STUDIES ON THE EXOTOXIC FACTORS OF LEPTOSPIRA POMONA IN VITRO AND IN OVO

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INTRODUCTION

Disease is defined as a definite state of ill health having a characteristic train of symptoms, involving the whole body or any of its parts, and with the etiology, pathology and prognosis either known or unknown. Microorganisms which incite disease within the host are called pathogens, and the ability of any pathogen to produce disease is called its pathogenicity.

In order for an organism to cause infection it must first overcome the natural defenses of the host and must establish itself on or within the host and multiply (Raffel, 1961). The host presents a wide array of defenses against such invasions by microorganisms; likewise, the invading microbe may possess one or several aggressive systems to aid in gaining a foothold within the host. Hyaluronidase, fibrinolysin and leukocidin are prominent examples. Toxins produced by microorganisms, either in the form of exotoxins or endotoxins, kill or injure tissues and constitute perhaps the greatest threat to the health and well-being of the host. Classic examples of toxin producing microorganisms are Corynebacterium diphtheriae and Clostridium botulinum.

Much has been learned about the pathogenicity and virulence of many microorganisms. The leptospires, however, still remain available for much investigation in this respect, for very little is known regarding their disease-producing characteristics. This statement has added significance when one considers that over fifty leptospiral serotypes have been described, three-fourths of which have been associated with disease in man (Alston and Broom, 1958).

The first notable step in solving the key to leptospiral infections occurred in 1956 when a "hemolysin" was demonstrated in certain pathogenic serotypes. Injection of this hemolytic component into susceptible animals produced a syndrome comparable to the actual symptoms of the disease itself (Sleight and Langham, 1962).

Other serotypes of pathogenic leptospires, however, do not produce any demonstrable <u>in vitro</u> hemolytic activity. Perhaps, then, the hemolytic factor is not solely operative in the course of the disease and an additional factor, or factors, may be present.

The object of this study, therefore, was two-fold. First, to further characterize the "hemolysin" produced by certain leptospires, primarily through electrophoretic and gel

filtration technics. Second, it was hoped that the presence or absence of toxic substances other than "hemolysin" could be demonstrated.

LITERATURE REVIEW

Leptospirosis

Spirochetal fever was first described in 1886 as a febrile disease associated with jaundice and characterized by involvement of the spleen and kidney (Dubos, 1958). In 1915 Inada et al. (from Alston and Broom, 1958) described Leptospira icterohemorrhagiae as the causative agent of Weil's Disease in man. Since Inada's first descriptions on the mode of infection, etiology, and specific therapy of "spirochaetosis icterohaemorrhagica" many workers have investigated the leptospires in an attempt to unravel the mysteries of their pathogenicity. Fukushima et al. (from Van Theil, 1948) demonstrated the effects of a leptospiral toxin on guinea pigs. When introduced into the animal this toxin produced hyperemia and fever. Stavitsky (1948) could not find any trace of this toxin, nor could he find any detectible amounts of hyaluronidase, fibrinolysin, leukocidin or coagulase.

A significant break-through occurred when Alexander et al. (1956) described the presence of a hemolysin in the supernatant fluids of certain leptospiral cultures. This hemolysin was water-soluble, non-dialyzable, thermolabile and oxygen-stable (Russell, 1956; Alexander et al., 1956).

Levels of hemolysin were highest one to three days following optimal growth (Alexander et al., 1956). These workers theorized the hemolytic activity was due to a toxin produced by the organism. Russell (1956) noted that most hemolytic activity occurred at 37 C and that the hemolysin was heat-labile but unaltered by repeated freezing and thawing. This hemolysin easily lysed the erythrocytes of cattle, sheep and goats, but had no effect on guinea pig erythrocytes (Bauer and Morse, 1958).

Dozsa et al. (1960) determined the hemolytic activity of leptospiral hemolysin against the erythrocytes of 44 wild and domesticated animal species. Only the erythrocytes of the Pecora (red deer, roe deer, black buck, Mongolian gazelle, mouflon, Barbary sheep, goat, Alpine ibex, cattle, buffalo and zebu cattle) were susceptible.

The effect of hemolysin on lambs was investigated by Kemenes (1958). A single intravenous injection of 150 to 200 ml of the supernatant fluid of Leptospira pomona cultures, or repeated injections of smaller amounts, resulted in anemia. Bauer et al. (1961), using concentrated and partially purified hemolysin, produced a hemolytic anemia in lambs evidenced by icterus and hemoglobinuria.

Bauer et al. (1961) also studied the in vivo hemolytic process following infection with washed L. pomona cells. This study indicated that hemolysin was associated with a leptospiremia, and hemolysis continued up to 2 days in the presence of agglutinins, suggesting that the hemolytic process was irreversibly established before antibody was produced.

Rogols et al. (1959) suggested that the hemolysin might be a phospholipase since phospholipids acted as inhibitors of hemolysin.

Bertók and Kemenes (1960) demonstrated that leptospiral lipase activity is present in non-pathogenic and pathogenic strains. This enzyme had different properties than hemolysin. Lipase was more heat stable than hemolysin and appeared earlier and persisted longer in cultures. Like hemolysin, it could not be dialyzed. Lipase, specifically tributyrinase, activity was present in virulent serotypes, e.g., L. icterohemorrhagiae, L. canicola, L. pomona, and others.

Wolff (1954) found that pathogenic leptospires decreased in virulence upon repeated transfer in culture media. Bauer and Morse (1958) could find no correlation between hemolysin production and virulence. Bertók and Kemenes (1960) stressed that lipase and hemolysin production, per se, cannot be associated with virulence.

Most of the hemolytic activity associated with the growth of leptospires is found in the culture supernatant fluid (Russell, 1956). Imamura et al. (1957), however, subjected L. icterohemorrhagiae to sonic vibration and injected the cell-free portion of the sonic extract intracutaneously into rabbits and guinea pigs. Large, erythematous areas approximately 20-30 mm in diameter developed. The non-protein nature of the substance producing this erythema was apparent due to it its positive Molisch and Bial's orcinol reaction and its negative biuret reaction.

Electrophoresis

Electrophoresis is the movement of charged particles suspended in a liquid medium, under the influence of an applied electric field (Overbreek and Lijklema, 1959). A variety of supporting media is available to the investigator including starch gel, starch block, filter paper, agar gel and sponge rubber (Smith, 1960). Kohn (1957) introduced cellulose acetate as a supporting medium for electrophoresis. Cellulose acetate is advantageous in electrophoretic analysis since the time of the electrophoretic run is greatly decreased from that of conventional filter paper strips. Also, adsorption is minimal, thus resulting in practically no

"tailing." Fractions which do not separate well on filter paper may be demonstrated on cellulose acetate. Of particular biological significance is the separation of serum proteins by this method (Putnam, 1960). Flynn and DeMayo (1951) and Owen (1956) have pioneered much work in the electrophoretic studies of serum protein. Aaronson and Grönwal (1957) identified twelve separate serum fractions using cellulose acetate electrophoresis.

Gel filtration

While electrophoresis is based on migration of particles in an electrical field, gel filtration is based entirely on the separation of substances according to their molecular dimensions. Sephadex (Pharmacia), composed of small grains of hydrophilic, insoluble cross-linked dextran, represents a great advance in molecular investigations (Pharmacia, 1959). Treatment of the sample undergoing gel filtration is exceptionally mild since the only fractionating process occurring is one of filtration. Sephadex possesses the added advantage of automatic regeneration after the sample has passed through the column (Pharmacia, 1959). This is easily accomplished by washing the column thoroughly with an eluent, such as water or a suitable buffer. Sephadex is also useful in

effecting a concentration of a given substance (Flodin, et al., 1960). Due to the hydrophilic nature of the gel, water is easily removed and the activity of the substance undergoing concentration remains unaltered. The use of Sephadex also eliminates lengthy dialysis procedures such as those inherent in the preparation of fluorescent antibody reagents (Killander, et al., 1961). Sephadex has been used successfully for the purification of a variety of substances which include virus suspensions (Matheka and Wittmann, 1961), fractionation of human sera (Epstein and Tan, 1961), enzyme preparations (Gelotte and Krantz, 1959), and rattlesnake venom (Bjork and Porath, 1959).

Tissue culture technics

Tissue culture, per se, is the growth and maintenance of cells in vitro on a suitable supporting menstrum (Cunningham, 1960).

As early as 1913 Steinhardt et al. (from Dubos, 1958) demonstrated that vaccinia virus survived several weeks in a tissue suspension made from corneas of rabbits and guinea pigs. Rous and Jones (1916) devised a method for the preparation of suspensions of tissue cells by treatment with trypsin.

This treatment facilitated the growth of monolayer cultures and introduced uniformity into tissue culture preparations by insuring an approximately equal number of cells in each culture flask. Youngner (1954) observed better results in preparing tissue cultures by subjecting cells undergoing trypsinization to mechanical agitation. Dosser (1961) stressed the importance of the inoculation of the optimum number of cells for a given amount of medium as a strict prerequisite for obtaining suitable monolayer cultures.

