesize enough protein that would give a precipitation reaction in agar gel. The fact that positive results were obtained with this technique indicated that the Ouchterlony technique could be used to identify the protein(s) synthesized by single antibody producing cells.

Ouchterlony type gel diffusion and agar gel immunoelectrophoresis studies

A series of tests was done to determine whether the precipitation lines formed in gel diffusion studies (Figures 17 and 18) were due to the production of immunoglobulins by the cells or to some other factor. An immunoelectrophoretic analysis of guinea pig serum, used as the source of complement (See text), revealed that there was a protein in the guinea pig serum which cross-reacted with the rat serum. Fig. 19 shows the immunoelectrophoresis of the guinea pig serum against the anti-rat serum; two faint lines are visible. However, when the guinea pig serum was tested against the rabbit anti-rat globulin only one line was visible (Figure 21). Moreover, the protein present in the guinea pig serum which produced the precipitation line was not cross-reacting

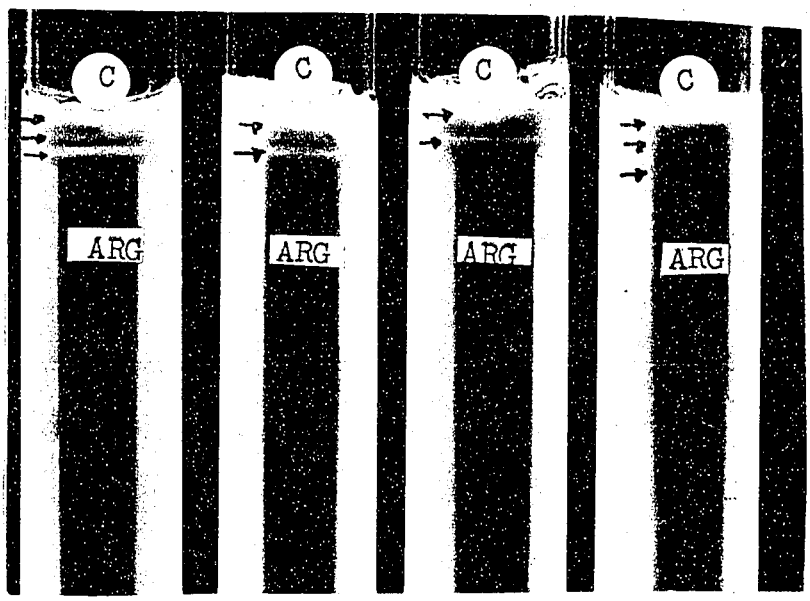


Figure 16

Precipitation lines as seen in Oudin type gel diffusion technique. The agarose contained anti-rat globulin; and the plaque was placed on the surface of the agarose. The plaque was removed after suitable incubation.

C : Plaque containing single cell
ARG: Rabbit anti-rat globulin

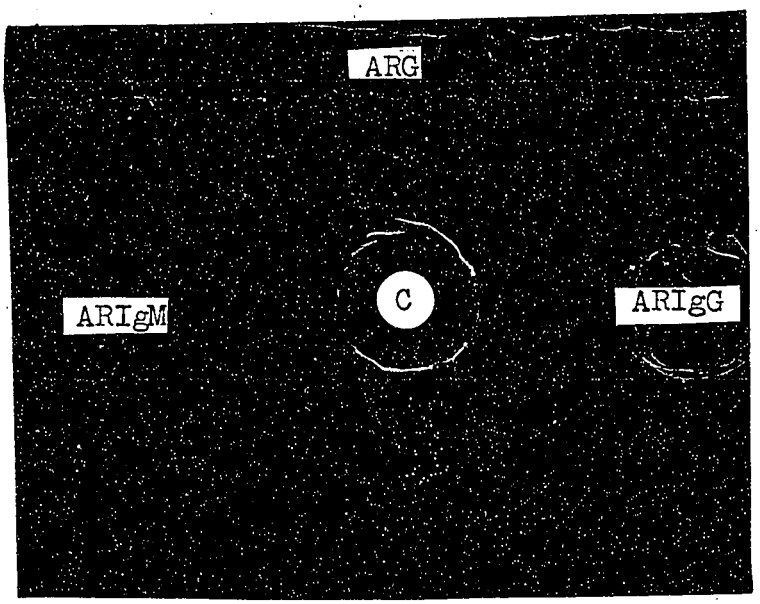


Figure 17

Ouchterlony type gel diffusion plate showing precipitation lines between the proteins released by a single cell and specific antisera (rabbit anti-rat globulin and rabbit anti-rat IgG).

