AN INVESTIGATION OF A HEMOLYTIC TOXIN FROM HISTOPLASMA CAPSULATUM

by

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INTRODUCTION

In order to understand the fundamentals of the disease process, it is necessary to study the mechanisms of the conflict between the parasite and host. Such mechanisms are often difficult to study due to the participation of multiple factors; however, it is, fortunately, often possible to isolate from the parasite single factors of potential significance in virulence and to study the relation of such a material to the host and its defense mechanisms.

This investigation was initiated in order to study toxic substances produced by the pathogenic fungus, Histoplasma capsulatum. The major part of the study has been concerned with the isolation and partial characterization of a hemolytic material from yeast phase cells of this fungus. Secondarily, it has been shown that serum from non-immunized animals possesses the ability to neutralize this hemolytic toxin in vitro.

HISTORICAL

As early as 1888 Roux and Yersin (1) and soon afterward Kitasato (2) recognized the production of toxins by bacteria. In many diseases these toxins are a major part of the parasite's invasive mechanisms. However, in other diseases the role of toxins is somewhat obscure. Bacterial diseases may vary from those characterized by a parasite of high invasiveness as in anthrax to complete toxic death as in botulism. For the complete understanding of any disease, however, the virulence factors produced by the parasite must be studied.

Toxins are generally classified as endotoxins or exotoxins depending upon their relationship to the cells from which they are derived. Toxins released into the medium are classified as exotoxins while those obtained only by breaking the cells are classified as endotoxins. This classification becomes somewhat arbitrary when it is realized that some "exotoxins" are derived only from old cultures which are undergoing autolysis. Typically however, exotoxins are obtained from cultures in their logarithmic growth phase just before reaching maximum growth.

A more precise and descriptive classification of toxins is based upon their activity. This includes not only lethal toxins but those showing any of a number of characteristic reactions upon the host. Neurotoxins are those which attack the nervous tissue of the host. The blood constituents are attacked by hemolysins, destroying erythrocytes; fibrinolysins, destroying clots; coagulase, clotting plasma; and leucocidins, destroying

leucocytes. Enzymes of various substrates are recognized as toxins since they perform a specific function detrimental to the host. These are the proteolytic enzymes and hyaluronidase.

Perhaps the most prolific toxin producing organism is Clostridium perfringins. At least more toxins have been identified and studied from that organism than any other. It is known to produce five major toxins; alpha, beta, gamma, delta, and epsilon. The most important of these in infection is the alpha toxin which is responsible for the toxemia in gas gangrene.

The alpha toxin was shown by Glenny (3) to be lethal, dermonecrotic, and hemolytic. The chemistry of the hemolytic reaction has been fully studied so it can now be described as a chemical reaction. Nagler (4) and Seiffert (5) first discovered the release of fat from serum from in vitro cultivation of Cl. perfringins. This same reaction of release of fat from egg yolk was reported by MacFarlane (6) who showed the serum reaction, egg yolk reaction and hemolysis were all due to the alpha toxin. Further chemistry (7, 8) showed certain ion dependencies of the reaction and finally MacFarlane and Knight (9) in 1941 showed the toxin to be a lecithinase. To date this stands as the only toxin whose action can be chemically described.

Two other hemolytic toxins are produced by Cl. perfringins, those being the delta and theta toxins. Of these, theta is the more investigated and appears to be related to the oxygen-labile hemolytic toxins of other bacteria. It has been identified (10) as a "hot-cold" hemolysin. That

is, one which must be incubated at 37 C for a period and then in the cold for a period in order to obtain its maximum activity.

Ganley (11) was able to show a correlation between toxic activities of fractions from cultural filtrates of Cl. perfringins and symptoms of gas gangrene. By use of heavy metal-ethanol precipitation techniques, he obtained a fraction which caused cardiovascular collapse but was not alpha or theta toxin. A second fraction was obtained which caused a marked phagocytosis inhibition but showed no other toxic activities and therefore was considered not one of the classical toxins. Each of these activities (cardiovascular collapse and phagocytosis inhibition) is characteristic of gas gangrene.

The staphylococcus toxins have been known for many years. As early as 1894 van der Velde (12) observed the filterable activities of the staphylococci and was supported soon after by Denys and Havet (13), von Lingelsheim (14), Kraus and Clairmont (15) and Neisser and Wechsburg (16). Three hemolysins are known. Of these, only the alpha toxin is thoroughly studied and is dermonecrotic, lethal and hemolytic. It has been shown (17, 18) that the production of this toxin is directly related to pathogenicity and rapid methods of identification of this toxin have been devised by Gillespie and Simpson (19) and Elek (20).