Fischer et al. (1948) noted that embryo extract alone could not furnish all substances necessary for normal cell growth. Ebeling (1921) used a medium containing fibrinogen, serum, and tissue juice and noted that the cells grew as well in this mixture as in plasma and embryo extract.

Baker (1929) suggested that a definite oxidation-reduction potential in the medium might be as important for the continuous proliferation of fibroblasts as a given hydrogen ion concentration or osmotic pressure.

Baker and Carrel (1925) have shown that the growth-inhibiting action of serum was due largely to serum lipids.

Swim and Parker (1955) reported storage of HeLa cells at 4 C for six weeks without replacement of nutrient during storage and even several days after the cells were returned to 37 C.

Antibiotics have had great importance in tissue culture investigations. When present in the growth and maintenance media, they help prevent bacterial contamination and allow greater availability of number and size of tissue cultures (Goldberg, 1959). Penicillin and streptomycin are still the antibiotics of choice and are commonly added in concentrations of 100,000 units per liter and 1,000 mg per liter, respectively (Goldberg, 1959; Cunningham, 1960).

Embryonating egg technics

The history of embryonating egg technics dates back to nearly the same time as the first pioneering efforts in tissue culture. Rous and Murphy (1911) used embryonating avian eggs to study chicken sarcomas. Gay and Thompson (1929) noted an increase in vaccinia virus introduced into the yolk sac of a developing embryo. Woodruff and Goodpasture (1931) successfully infected the chorio-allantoic membrane with fowlpox virus. Goodpasture and Buddingh (1933) and Stevenson and Butler (1933) nearly simultaneously reported the production of a vaccine against smallpox which was prepared from extracts of infected chorio-allantoic membrane.

Leptospires and the embryonating egg technic

The leptospires and embryonating egg technics have only recently been united into a feasible research tool. Most of the research has been limited to studies on newly hatched chicks. However, Byrne et al. (1955) experimented with embryonating chicks by injecting live leptospiral cultures into the yolk sac of 17-day-old embryos. Upon hatching, the leptospires could be recovered in blood or urine cultures. These chicks died four to five days after hatching. Injection of 1-day-old chicks with L. canicola produced a leptospiremia of approximately 28 days duration. During this time, no other evidence of leptospirosis was observed. Ringen and Okazaki (1956) studied 2-day-old chicks to determine their susceptibility to infection. Hoag et al. (1953) reported that a leptospiremia persisted in all chicks examined through the ninth day following inoculation and often as late as the twenty-first day.

MATERIALS AND METHODS

A. Hemolysin Studies

Organisms

Two strains of Leptospira pomona obtained from stock laboratory cultures were used in these experiments. The Lethal Wickard strain (LW), originally isolated in 1956, was employed in the production of hemolysin. Johnson (J), a non-hemolysin-producing strain, served as a control culture. Large volumes of culture supernatant fluid were obtained by inoculating 5 ml of a 7-10 day culture into 450 ml Stuart's medium (Difco) containing 50 ml sterile, inactivated rabbit serum. The cultures were incubated at 29 C for approximately 14 days, or 1-2 days after maximum turbidity was observed. The organisms were then removed by positive pressure through a Seitz-type filter.

Preparation of hemolysin

Hemolysin was precipitated from the culture filtrate by 40 per cent saturation with ammonium sulfate at 4 C for 12 hours (Bauer et al., 1961; Carter, 1957). The precipitate was harvested by centrifugation in the cold at 10,000 rpm for 30 minutes in an I. E. C. Model HR-1 refrigerated centrifuge

with an 858 angle head. The precipitate was resuspended to 5 per cent of the original culture volume in 0.15 M phosphate buffered saline (PBS), pH 7.2, ionic strength 0.15, and dialyzed in the cold against PBS until no sulfate ion could be detected in the dialysate when tested with a 10 per cent barium chloride solution. Merthiolate (Lilly) was added until a final concentration of 1:10,000 was reached and the solution of crude hemolytic extract was stored at 4 C until further use.

Alternative method of hemolysin preparation

An alternative method of hemolysin preparation was also employed in these studies. The technics used were identical to those above except the crude hemolytic extract was dialyzed 2 hours against distilled water and 72 hours against frequent changes of 0.005 M phosphate buffer, pH 8.1, ionic strength 0.005, in order to precipitate the euglobulin portion of the hemolytic extract. Following this precipitation the contents of the dialysis tubing were centrifuged at 10,000 rpm for 15 minutes at 4 C, again in the 858 angle head. The resulting euglobulin precipitate and the supernatant fluid were each tested for hemolytic activity.

Hemolytic activity assay method

The method of Bauer and Morse (1958) was employed for the assay of hemolytic activity and was as follows: A series of two-fold hemolysin dilutions was made in PBS to a total volume of 1 ml per tube. Dilutions ranged from 1:2 to 1:512. To each tube 1 ml of a 1 per cent suspension of washed sheep erythrocytes was added. The tubes were incubated for 30 minutes in a 37 C water bath followed by 60 minutes at 4 C. The tubes were then centrifuged in an I.E.C. Model CL centrifuge with a 215 head for 5 minutes at 1,500 rpm to sediment the erythrocytes. The optical density of the supernatant fluid was determined in a Bausch and Lomb Spectronic 20 colorimeter at 540 mμ. In each instance a set of controls was employed. The positive hemolysis control consisted of 1 ml of a saponin solution plus 1 ml of the erythrocyte suspension. The erythrocyte control consisted of 1 ml of PBS and 1 ml of the erythrocyte suspension. The optical density of the hemolysis control was assigned a value of 100 per cent hemolysis. The optical densities of the hemolysin dilutions were converted to per cent hemolysis. The reciprocal of the dilution producing 50 per cent hemolysis was designated as the number of hemolytic units (HU) per ml of hemolysin.

Electrophoresis

For the electrophoretic studies a Shandon Universal Electrophoresis Apparatus and VoKam Constant Voltage/ Constant Current D. C. Power Supply were used. Cellulose acetate strips (Colab), 2.5 x 12 cm, were used as the supporting medium. The principal electrophoretic buffer employed was Oxoid barbitone acetate buffer (Colab) and was prepared by dissolving 8.8 grams of buffer crystals in one liter of distilled water. The pH of this buffer was 8.6 with an ionic strength of 0.07. Tris-E.D.T.A.-Boric Acid buffer (Aaronson and Grönwal, 1957) with a pH of 8.6 and an ionic strength of 0.05 was also used.

An electrophoretic run consisted of applying 0.005 ml of the sample of crude hemolytic extract to a cellulose acetate strip impregnated thoroughly with buffer solution and fastened securely in position on the strip supports of the electrophorator. The sample was applied directly above the negative electrode. All electrophoretic analyses were carried out at constant amperage at a current level of one milliampere (1 mA) per strip. The runs were performed at 4 C to minimize heating effects. The duration of each run was 2 hours with the barbitone acetate buffer and 45 minutes with the Tris-E.D.T.A.-Boric Acid buffer. At the end

of the designated times the current was switched off and the strips were removed with forceps and placed immediately in a 0.2 per cent Ponceau S (National Aniline) in 3 per cent trichloracetic acid solution and stained for approximately 10 minutes (Colab, 1960). The strips were thoroughly rinsed in a 2 per cent acetic acid solution to remove any unadsorbed dye and dried between sheets of paper toweling. In cases where staining was insufficient with Ponceau S, a 0.001-.002 per cent Nigrosin stain was used (Smith, 1960; Colab, 1960).

In an effort to determine the effects of electrophoresis on the hemolytic activity of the crude hemolytic extract a number of strips were removed from the electrophorator, sectioned into three equal pieces, and transferred directly to blood agar plates, consisting of blood agar base and defibrinated bovine blood. These plates were incubated at 37 C and observed a total of 48 hours for hemolysis.

As a hemolysis control 0.005 ml of the crude hemolytic extract was introduced directly onto the surface of a control blood agar plate and incubated under the same conditions.

Gel filtration

The gel filtration column was prepared by introducing approximately 30 grams of dry Sephadex G-200 into a

sufficiently large volume of PBS and allowing it to swell and settle. The excess buffer was decanted to remove the "fines" and the mixture was then introduced gradually into a 4 x 60 cm chromatographic column and allowed to remain undisturbed until the gel completely settled. A disc of filter paper was carefully laid in place on top of the gel to help prevent agitation of the column contents. A three liter reservoir containing PBS was placed above the column and connected to it by rubber tubing fitted with a clamp which served to regulate the flow of buffer into the system. Buffer was introduced carefully onto the top of the gel to a depth of approximately three inches. The flow rate of the column was adjusted so that the amount of buffer entering the top of the column was equal to the amount of fluid leaving the bottom of the column. The apparatus was allowed to undergo final settling by permitting the buffer to flow through the system overnight at 4 C.