ARG : Rabbit anti-rat globulin
ARIgG: Rabbit anti-rat IgG
C : Plaque containing a single cell
ARIgM: Rabbit anti-rat IgM (See text for explanation)

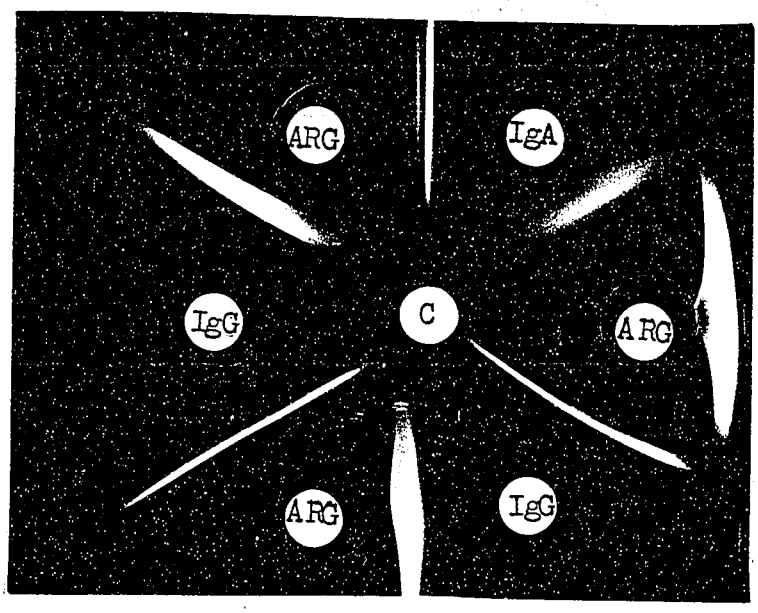


Figure 18

Ouchterlony type gel diffusion plate showing precipitation lines between the protein released from a single cell and specific antiserum (rabbit anti-rat globulin). Rat IgG and IgA are used as internal standards.

ARG: Rabbit anti-rat globulin
C : Plaque containing a single cell
IgG: Immunoglobulin type G
IgA: Immunoglobulin type A

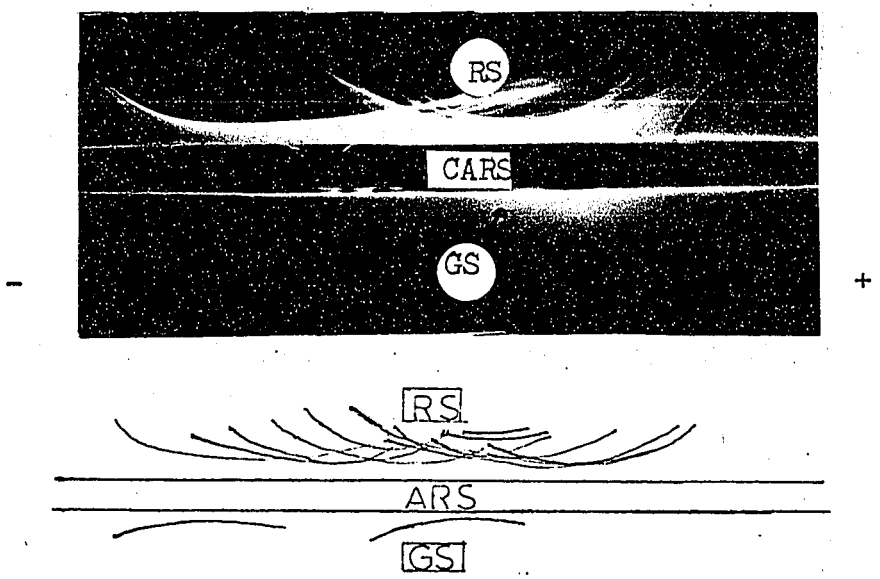


Figure 19

Agar gel immunoelectrophoresis of guinea pig serum. Anti-rat serum was used in the central trough for determination of cross reacting protein between the guinea pig serum and rat serum.