The staphylococcus beta toxin is a typical example of a "hot-cold" hemolysin and was first described by Bigger et. al. (21) in 1927. Its lytic activity is primarily upon the cells of sheep and the ox with only slight activity on cells of other species (22, 23). Gamma and delta

toxins have not been well studied but are known to have a wider erythrocyte spectrum than the beta toxin.

Perhaps the most studied of the hemolytic toxins are streptolysin "O" and streptolysin "S". These are toxins produced by the streptococci and several other bacterial species (24, 25, 26, 27). Streptolysin O is an oxygen-labile compound produced by several groups of the streptococci. Herbert and Todd (28) showed that reduction of the oxygen-inactivated toxin would restore its activity. It was also found (29) that these oxygen-labile compounds could be inhibited by cholesterol.

Streptolysin "S", first isolated by Weld (30, 31), was so named by Todd (32) because it was produced only in the presence of serum. He suggested that O is produced with or without the presence of serum but S would only be produced in the presence of serum. It was shown (33) that washed streptococci would lyse erythrocytes. Okamoto (34) and later Bernheimer (35, 36) demonstrated that the cells would produce no hemolysin until brought into contact with certain factors such as ribonucleic acid.

Non-specific neutralization of the toxins produced by bacteria has been suggested for many years. As early as 1930 Kerrin (37) noted the antihemolytic effect of normal serum from rabbit, horse and humans. Then in 1939 Todd (38), testing cross neutralization of streptolysins, found that in some cases there was as good protection using normal serum as there was when he used immune serum. Humphrey (39) suggested that the antistreptolysin of various species was not a true

antibody but rather a lipoprotein component of the serum. Stallerman and Bernheimer (40) studied in detail this neutralization of streptolysin S in serum from persons with rheumatic fever and acute streptococcal pharyngitis. The results of this and a subsequent study (41) suggested to them that the active agent was a lipoprotein fraction of the blood. The activity was not destroyed at 56 C for 2 hours and was increased when heated at 60 C. They found the activity to be located in the albumin or alpha globulin fraction by ammonium sulfate precipitation and in the fraction IV by the Cohn ethanol precipitation.

Other serum components have been implicated as protective agents against toxic materials. Davis and Dubos (42) suggested serum albumin as a protective agent for binding fatty acids.

As was pointed out earlier, the streptolysin O is inhibited by cholesterol. Serum cholesterol will not inhibit except in old serum. Hewitt and Todd (43) showed that this is possibly due to contaminating enzymes or normal breakdown of the cholesterol in aging serum. The form of the cholesterol is changed from what is normally found to a form which is active in inhibiting hemolytic activity of streptolysin O.

From the clinical picture it would appear that there are several toxins associated with H. capsulatum. There are two main types of histoplasmosis: primary and progressive. At least 95 per cent of these cases are asymptomatic. They may show any grade of lung involvement as seen by x-ray with no more severe symptoms than a mild fever and a cough. Calcification in the lungs seems to be slower

than in primary tuberculosis.

Progressive histoplasmosis, though usually fatal, results from less than 0.1 per cent of the primary infections. The primary lesion in the progressive form is usually extrapulmonary being on the lips, tongue, ears, pharynx, or larynx. The intestinal tract is a common portal of entry for children while in adults, the most frequent is the skin. The primary lung lesion is difficult to differentiate from primary tuberculosis. As the disease progresses there results an enlargment of the liver and spleen with the development of leucopenia and anemia. The presence of anemia might suggest a hemolysin and the presence of the leucopenia a leucocidin.

Fungus toxins have, for the most part, been neglected until recently. As early as 1902 toxin was extracted from spores of Aspergillus fumigatus (44) and a few years later Bodin (45) obtained a toxin from an 18 day culture filtrate. Henrici (45) obtained an endotoxin from the mycelium. Toxic substances have also been demonstrated in the saprophytic fungi (47, 48).

The pathogenic fungi have not been investigated thoroughly. Drouhet and Segretain (49) reported a toxin from Cryptococcus neoformans which inhibited leucoyte migration. Salvin (50) reported a lethal dosage for killed cells of Candida and various other pathogenic fungi including blastomyces and histoplasma. More recently (51) he has reported hemolytic toxins from disintegrated cells of these organisms. He made no attempt to purify the toxins.