Before any analytical work could be performed the column required standardization to determine when molecules of a known size would leave the system. Normal chicken serum was chosen as the standardizing agent.

The flow of buffer from the reservoir was stopped and the column was allowed to drain until the level of the

buffer was even with the filter paper disc covering the gel. Ten ml of chicken serum were carefully introduced onto the filter paper pad with a Pasteur pipette and allowed to absorb into the top of the column. When the level of sample introduced equaled the height of the paper disc the supply of buffer from the reservoir was carefully reintroduced into the system, again to a depth of about three inches. The flow rate was readjusted and the sample passed through the gel into collection tubes. All fractionation procedures, including chicken serum and hemolysin preparations, were carried out at 4 C.

The various fractions resulting from the column treatment were collected in 18 x 150 mm test tubes contained in an R.S.Co Model 1205A fraction collector. These fractions were analyzed in a Bausch and Lomb Spectronic 20 colorimeter at 750 mμ for their protein content as determined by the Lowry modification of the Folin technic (Lowry, et al., 1951).

Electrophoretic analysis of the normal chicken serum fractions

Determination of the electrophoretic mobility of the resulting fractions of normal chicken serum following

treatment with gel filtration was also undertaken. Random samples of 0.005 ml each were taken from the collection tubes and subjected to cellulose acetate electrophoresis, using barbitone acetate buffer. At the end of the 2 hour electrophoretic run the strips were removed and stained for 12 hours in a 0.001-0.002 per cent Nigrosin stain.

An identical amount of normal chicken serum was fractionated electrophoretically in the same manner and stained 10 minutes with Ponceau S. The strip of normal chicken serum and strips from the randomly selected fractions were then compared.

Based on known molecular weights of various serum components (Putnam, 1960), as well as the electrophoretic mobility of the various fractions as compared to whole normal chicken serum, it was easily determined when different molecular weight entities made their exit from the column.

The methods used in standardizing the column were reemployed in the analyses of the crude hemolytic extract. In this case 10 ml of sample were introduced onto the column and fractions were collected and analyzed for protein content. Those collection tubes having a high protein content were pooled and concentrated by pervaporation at

4 C. These preparations were then analyzed for possible hemolytic activity.

Effect of pH on the stability of hemolysin

The stability of the crude hemolytic extract at various pH levels was also determined. Routine hemolytic assays were performed as before, except the pH of the buffer diluent and erythrocyte suspension was changed in each assay. pH values ranging from 5 to 9 were employed. Control systems were subjected to the same pH variation.

Effect of storage on the stability of hemolysin

The stability of the hemolytic activity of the crude hemolytic extract was also examined. Samples were stored at 4 C and -20 C and examined for hemolytic activity at 7, 14 and 60 day intervals.

Lipase determinations

The protein fractions resulting from gel filtration of the crude hemolytic extract were analyzed for lipase activity. The test was performed in the following manner: (Sigma, 1961).

1. A substrate consisting of 2.5 ml water; 3.0 ml Sigma Lipase Substrate, Stock No. 800-1; and 1.0 ml Tris

Buffer, Stock No. 800-2 was prepared for each sample to be analyzed.

2. 1.0 ml of sample was added to each substrate tube.
3. Tubes were shaken vigorously for 5 seconds and incubated for 6 hours in a 37 C water bath.
4. At the end of the incubation period the contents of each tube were poured into clean 50 ml Erlenmeyer flasks.
5. 3.0 ml of 95 per cent ethyl alcohol were added to each flask, followed by the addition of 4 drops of thymolphthalein indicator.
6. The contents of each test flask, plus a blank control flask, were titrated against 0.05 N NaOH until a faint blue color appeared.
7. The number of ml necessary to titrate the blank was subtracted from the amount necessary to titrate the sample.
8. The difference between these two numbers represented the values of lipase present.

The 0-40 per cent ammonium sulfate fractions from strain LW, strain J and from uninoculated Stuart's medium were also analyzed for lipase activity in the same manner.

B. Studies on Other Toxic Factors

Organisms

Again, two strains of Leptospira pomona were chosen as test organisms. Strain LW and strain J were grown as before. Following maximum culture turbidity each culture was filtered through a Seitz-type filter to remove the organisms.

Fractionation procedures

Ammonium sulfate was added to each culture filtrate until a 40 per cent saturation level was reached. The solutions were then allowed to stand 12 hours at 4 C. The resulting precipitates were, as before, centrifuged at 10,000 rpm for 30 minutes at 4 C and resuspended to 5 per cent of the original volume in PBS. To the remaining filtrates ammonium sulfate was added until a 60 per cent saturation level was achieved. Centrifugation and resuspension of this precipitate were carried out in an identical manner. Ammonium sulfate was again added to the remaining filtrates until an 80 per cent saturation level was reached, centrifugation and resuspension repeated, and, finally, ammonium sulfate was added to the remaining filtrates until 100 per cent saturation was attained. These

procedures resulted in obtaining four fairly concentrated protein fractions from each culture.

Uninoculated Stuart's medium containing 10 per cent inactivated rabbit serum underwent identical fractionation and served as a medium control, supposedly void of any toxic factors.

Each of the fractions was dialyzed against PBS until no trace of sulfate ion was detected in the dialysate. Each of the twelve fractions--four from the culture filtrate of strain LW, four from the filtrate of strain J and four from the uninoculated Stuart's medium--was assayed for hemolytic activity. Merthiolate was added and the solutions were stored at 4 C until further use.

Tissue culture experimentation

Bovine fetuses were obtained through commercial meat packing sources. The kidneys were removed aseptically and placed in Hank's balanced salt solution (BSS) (Cunningham, 1960) containing penicillin and streptomycin in suitable concentration. All solutions employed in tissue culture experimentation contained these two antibiotics as an added safeguard against possible bacterial contamination.

Cortical tissue was removed aseptically and placed in sterile petri dishes containing BSS. The tissues were then minced with scalpels, resulting in a finely diced tissue suspension. After repeated rinsings in BSS the suspension was placed in BSS containing 0.25 per cent trypsin 1:250 (Difco) and trypsinized 1 hour at room temperature. The cells were allowed to settle in the flask and the trypsin solution was carefully decanted. Fresh trypsin solution was added and the mixture was again trypsinized an additional 30 minutes at 37 C. Following trypsinization the cells were washed in BSS and centrifuged at 1,500 rpm for 5 minutes. This washing and centrifugation procedure was repeated twice and the resulting mixture was filtered through sterile gauze. The filtrate was again centrifuged at 1,500 rpm for 5 minutes to sediment the cells. Approximately 0.4 ml of the packed cells were added to 200 ml of growth medium, which consisted of BSS solution containing 0.5 per cent lactalbumin hydrolysate plus 10 per cent sterile bovine serum. Five ml aliquots of this mixture were added to sterile 2-ounce prescription bottles, resulting in a total of 40 separate cultures. These were incubated at 37 C and observed routinely for monolayer formation.

Following monolayer formation the growth medium was removed and the cells were maintained by the addition of a maintenance medium consisting of BSS solution containing 0.5 per cent lactalbumin hydrolysate plus 2 per cent sterile bovine serum.

The monolayers were then subjected to the following inoculation procedures: Varying amounts, ranging from 0.1 ml to 0.5 ml, of the leptospiral culture filtrate fractions and the uninoculated Stuart's medium fractions were introduced onto the monolayers. A total of 36 monolayer cultures were inoculated. A set of controls was also employed in these experiments and consisted of introducing sterile Stuart's medium containing 10 per cent inactivated rabbit serum nto two monolayer cultures. The two remaining cultures were uninoculated. The cultures were then incubated further at 37 C and observed for cytopathic effects.

Chicken embryo experimentation

Embryonating chicken eggs were obtained through a commercial poultry raising concern. The two routes of inoculation chosen were yolk sac and intravenous. For yolk sac studies inoculations of 0.5 ml into 7-day-old embryos were employed. Intravenous studies, utilizing 0.05 ml inoculums,

were performed on 15-day-old embryos (Cunningham, 1960). In each trial a minimum of 10 embryos was inoculated with each of the 12 protein fractions prepared. This resulted in a total of 120 embryos for each trial. The eggs were incubated an additional 6 days in the case of the yolk sac inoculation route, and 5 days for the intravenous route in a Jamesway Model 258 incubator at 38 C and observed daily.

Heat lability studies

All fractions toxic to tissue cultures and/or embryonating eggs were suspended in a 56 C water bath for 5, 10, 15, 20 or 30 minutes. At the end of the designated times an aliquot of each fraction was removed from the bath, cooled, and injected into embryos via the yolk sac route. The embryos were incubated and observed as before.