ARS: Anti-rat serum
GS : Guinea pig serum
RS : Rat serum

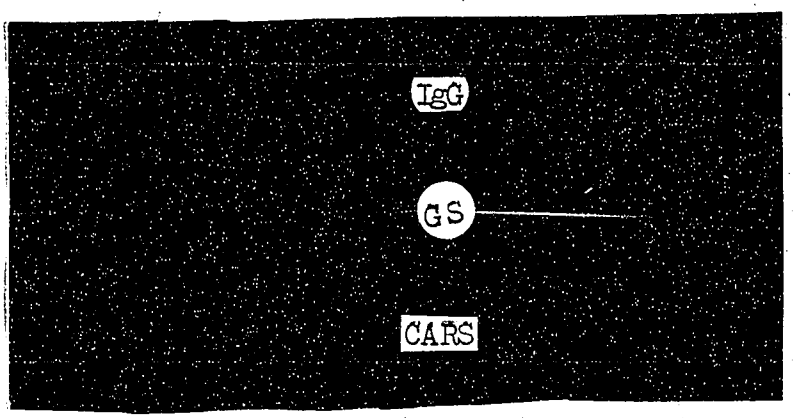


Figure 20

Agar gel immunoelectrophoresis of guinea pig serum. Anti-rat serum was used in one trough (top trough in the photograph) for determination of cross reacting protein between the guinea pig serum and rat serum. Rat IgG was used in the other trough (bottom trough) to determine whether the guinea pig serum cross reacted with the rat IgG.

ARS: Anti-rat serum
GS : Guinea pig serum
IgG: Rat immunoglobulin type G

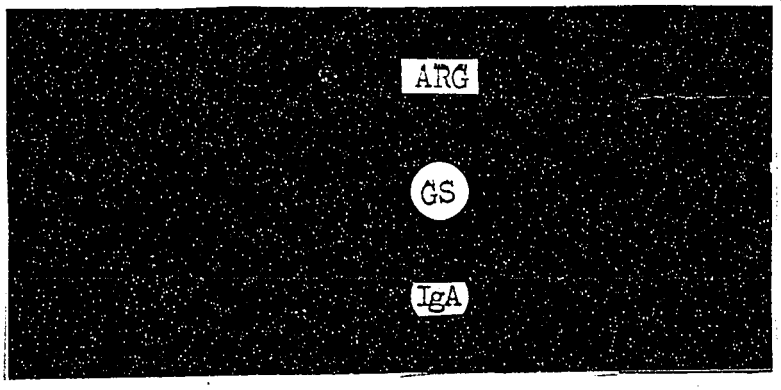


Figure 21

Agar gel immunoelectrophoresis of guinea pig serum. Rabbit anti-rat globulin was used in one trough (top trough in the photograph) for determination of cross reacting protein between the guinea pig serum and rat globulin. Rat IgA was used in the other trough (bottom trough) to determine whether the guinea pig serum cross reacted with the rat IgA.

ARG: Rabbit anti-rat globulin serum
GS : Guinea pig serum
IgA: Immunoglobulin type A

with rat IgG or IgA; this can be seen in Figures 20 and 21 since the precipitation lines for either IgG or IgA did not fuse with the guinea pig cross-reacting protein. The rabbit anti-rat IgG and rabbit anti-rat IgA sera were also tested against the guinea pig serum; no precipitation lines were observed indicating that there was no reaction between these two sera and the guinea pig serum.

Table 1 (A) shows the total number of plaques isolated and the number of plaques that were damaged or destroyed. Table 1 (B) lists the number of plaques showing precipitation lines with the rabbit anti-rat globulin; it also shows how many plaques produced either no lines, one line, two lines, or three lines. Table 1 (C) lists the number of nucleated cells in those plaques which showed more than one precipitation line with the rabbit anti-rat globulin serum.

Table 2 lists the percentage of single cells which showed more than one precipitation line with the anti-rat globulin serum; also, it lists the number of plaques containing a single nucleated cell which produced either two or three precipitation lines.

Table 3 (A) points out the total number of plaques examined by agar gel immunoelectrophoresis studies. Table 3 (B) lists the number of plaques which show either no precipitation line, one precipitation line or more than one precipitation line when studied by agar gel immunoelectrophoresis using rabbit anti-rat globulin serum to identify the protein(s). Similarly, Table 3 (C) lists the number of plaques which show none, one, or more than one precipitation

line(s) using either anti-rat IgG or IgA serum to identify the protein.

The results of these gel diffusion experiments indicate that the present method is capable of detecting immunoglobulin production by single cells. In total, 15.7% of those plaques isolated contained single cells which were detected as producing at least one type of immunoglobulin. Furthermore, 7.5% of the single cells which showed immunoglobulin production were found to produce more than one type of immunoglobulin.

Table 1

Gel Diffusion Studies Of Single Cells

A) Total Plaques Isolated

343

Total: 253

Plaques Destroyed or Damaged

90

B) Number Of Plaques Showing Precipitation Lines With Anti-rat Globulin Serum

No line	One line	Two lines	Three lines
28	166	55	4

C) Number Of Nucleated Cells In Plaques Showing 2 Or 3 Precipitation Lines

No cell detectable	One cell	Two or more cells
11	40	8

Table 2

Percentage Of Single Cells Showing Two Or More Precipitation Lines In Immunoprecipitation Studies

Number of cells	% of total plaques isolated	% of total plaques showing more than one precipitation line	Number of precipitation lines	
			2	3
37	14.6	92.5	X	-
3	1.18	7.5	-	X

Total:40

- indicates absence

X indicates presence

Table 3

Immunoelectrophoresis Studies Of Single Cells

A) Total Plaques Isolated

Plaques Destroyed or Damaged
9

Total: 41

B) Number Of Plaques Showing Precipitation Lines With Anti-rat Globulin

No lines	One line	More than one line
12	21	0

C) Number Of Plaques Showing Precipitation Lines With Either Anti-rat IgG Or Anti-rat IgA

No lines	One line	More than one line
8	0	0

DISCUSSION

A method for the detection of immunoglobulins by single antibody producing cells has been presented and supported with experimental evidence. The investigation shows that a small percentage (7.5%) of single antibody producing cells can produce different types of immunoglobulin at the same time. This finding is in accord with other investigators who have used fluorescent antibody techniques and other methods to study this problem.

Nossal (1964) found that about 14% of the cells, which were positive for antibody production produced both IgG and IgM type immunoglobulin. Mellors et al.(1963) found only a very few cells producing both IgG and IgM immunoglobulin in the same cell. However, Fahey et al. (1967) found that approximately 23% of positive immunoglobulin producing cells contained both γ and μ heavy chains. In the present investigation only 7.5% of those cells which were considered positive for immunoglobulin production produced two different immunoglobulins at the same time. This is a lower percentage than Fahey or Nossal's results. There are a few plausible reasons for this apparent discrepancy.

First, Fahey et al. used malignant lymphoid cell lines; these normally produce a greater abundance of protein than normal cell lines. A second reason for the difference depends upon the method of detection of the immunoglobulins present in the cell. In the present investigation immunoprecipitation techniques were used to detect the immunoglobulins

synthesized by the cells. Immunoprecipitation techniques depend on the formation of a precipitation line; "but the intensity of a precipitate, and therefore its potential visibility, depends primarily upon the quantity of antibody forming it .... Normally the potential sensitivity of the most sensitive unsupplemented immunodiffusion tests should be such to detect about 0.01 µg of either antigen or antibody", (Crowle, 1961). Other reports have since shown that immunodiffusion techniques can detect as little as 0.002 µg of protein (Van Furth, 1966). This means that the immunoprecipitation techniques employed in the present method are as sensitive as the strength of the antiserum used. The rabbit anti-rat globulin serum used in the investigation could detect 0.283 µg of rat IgG and 0.205 µg of rat IgA. The question that arises is whether a single cell can synthesize enough immunoglobulin which can be detected by the rabbit anti-rat globulin serum?