Electrophoresis

Fractions of the culture filtrates, other than the crude hemolytic extract, which were lethal to the tissue culture monolayers and/or the embryonating chicken eggs were analyzed electrophoretically on cellulose acetate strips. The entire electrophoretic procedure employed in these particular experiments was identical in all respects to that used in analysis of the crude hemolytic extract, including amount

of sample analyzed, time of electrophoretic run, applied field strength, buffers and staining technics.

Sterility controls

In all embryonic work a series of sterility controls were utilized. These consisted of inoculating an amount of sample, equal the amount injected into the embryonating chicken eggs, into thioglycollate broth (Difco) tubes. These tubes were incubated at 37 C and observed for 72 hours for evidence of bacterial growth.

Treatment of the 60-80 per cent fractions with Sephadex G-50

A 2.5 x 40 cm chromatographic column of Sephadex G-50 was prepared in a manner identical to that of the G-200 column used in the investigation of the hemolysin. Fractions of culture filtrates, other than the crude hemolytic extract, found to be lethal to embryos, were passed through this column at 4 C. Protein analyses of the resulting fractions were performed as before. The collection tubes under each protein peak were pooled and pervaporated at 4 C. The resulting concentrates were stored at 4 C until further use.

Lipase determinations

The fractions resulting from the gel filtration of the 60-80 per cent culture filtrate fractions were analyzed for lipase activity. The same procedure used in the analysis of the hemolysin fractions was re-employed in these particular studies.

RESULTS

Treatment of the various culture extracts and uninoculated Stuart's medium with ammonium sulfate gave rise to the following fractions:

Strain LW	0-40	per cent	ammonium	sulfate	fraction
	40-60	per cent	"	"	"
	60-80	per cent	"	"	"
	80-100	per cent	"	"	"
Strain J	0-40	per cent	"	"	"
	40-60	per cent	"	"	"
	60-80	per cent	"	"	"
	80-100	per cent	"	"	"
Uninoculated Stuart's medium	0-40	per cent	"	"	"
	40-60	per cent	"	"	"
	60-80	per cent	"	"	"
	80-100	per cent	"	"	"

For purposes of clarification the results will be divided into two parts: First, the studies with crude hemolytic extract (strain LW 0-40 per cent fraction); and second, studies on the other toxic factors.

Hemolytic activity of the euglobulin precipitate of the 0-40 per cent ammonium sulfate fraction of strain LW

Routine hemolytic assays demonstrated that almost all of the hemolytic activity associated with the crude hemolytic

extract from strain LW was present in the euglobulin precipitate. Hemolytic titers of this precipitate gave values of 128 HU/ml, while those of the supernatant fluid were only 2 HU/ml. Results are listed in Table 1.

Table 1

Comparison of hemolytic activity of the euglobulin precipitate and supernatant fluid from the 0-40 per cent ammonium sulfate fraction of strain LW culture filtrate

Sample	Hemolysin dilution in PBS (pH 7.2)								
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Euglob. ppt.	+	+	+	+	+	+	+	-	-
Super-natant	+	-	-	-	-	-	-	-	-

Key:

\+ : Greater than 50 per cent hemolysis

\- : Less than 50 per cent hemolysis

Hemolysin studies in tissue culture

Bovine embryo kidney monolayers were completely destroyed within 24 hours following introduction of the crude hemolytic extract. The cells had been completely disrupted from the wall of the culture flask and were floating within the culture medium. The phenol red indicator had not changed

appreciably from its original red color, thus indicating that very little, if any, cellular respiration had occurred.

Control cultures in these experiments responded normally and continued to maintain themselves. Cells from these cultures appeared normal when stained with Giemsa stain, while those subjected to the hemolysin preparation possessed deeply stained pyknotic nuclei and shrunken cytoplasm.

Hemolysin studies with embryonating chicken eggs

Introduction of the hemolytic extract from the culture filtrate of strain LW, either by yolk sac or by intravenous injection, killed all embryos approximately 96 hours postinoculation. Criteria for death were the apparent curling and dwarfing of the embryo, as well as lack of any visible motion. Death was verified in each case by gross examination of the exposed embryo. Results are listed in Tables 4, 5, and 6.

Those embryos dead within 24 hours postinoculation were considered traumatized.

Controls employed in these experiments were identical to those used in the tissue culture studies. All control embryos were unaffected.

Standardization of the Sephadex G-200 column

Analysis of protein fractions obtained when 10 ml of normal chicken serum was eluted from the column gave rise to three distinct protein peaks. The first peak included molecules having molecular weight of 200,000 or greater, while the second and third peaks contained molecules of approximately 200,000 to 160,000 molecular weight and molecules of less than 160,000 molecular weight, respectively. These findings were verified by electrophoresis of random samples chosen from collection tubes as compared to electrophoresis of normal chicken serum. It was ascertained from these studies that the first peak contained primarily $alpha_1$, $alpha_2$ and beta globulins, while the second peak contained a single beta globulin and gamma globulins. The third peak was composed primarily of serum albumin. The results are represented graphically in Figure 1.

Gel filtration of the crude hemolytic extract

As seen in Figure 2, elution of the crude hemolytic extract from the column again led to three distinct protein peaks, as determined by the Lowry modification of the Folin technic. Analysis showed that hemolytic activity was present only in the fraction under the first peak. This

indicated the hemolytic activity was associated with a molecule, or molecular unit, of molecular weight 200,000 or greater.

No other eluted fraction exhibited any hemolytic activity.

Heat lability of the hemolysin

Experiments determining the heat lability of the crude hemolysin extract indicated that the hemolytic activity was destroyed by heating for 5 minutes at 56 C. Experiments with embryonating chicken eggs and routine hemolytic assay technics gave identical results in this respect in that no embryos were killed with heat-treated hemolysin, and no hemolytic activity was observed *in vitro*. Results are listed in Table 3.

Storage stability of the hemolysin at 4 C

Beginning with an original hemolytic titer of 256 HU/ml the titer dropped to 128 HU/ml at the end of 7 days and finally reached a low point of 16 HU/ml after 60 days. Results are shown in Figure 3.

Storage stability of the hemolysin at -20 C

The original titer of 256 HU/ml dropped to 128 HU/ml after 7 days. At the end of 60 days the titer still

remained at 128 HU/ml. Results are show in Figure 3.

pH stability of the hemolysin

The pH range of hemolysin stability is between pH 7.0 and 8.0 with an apparent optimum value of pH 7.2. A sharp drop in hemolytic activity occurred on either side of this value, as seen in Figure 4.

Electrophoretic analysis of the hemolysin

Results in these experiments were inconclusive, in that no differences in the electrophoretic mobility of the 0-40 per cent ammonium sulfate fractions of strain LW, strain J or uninoculated Stuart's medium were apparent.

Also, no hemolysis occurred when the electrophoretic strips containing the crude hemolytic extract were incubated on the blood agar plates. Hemolysin not subjected to electrophoresis, however, did produce a detectible hemolysis within 24 hours after its application to the control blood agar plate.

Lipase activity of hemolysin

Crude hemolytic extract from strain LW contained 7.1 Sigma-Tietz lipase units per ml. Of the three protein fractions resulting from the gel filtration of this crude

hemolytic extract significant lipase activity was demonstrated in the first of these three peaks. This indicates that the lipase has a molecular weight of 200,000 or greater. This first peak contained 7.2 Sigma-Tietz lipase units per ml, as opposed to 4.8 and 4.4 for peaks two and three, respectively.

The control blank contained 4.3 Sigma-Tietz lipase units per ml. Values greater than 1.5 units over the value of the blank are considered significant.

Lipase activity of the 0-40 per cent ammonium sulfate fractions of strain J and uninoculated Stuart's medium

The 0-40 per cent fraction of strain J contained 4.9 Sigma-Tietz lipase units per ml. The identical fraction of uninoculated Stuart's medium contained 4.5 lipase units per ml.

Results of the above data are presented in Table 7.

Studies on ammonium sulfate culture fractions other than the 0-40 per cent fraction from strain LW (crude hemolytic extract)

Hemolytic activity of the various fractions

No hemolytic activity was observed in any fraction when investigated by routine hemolytic assay technics, as seen in Table 2.

Table 2

Hemolytic activity of ammonium sulfate fractions from strain LW, strain J and uninoculated Stuart's medium

Culture filtrate	Ammonium sulfate concentration			
	0-40%	40-60%	60-80%	80-100%
LW	+	-	-	-
J	-	-	-	-
Uninoc. Stuart's medium	-	-	-	-

Tissue culture studies

A common toxic factor for bovine embryo kidney monolayer tissue cultures was found in the 60-80 per cent ammonium sulfate fractions of the culture fluids of both strain LW and strain J, but was not found in the 60-80 per cent fraction of uninoculated Stuart's medium. This toxic factor acted within 48 hours postinoculation. Criteria for cytopathic effect were identical to those employed in the studies of the effect of hemolysin on tissue cultures.