A number of experiments have been done to determine the amount of antibody present in a single cell. Roberts et al. (1955) reported that a single cell could produce 30×10^{-12} g of antibody in 24 hours. Humphrey et al. (1958) found $1.25\text{-}12.5 \times 10^{-13}$ g of antibody per cell; whereas Nathans et al. (1958) found $4.05\text{-}13.5 \times 10^{-13}$ g of myeloma protein per cell. Berenbaum (1958) found 12.5×10^{-13} g per cell. The findings of all these investigations were based on a number of assumptions which may or may not be valid. However, they indicate that single cells contain a smaller concentration of protein than can be detected by normal immunoprecipitation techniques. Yet, the present study shows that immunoglobulins were detected in single cells by

immunoprecipitation techniques (albeit at a lower rate than fluorescent studies). A possible explanation to this phenomenon is that those cells which showed immunoglobulin production were producing more antibody than considered normal by other reports (Humphrey et al. 1958, Roberts et al. 1955, Nathans et al. 1958, Berenbaum 1958). The fact that only a small percentage (15.8%) of the plaques, which were isolated, showed immunoglobulin production might indicate that the other isolated cells were producing normal levels of antibody; and could not be detected by the immunoprecipitation techniques. It should also be pointed out that all the other investigators reported the amount of antibody present in a single cell at any one time. However, in the present investigation the cell is actively synthesizing antibody which is diffusing into the surrounding medium. Therefore, the cell would have a total antibody production in excess of that reported by the other investigators.

What are some of the advantages and disadvantages of the present method for the detection of immunoglobulins compared to other methods? Other investigators have used immunofluorescent techniques to detect the type of immunoglobulin present in antibody producing cells (Fahey et al. 1967, Yagi 1967, Cebra 1969). Immunofluorescent techniques have a major disadvantage over the method used in the present investigation. This disadvantage lies in the fact that fluorescent antibody techniques require the cell to be killed in the staining procedure. However, in the present method the cell remains viable, actively synthesizing protein (immunoglobulin), for a period of time. Cells

have been shown to actively synthesize protein for a period of at least 20 hours (Clafin et al. 1967). Moreover, the use of the agar plaque technique (Jerne et al. 1963) affords an opportunity to screen a large number of cells for active antibody producers. Furthermore, the morphology of single antibody producing cells can be studied with respect to the type of immunoglobulin(s) that they are actively synthesizing.

The only seemingly disadvantage to the present method is the fact that it does not seem to be as sensitive in detecting immunoglobulin(s) as the fluorescent studies. However, the sensitivity can be increased by the use of autoradiographic techniques. In general, the advantages of the present method outweigh this disadvantage.

In what manner can the present method be altered to increase its efficiency? First, the complement employed (See text) could be from the same species of animal as the specific antiserum; this would prevent a cross-reaction between the complement and the rat serum. Second, the incubation period of the spleen cells in the blood-agar mixture could be varied to allow the cells to remain viable for a longer time. Third, the antiserum used for the detection of the immunoglobulins could be concentrated to increase its avidity (combining capacity).

Although the method presented in this investigation has demonstrated that it can be used for the detection of immunoglobulins in single cells, only the initial ground work had been laid. There remains much more to do: 1) a study to determine whether single antibody producing

cells can synthesize both 19S and 7S antibody with the same specificity; 2) a study to determine whether single antibody producing cells can synthesize both 19S and 7S antibody with more than one specificity; 3) a study of the pluorpotential ability of single antibody producing cells with respect to the specificities of the antibody produced (that is, whether a single cell can produce antibodies with distinct specificity to two or more different antigens); 4) a study of malignant lymphoid and plasmacytoid cells with respect to the type of immunoglobulins they synthesize; 5) a study of the amount of protein (immunoglobulin) a single antibody producing cell can synthesize over a period of time. The present method has been developed to lay the foundations for subsequent studies involving single cell antibody production.

SUMMARY

The present investigation has presented a method for studying immunoglobulin production by single cells. Jerne's agar plaque technique was used as a basis for isolating single antibody producing cells; in this technique the cells remained viable, actively synthesizing protein, for a period of time.

The results of the experiments, using the present method, show that 15.8% of those plaques which were isolated (343 plaques in total) contained single cells which produced at least one type of immunoglobulin; 7.5% of these cells (which showed immunoglobulin production) contained single cells which were found to be producing two different immunoglobulins at the same time. In total, 40 plaques contained single cells which synthesized immunoglobulin; 3 of these 40 contained single cells which produced two different immunoglobulins.

The present method was found to be somewhat less sensitive than the fluorescent technique in the detection of immunoglobulin production. Fahey et al. (1967) found that 23% of the single cells studied produced heavy chains of two different immunoglobulins.