Embryonating egg studies

The same toxic manifestation which appeared in the tissue culture studies was also observed in embryonating chicken eggs. The criterion here, as in the studies with hemolysin, was death of the embryo. Death resulted in an average of 7 out of a total of 10 untraumatized embryos with the 60-80 per cent ammonium sulfate fraction from strain LW, and in 5 out of 9 untraumatized embryos with the identical fraction of strain J. Death occurred approximately 96 hours post-inoculation. No such toxic effect was noted when the 60-80 per cent ammonium sulfate fraction from uninoculated Stuart's medium was employed. All embryo controls and sterility controls remained normal.

Heat lability studies

Heating for 10 minutes at 56 C negated the toxic effect of all fractions. Controls consisted of embryos inoculated with unheated fractions. Sterility controls were also employed. All were normal. The results are shown in Table 3.

Table 3

Embryo lethality following yolk sac inoculation of 56 C treated toxic ammonium sulfate fractions

Ammonium sulfate fraction	Length of heat treatment (minutes)					Unheated controls
	5	10	15	20	30	
LW 0-40%*	10/10	10/10	10/10	10/10	10/10	1/10
LW 60-80%	7/10	9/9	9/9	9/10	10/10	5/10
J 60-80%	7/10	10/10	10/10	8/9	9/9	4/10

*crude hemolytic extract

Key:

Numerator: Number of embryos surviving.

Denominator: Total number of embryos inoculated (10) less those traumatized.

Electrophoretic analysis

In the electrophoretic analysis of the 60-80 per cent fractions of strain LW, strain J and uninoculated Stuart's medium there was no significant difference in their respective migration patterns when barbitone acetate buffer, ionic strength 0.07 was used. However, when the Tris-E.D.T.A.-Boric Acid buffer system, ionic strength 0.05, was used a line common to the 60-80 per cent fractions of both strain LW and strain J was observed. This line was not found in the

identical fraction of uninoculated Stuart's medium. Results of these electrophoretic examinations are shown in Figures 5 and 6.

Lipase activity of the 60-80 per cent ammonium sulfate fractions of strain LW, strain J and uninoculated Stuart's medium

No significant lipase activity could be demonstrated in any of the protein peaks resulting from gel filtration with Sephadex G-50. Results are shown in Table 7.

Table 4

Embryo lethality following yolk sac inoculation of culture filtrate ammonium sulfate fractions (Trial #1)

Culture filtrate	Ammonium sulfate concentration			
	0-40%	40-60%	60-80%	80-100%
LW	0/10	8/9	3/10	10/10
J	8/9	9/9	4/9	10/10
Uninoc. Stuart's medium	9/9	9/9	9/9	6/6

Key:

Numerator: Number of embryos surviving.

Denominator: Total number of embryos inoculated (10) less those traumatized.

Table 5

Embryo lethality following yolk sac inoculation of culture filtrate ammonium sulfate fractions (Trial #2)

Culture filtrate	Ammonium sulfate concentration			
	0-40%	40-60%	60-80%	80-100%
LW	0/10	9/10	4/10	10/10
J	10/10	10/10	6/10	10/10
Uninoc. Stuart's medium	10/10	9/10	10/10	10/10

Key:

Numerator: Number of embryos surviving.

Denominator: Total number of embryos inoculated (10) less those traumatized.

Table 6

Embryo lethality following intravenous inoculation of culture filtrate ammonium sulfate fractions

Culture filtrate	Ammonium sulfate concentration			
	0-40%	40-60%	60-80%	80-100%
LW	2/5	5/5	3/5	5/5
J	4/5	5/5	3/8	4/5
Uninoc. Stuart's medium	5/5	4/5	5/5	5/5

Key:

Numerator: Number of embryos surviving.

Denominator: Total number of embryos inoculated less those traumatized.

Table 7

Lipase activity of various ammonium sulfate fractions

Fraction	Lipase activity (Sigma-Tietz units/ml)
Control blank	4.3
Gel filtrated hemolysin	
Protein peak #1	7.2
" " #2	4.8
" " $3	4.4
0-40 per cent fractions	
Strain LW	7.1
Strain J	4.9
Uninoculated Stuart's medium	4.5
Gel filtrated 60-80 per cent fractions	
Strain LW	
Protein peak #1	4.3
" " #2	4.1
" " #3	4.3
Strain J	
Protein peak #1	4.3
" " #2	4.4
" " #3	4.1
Uninoculated Stuart's medium	
Protein peak #1	4.0
" " #2	4.3
" " #3	4.3

Figure 1

Protein analysis of the fractions of normal chicken serum after passage through Sephadex G-200

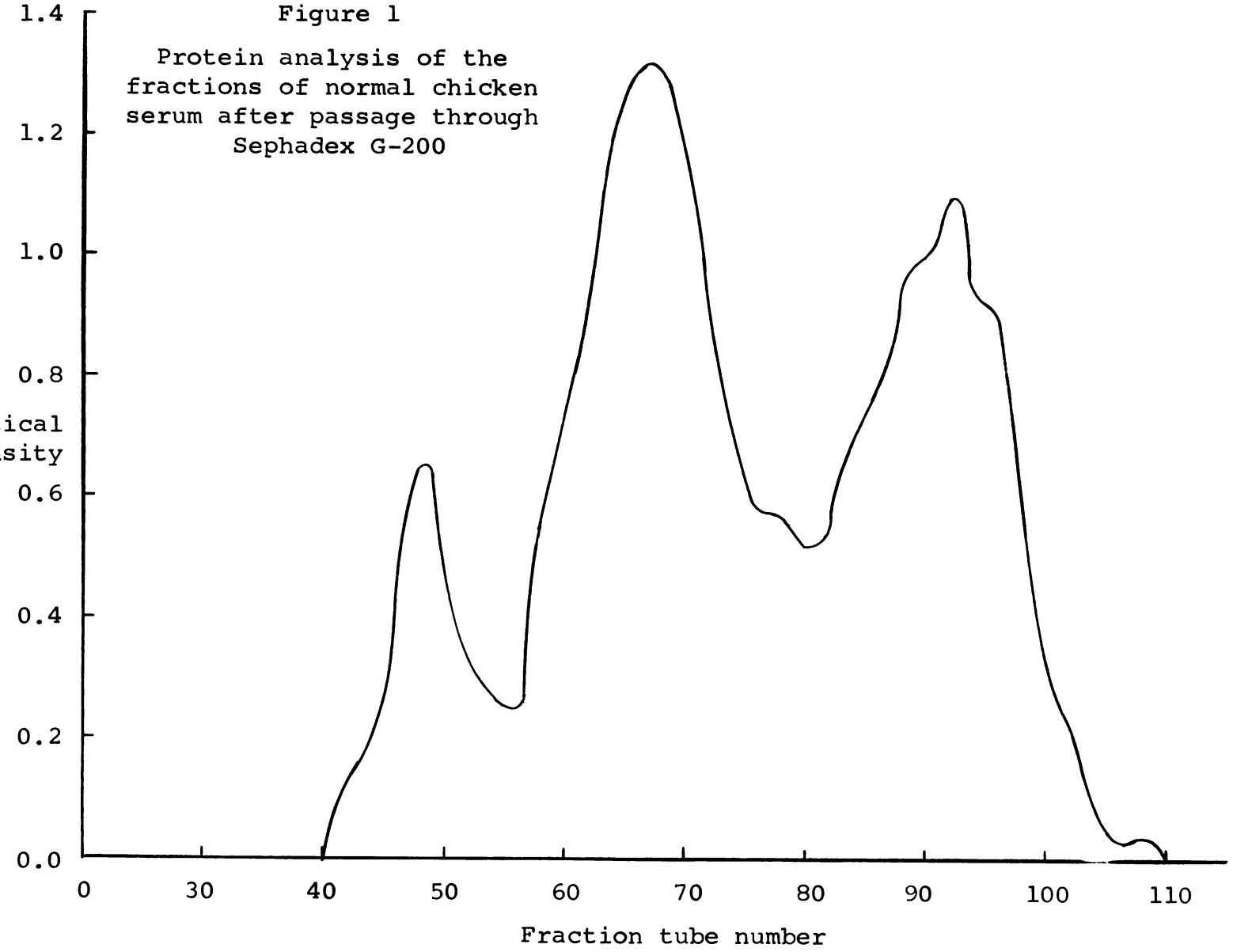


Figure 2

Protein analysis of the fractions of crude hemolysin
(strain LW 0-40% ammonium sulfate fraction)
after passage through Sephadex G-200

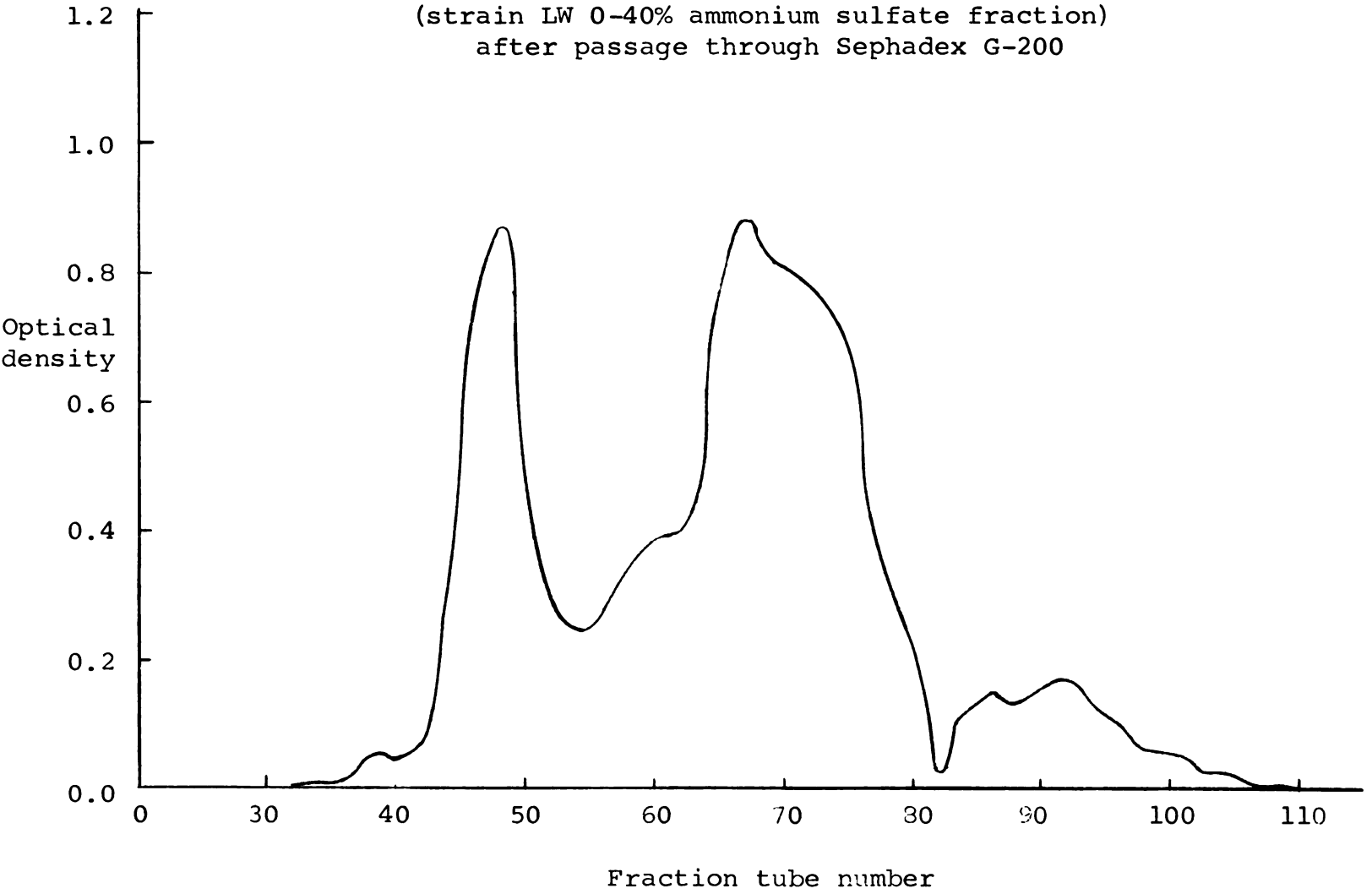
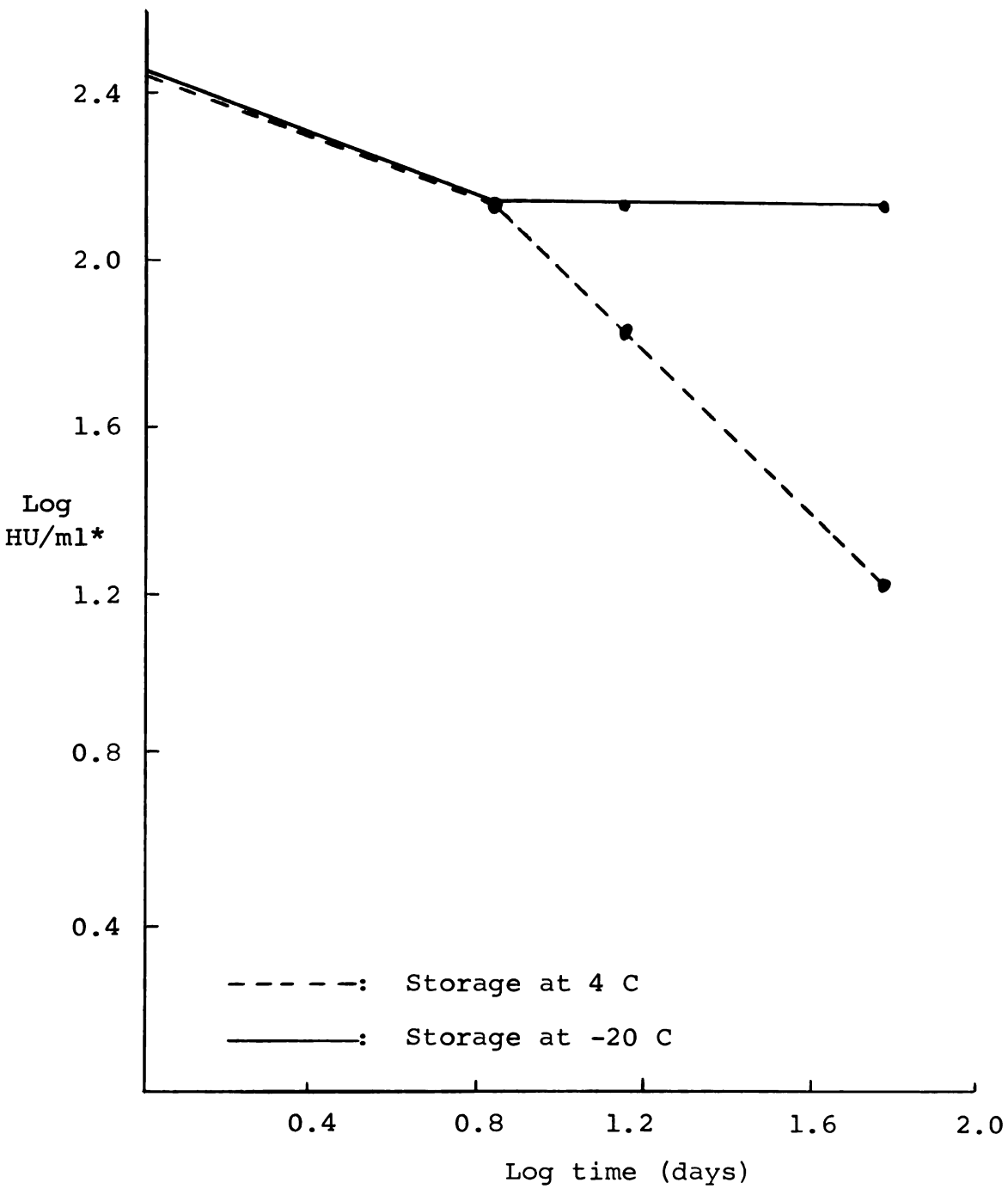