However, the present method has a distinct advantage over fluorescent techniques in that the cell, located in the plaque, is actively synthesizing antibody (immunoglobulin) which diffuses into the surrounding medium.

A number of ways were discussed to improve the method and make it more sensitive. Also, a number of problems dealing with single

cell antibody production were suggested in which the present method could be applied.

APPENDIX

1) Sheep Erythrocytes

Sheep blood in Alsever's solution (Department of Agriculture, Hull, Quebec).

2) Veronal Buffer Stock (Nastuk et al, 1969)

5, 5-diethyl barbituric acid (A)	5.75 g
Sodium 5, 5-diethyl barbiturate (B)	3.75 g
Sodium chloride (C)	85.00 g
Distilled water	2000 ml

The barbituric acid (A) was dissolved in 500 ml of hot distilled water, and was allowed to cool. Sodium barbiturate (B) and sodium chloride (C) were dissolved in one litre of distilled water. The barbituric acid (A) was added to this solution (B + C) and then diluted to a total volume of two liters.

Stock Calcium Chloride

Calcium chloride.....	0.15 M

Stock Magnesium Chloride

Magnesium chloride.....	0.50 M

Veronal Buffer for Complement Studies

Stock veronal buffer	50 ml
Stock calcium chloride	0.25 ml
Stock magnesium chloride	0.25 ml
Distilled water	200 ml

3) Eagle's Minimal Essential Medium (Eagle, 1959)

A. Amino Acid Stock Solution

Bacto-TC Amino Acids Minimal Eagle-Dried, 5856, Difco Laboratories, Detroit, Michigan, U.S.A.

B. Vitamin Stock Solution

Bacto-TC Vitamins Minimal Eagle-Dried, 5730, Difco Laboratories, Detroit, Michigan, U.S.A.

C. Stock Salt Solution (10 fold concentration)

Sodium chloride	34.00 g
Potassium chloride	2.00 g
Magnesium chloride (6 H_2O)	1.00 g
Sodium dihydrogen phosphate (2 H_2O)	7.50 g
Distilled water	500 ml

The solution was stored frozen in 100 ml aliquots.

D. Glutamine Stock Solution

Glutamine	2.50 g
Distilled water	50 ml

The solution was stored frozen.

E. Stock Antibiotic Solution

TC Penicillin-Streptomycin Desiccated, Difco Laboratories, Detroit, Michigan, U.S.A.

F. Eagle's Minimal Essential Medium Working Solution

Stock Amino Acids	0.51 g
Stock vitamins	100.00 mg
Stock salt solution	100 ml

Stock antibiotic solution	10.00 ml
Stock glutamine	5.85 ml
Glucose	1.00 g
Sodium bicarbonate	2.00 g

The salt solution was dissolved in 700 ml of warm distilled water. The amino acids were then dissolved in this solution followed by the vitamins. After the vitamins had dissolved, the glutamine, glucose and antibiotics were added in that order. The pH was adjusted using 1.4% sodium bicarbonate. The solution was then made up to one liter with distilled water. The solution was sterilized by Seitz filtration and dispensed in 50 ml aliquots in sterile vials. They were stored at 4° C.

4) Agar-Gel Diffusion

A. Phosphate Buffer for Gel Diffusion

a) Disodium hydrogen phosphate (2 H_2O) 0.01 M	1500 ml
b) Sodium dihydrogen phosphate 0.01 M	200 ml
c) Sodium chloride	34.00 g

The solution was adjusted to pH 7.4 by adding extra (a) or (b). The volume was then made up to four liters with a buffered solution of (a) and (b) in a ratio of 8:3 volumes respectively.

B. Agar Preparation in Phosphate Buffer

a) Phosphate buffer	100 ml
b) Agar (purified special grade, Mann Research Laboratories)	1.00 g

c) Sodium azide (Fischer Scientific) ..... 0.01 g

The 1% agar was stored in 20 ml aliquots in sterile vials at 4° C.

5) Agar-Gel Electrophoresis

A. Electrode Buffer (μ= 0.05)

Sodium barbital ..... 0.50 M

Hydrochloric acid ..... 1.00 N

The sodium barbital was adjusted to a concentration of 0.048 M with distilled water. The pH was adjusted to 8.2 using the hydrochloric acid.