Figure 3

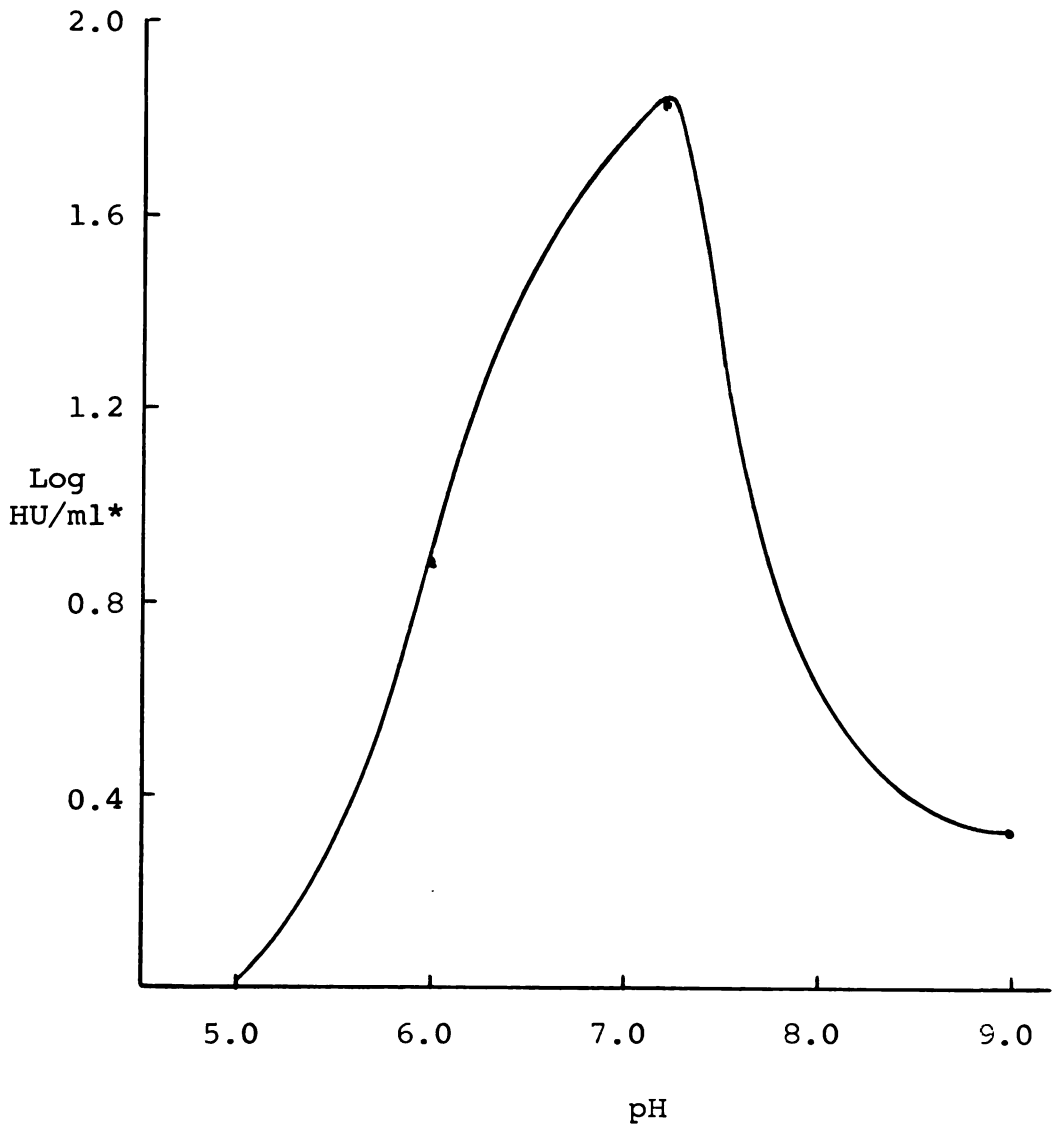
Storage stability of hemolysin
at 4 C and at -20 C



*HU/ml: hemolytic units/milliliter

Figure 4

Measurement of hemolytic activity
in vitro with varying pH



*HU/ml: hemolytic units/milliliter

Figure 5

Electrophoretic migration patterns of the 60-80 per cent ammonium sulfate fractions in barbitone acetate buffer.

A = strain LW; B = strain J;
C = uninoculated Stuart's medium

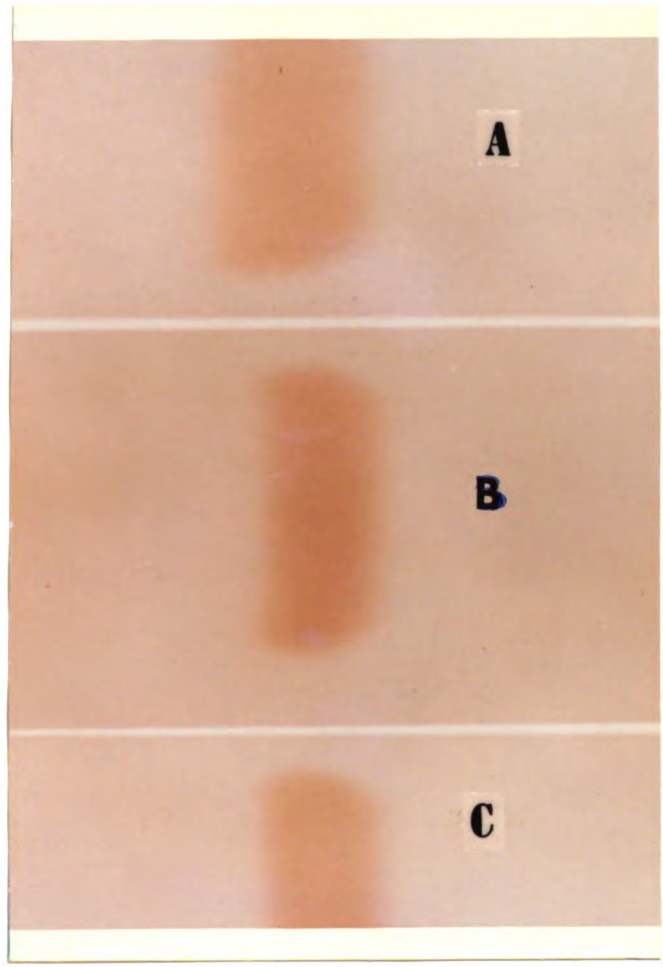
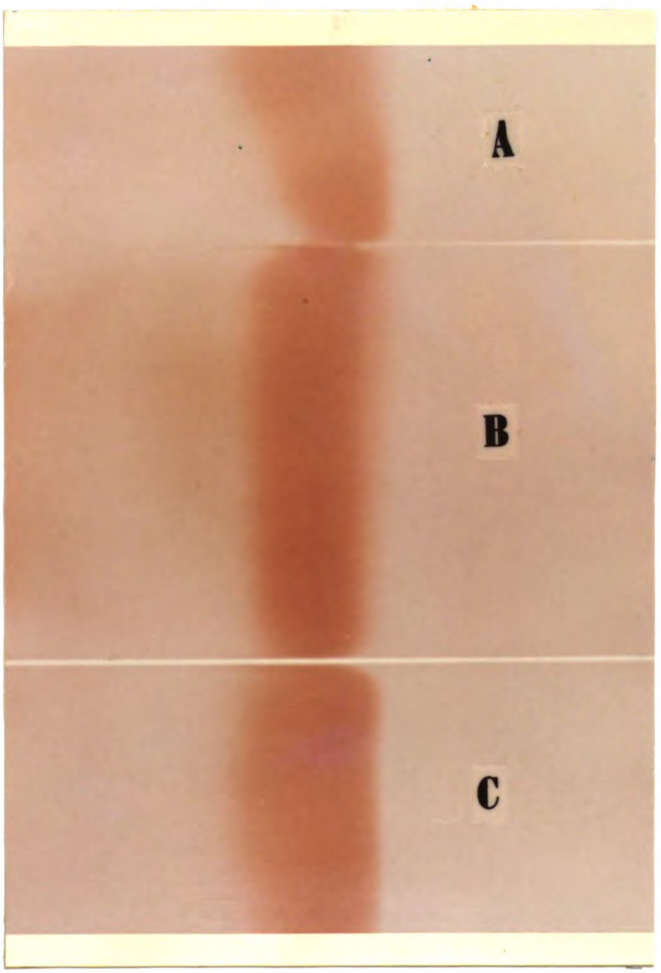


Figure 6

Electrophoretic migration patterns of the 60-80 per cent ammonium sulfate fractions in Tris-E.D.T.A.-Boric Acid buffer

A = strain LW; B = strain J;
C = uninoculated Stuart's medium



DISCUSSION

Studies on the crude hemolytic extract

Based on the analysis of crude hemolytic extract from the culture fluid of strain LW by Sephadex G-200 gel filtration a molecular weight of 200,000 or greater has been assigned to the hemolysin molecule, or molecular unit. From evidence presented here one cannot definitely state whether the hemolysin is a single molecule or a biological dimer or polymer. There may be a single, large molecule to which is attached a smaller "schlepper" unit, the two of which work in combination to produce the hemolytic activity demonstrated. It is not beyond reason to assume that the hemolytic activity may be manifest in an aggregation of several smaller biological monomers, the total molecular weight of which is greater than 200,000. Ultracentrifugation studies may be of benefit in settling this dispute in that a single, large sedimentation peak followed by one or more trailing peaks would indicate the presence of a relatively large molecule with several small molecules attached.

Certain studies have led to the conclusion that the hemolysin may be a molecular unit, rather than a single molecule. Sleight and Dardas (1960, unpublished data)

subjected the crude hemolytic extract to DEAE cellulose chromatography. Two of the resulting fractions, one fairly large and the other a smaller trailing fraction, both possessed hemolytic activity when analyzed by the standard hemolytic assay method. This apparently indicates that a fairly readily adsorbed molecule, as well as a less readily adsorbed molecule, are active in the production of hemolytic activity. These results, however, were not repeated with any degree of significance.

Detection of hemolysin using cellulose acetate electrophoresis rendered no concrete findings. No hemolytic activity was found in any of the resulting fractions. Here it seems possible that, if a molecular unit existed, the presence of an applied electric field induced the separation of the molecular species, resulting in the inactivation of the hemolysin. The possibility also exists that the hemolysin may have migrated completely off the electrophoretic strip during the course of the run.