B. Agar Preparation in Electrode Buffer

Electrode buffer ..... 100 ml

Agar (Mann Research) ..... 2.00 g

Sodium azide ..... 0.02 g

The 2% agar solution was diluted 1:1 with distilled water before use. This resulted in a 1% solution with an ionic strength of 0.025.

6) Sephadex Column Chromatography

A. Sephadex G-200 (Pharmacia, Upsala, Sweden)

B. Elution Buffer

a) Sodium dihydrogen phosphate 0.01 M ..... 650 ml

b) Disodium hydrogen phosphate 0.01 M ..... 3350 ml

c) Sodium chloride ..... 36.00 g

The preceding ratio of (a), (b) and (c) gave a pH of 7.4

7) Diethylaminoethyl (DEAE) Cellulose chromatography

A. DEAE Cellulose (Mann Research Laboratories)

B. Buffers for DEAE Cellulose (Oh & Sanders, 1966)

Sodium Phosphate buffer (0.01 M) pH 7.8

0.035 M NaCl in Sodium Phosphate buffer (0.01 M) pH 7.0

0.070 M NaCl in Sodium Phosphate buffer (0.01 M) pH 6.5

0.200 M NaCl in Sodium Phosphate buffer (0.01 M) pH 6.5

0.500 M NaCl in Sodium Phosphate buffer (0.01 M) pH 6.5

C. Generation of DEAE cellulose before use

DEAE cellulose (dry) ..... 200 g

Hydrochloric Acid 0.5 N .....3000 ml

Sodium Hydroxide 0.5 N .....6000 ml

The DEAE was suspended in the HCl and left for thirty minutes. The supernatant was poured off, and the DEAE washed with distilled water until pH 4.0. Then the DEAE was suspended in three liters of the NaOH and left for thirty minutes. The supernatant was removed and the entire process (NaOH and HCl) was repeated. Finally the DEAE was washed with distilled water until pH 6.0-7.0.

8) Alum Precipitate Antigen

Protein solution ..... 7 ml

Distilled water ..... 16 ml

Alum sulphate $KAl(SO_4)_2 \cdot 12H_2O$ ..... 18 ml

The pH was adjusted to 6.5 with NaOH 5 M. The precipitate was washed three times and made up to 25 ml with 0.9% NaCl.

9) Freund's Adjuvant

Bacto Adjuvant complete, Freund, Difco
Laboratories, Detroit, Michigan ..... 5 ml
Protein solution ..... 5 ml

The two were mixed thoroughly by drawing the solution into and out of a small bore syringe. Mixing was continued until the solution became thick and creamy.

10) Coomassie Blue Stain (Mann Research Laboratories, New York, N.Y. 10006, U.S.A.)

Coomassie Blue ..... 1 g
Deionized water .....100 ml

11) Thiazine Red Stain (Allied Chemical Corporation, 40 Rector Street, New York 6, N.Y., U.S.A.)

Thiazine Red .....0.1 g
Acetic acid (1.0%) .....100 ml

12) Immunofluorescent Stain

Fluorescein isothiocyanate (Mann Research)

13) Wright-Giemsa Stain (Fischer Scientific Company)

A. Giemsa Stain

Giemsa Stain ..... 2 g

Glycerol .....100 ml

The stain and the glycerol were heated in a water bath, 55-60° C for 2 hours with stirring at frequent intervals. Care was taken to avoid absorption of moisture by covering the mouth of the flask with paper secured in place by elastic bands.

B. Stock Solution of Wright-Giemsa Stain

To the 100 ml of Giemsa staining solution prepared in (A) 100 ml of Wright's staining solution (2 g per 1000 ml of methanol) was added and allowed to stand overnight. The next day an additional 900 ml of Wright's solution was added, and the entire 1100 ml of Wright-Giemsa stain was filtered.

C. Working Solution

Wright-Giemsa stock solution ..... 1 part

Neutral Phosphate buffer pH 7.0 ..... 9 parts

The slides were stained for ten minutes.

14) Goat Antiserum to Rat Globulin (Hyland, Division of Travenol Laboratories, California, U.S.A.)

15) Rabbit Antiserum to Rat Serum (Hyland)

16) Rabbit Anti-Rat Gamma Globulin Serum (Pentax, Kankakee, Illinois, U.S.A.)

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