That the hemolytic unit is of large molecular dimensions is shown both in the studies with Sephadex G-200 and by the presence of hemolytic activity in the euglobulin precipitate of the 0-40 per cent ammonium sulfate fraction of strain LW, as opposed to the pseudoglobulin supernatant. Euglobulins

have molecular weights of upwards of 1,000,000 (Fruton and Simmonds, 1958). The fact that a greater percentage of hemolytic activity is found within this euglobulin precipitate is in disagreement with Bauer (1958), but its demonstration in these current studies is of a definite correlative nature.

In previous unpublished studies (Belding and Lacey, 1962) the antigenicity of the hemolytic unit was tested in rabbits with rather unencouraging results. Two out of five rabbits inoculated with the hemolytic extract produced only a slightly detectible amount of antibody to the hemolysin as measured by the Ouchterlony technic (Ouchterlony, 1948).

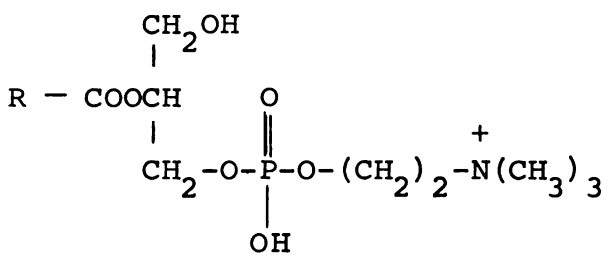
The possibility of performing L-plate studies as further correlative evidence of the molecular size of the hemolysin was not considered feasible due to the lack of knowledge regarding relative concentrations of the antigen (hemolysin preparation) and antibody (rabbit anti-hemolysin antiserum).

Lillevik (1962, personal communication) suggested that the hemolysin may be a lysolecithin. Studies to determine the validity of this assumption were not performed, but it is known that lysolecithins are powerful hemolytic agents which rapidly lyse erythrocytes, and are considered responsible for the harmful physiological effects of certain snake venoms (West and Todd, 1962).

The basic structure of a lysolecithin, however, does not endorse a chemical relationship between a lysolecithin and the hemolytic phenomenon associated with certain leptospiral serotypes. Sephadex gel filtration has indicated that the hemolysin unit has a molecular weight of 200,000 or greater. The basic structure of a lysolecithin is given below in Figure 7.

Figure 7

Schematic representation of the structure of a lysolecithin



Exclusive of the R-group, the molecular weight of the above compound is 285. If hemolysin is a lysolecithin, and possesses a molecular weight of 200,000 or greater, then the dimensions of the R-group must be astronomical. Logic erases the possibility of an R-group of such proportions. Also, lysolecithins are not precipitated by ordinary protein precipitation technics.

The possibility exists that the hemolysin is a mucoprotein but this seems unlikely in that mucoproteins, too, are not easily precipitated by ordinary protein fractionating methods, and are quite heat stable (White et al., 1959).

The demonstration of leptospiral lipase activity by Bertók and Kemenes (1960) has been confirmed. This activity was noted only in the culture filtrate from strain LW, a hemolysin producer, and not in the culture filtrate from strain J, a non-hemolysin producer. The above results would tend to implicate the lipase directly with the hemolysin suggesting, perhaps, that the hemolysin and the lipase are the same chemical entity. Certain lipases, specifically phospholipase A, are known to be powerful hemolytic agents (Fruton and Simmonds, 1958). However, Bertók and Kemenes (1960) noted that the lipase had properties which differed markedly from the hemolysin, e.g., the greater heat stability of the lipase, and its earlier appearance and longer persistence in culture filtrates. Also, lipase activity has been demonstrated in both pathogenic and non-pathogenic strains.

The possibility exists that the hemolytic activity may be due to the mutual effect of a lipase and some other loosely bound chemical component. In non-hemolytic and in non-pathogenic strains only lipase activity is present. Lack

of hemolytic activity may be due to the failure of these organisms to produce this additional chemical component.

In the gel filtration of the crude hemolytic extract both lipase activity and hemolytic activity occurred in one protein fraction alone. Since Sephadex avoids the use of ion-exchange principles and involves only a filtration process, the intact "lipase-component" unit produced both lipase activity and hemolytic activity. When subjecting this "lipase-component" unit to ion exchange resins or to electrophoresis the "lipase-component" unit may be disrupted, leaving only lipase activity present. This is a tempting speculation and warrants further biochemical investigation.

In previous experiments, as well as in the present work, the degree of purity of the hemolysin was questionable. For example, when beginning with one liter of culture filtrate containing the hemolysin, and subjecting it to the fractionation, chromatographic and concentration methods employed in these experiments, a final volume of two ml of "concentrated hemolysin" resulted, based on <u>in vitro</u> hemolytic assay methods.

The exact chemical nature of the hemolysin has not been undertaken in these studies, primarily due to the insufficient quantities of purified hemolysin available. Once obtained in a relatively pure form the basic chemical

properties of the hemolysin should be studied. Amino acid analyses and ultracentrifugation studies would be adequate starting points in this respect.

Studies on other toxic factors

The original impetus for this work came from the experiments of Lundberg (1962, unpublished data). After extraction of the hemolysin from the culture filtrate, aliquots of approximately 10 ml of the remaining filtrate were injected intraperitoneally into young lambs. These lambs were observed daily for any possible toxic effects of the culture filtrate. Only slight temperature elevations were observed during the testing period. These were attributed to proteins in the rabbit serum medium supplement. It seemed feasible, however, that other toxic factors could be present and that effects were being masked due to dilution in such a large volume. It was decided to further fractionate this remaining filtrate and concentrate the resulting fractions in a manner similar to the methods used in the preparation of hemolysin.

The presence of a toxic factor in the 60-80 per cent ammonium sulfate fraction of both strain LW and strain J has been demonstrated. This factor was lethal to tissue

cultures and embryonating chicken eggs. This fact that this toxic factor was lethal to these two assay systems, however, does not mean that this same toxic effect would be operative in another system; namely, an animal infected with either strain LW or strain J of Leptospira pomona. Under the conditions of experimentation used in this study, however, the experimental results with the embryonating eggs were repeated with nearly identical findings, thus indicating some degree of significance.

The electrophoretic analyses using cellulose acetate as the supporting medium gave mixed results. Use of the barbitone acetate buffer system produced electrophoretic strips with nearly identical migrational patterns, within experimental limits. However, when a buffer system of lower ionic strength was employed a new protein band was noted which was common to both strain LW and strain J. This fraction migrated slightly ahead of the albumin fraction and was not present when the same fraction of uninoculated Stuart's medium was tested. This new band may be a serum pre-albumin although, if this were the case, it should also appear in the uninoculated medium. On the basis of these findings it is possible that this new band could be the toxic factor present in these culture filtrate fractions.

One might question whether the death of the tissue cultures and the embryos could be due to incomplete dialysis of the culture filtrate fractions. This is unlikely, however, since dialysis was carried out identically for each of the culture filtrate fractions, as well as for fractions of the uninoculated Stuart's medium. If dialysis was not complete, and the death of the tissue cultures and embryos was due to the presence of ammonium sulfate, the uninoculated Stuart's medium should also have been lethal to the assay systems. This, however, was not the case.

Again, the exact nature of this toxic factor may well be determined once a fairly pure sample is obtained. In addition to biochemical analyses, a thorough study on its gross pathological and hematological effects should be undertaken. At present, work is in progress attempting to fractionate this toxic factor further through use of gel filtration technics. Also, in addition to the further studies on the exotoxic factors of the leptospires an investigation into the realm of possible endotoxic factors should be performed.

SUMMARY

1. Further characterization of the hemolysin from strain LW of Leptospira pomona has been carried out, primarily through gel filtration and cellulose acetate electrophoresis. On the basis of the gel filtration experiments a molecular weight of 200,000 or greater has been assigned to the hemolysin.

2. This hemolysin has also been shown to be a euglobulin which is stable at or near pH 7.

3. The hemolysin is destroyed by heating for 5 minutes at 56 C, and also by electrophoresis.

4. An alternative method of hemolysin preparation, involving euglobulin precipitation technics, has been presented. This method of preparation resulted in obtaining a more concentrated hemolysin.

5, A lipase with a molecular weight of 200,000 or greater has been observed in the 0-40 per cent ammonium sulfate fraction of strain LW. No lipase activity was noted in analogous fractions of strain J or uninoculated Stuart's medium.

6. Studies on other possible leptospiral exotoxic factors have been undertaken through electrophoresis and also by tissue culture and embryonating egg technics.

7. The presence of a toxic factor, other than hemolysin, present in the 60-80 per cent ammonium sulfate fractions of culture filtrates of both hemolytic and non-hemolytic strains of L. pomona has been demonstrated. The assay media for this study were bovine embryo kidney monolayers and embryonating chicken eggs. This toxic effect was not noted in the identical ammonium sulfate fraction of uninoculated Stuart's medium.

8. Further biochemical investigation into these toxic factors is suggested.

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