METHODS AND MATERIALS

Culture - Throughout these studies, strain HC-12 of Histoplasma capsulatum was used. This culture was furnished by Dr. C. W. Emmons and was originally isolated from a human. Yeast phase cultures were maintained at 37 C on cystine heart agar (Difco) with 10 per cent human blood added and also in neopeptone dialysate broth under constant agitation at 37 C.

Preparation of crude toxin - Yeast phase cells of Histoplasma capsulatum were used exclusively. One liter quantities of neopeptone dialysate (52) were dispensed into two liter Erlenmeyer flasks and sterilized. Each flask was inoculated with approximately 50 ml. of a 6 day broth culture of the HC-12 strain. The flasks were then placed on a New Brunswick Rotary Shaker at 37 C and agitated at 230 R.P.M. for 6 days. At the end of this time, the flasks were removed from the shaker and checked for purity of culture. One per cent formalin was added and the flasks allowed to stand at room temperature for 48 hours to permit the cells to settle out. The majority of the supernate was siphoned off and the remainder centrifuged. The cells were washed 3 times with distilled water to remove any medium constituents.

After washing the cells, ten glass marbles and a volume of sand equal to the volume of packed cells were added to each bottle and the bottles rotated on a ball mill for 12 hours at 4 C to grind the cells. The ground cells from 14 liters of culture were transferred to one and a half

liters of distilled water, shaken thoroughly and allowed to stand at least 3 hours at 4 C.

The mixture was then centrifuged at 2000 R.P.M. for 20 minutes on a type 2 International Centrifuge. The supernate from this first centrifugation was centrifuged twice more to be sure all large particles had been removed.

The remaining supernate was an opaque white suspension which was stable over long periods of standing. It shall be referred to subsequently as "crude toxin." It was lyophilized to dryness and stored in a dessicator.

Purification of toxin - Unless otherwise stated, each fraction described was finally dissolved in distilled water, dialyzed against running tap water at 10 C for 48 hours, lyophilized and stored in a vacuum dessicator at 4 C.

Zinc acetate precipitation - Solid zinc acetate was added to a suspension of crude toxin in amounts from 0.05 M to 0.40 M and the pH adjusted to 7.0. This was accomplished by either adding each concentration of salt to an individual aliquot or by increasing the concentration of a single aliquot after removal of the precipitate from the previous salt addition. In either case, the precipitates were removed by centrifugation and redissolved in 4/15 M phosphate buffer at pH 7.3. The resulting insoluble zinc phosphate was removed by centrifugation and the supernate dialyzed and lyophilized.

Copper acetate precipitation - Aliquots of a suspension of crude toxin were tested to determine the quantity of saturated cupric acetate

necessary to cause miximal precipitation of the toxin. The whole suspension was then precipitated by the addition of the proper amount of saturated cupric acetate. The resulting bluish precipitate was removed by centrifugation and resuspended in distilled water. The toxin was freed from copper by either of two methods:

(a) The solution was placed into the central chamber of an electromigration vessel whose three chambers were separated by cellophane diaphragms. Water was placed in the two end chambers and electrodes introduced into these end chambers. Sufficient acetic acid was added to each chamber to allow a current flow of 5 ma. at 400 V potential. The dialysis continued at 4 C until there no longer existed a blue color in the central chamber. It was necessary to make several changes of water in the terminal chambers during this time. Each change of water was lyophilized and tested for toxic activity. When the bluish color had been removed, the material remaining in the central well was lyophilized to dryness and tested for activity.

(b) A more convenient and rapid method consisted of sorbing the copper with an ion exchange resin. Amberlite IRC-50 (Rohm and Haas), a weak cationic resin, was used as the sorbing agent. It is of large particle size which allows for rapid and easy removal from the solution by settling and decantation. The resin was first thoroughly washed by several changes of sodium hydroxide over a period of 24 hours to convert it to the sodium salt. About 25 per cent (v/v) of resin to solution was added and stirred continuously at 4 C for two hours.

Fresh resin was added and the procedure repeated for one resin change after no bluish color developed on the resin. The supernate was then treated as previously described.

Ammonium sulfate precipitation - A saturated ammonium sulfate solution was added to the toxin suspension in sufficient amounts to give the desired final concentration of ammonium sulfate. The addition was made at room temperature slowly with constant stirring. At each concentration the pH was adjusted to neutrality in order to obtain maximum precipitation. Each fraction was removed by centrifugation, redissolved and lyophilized.

Ethanol precipitation - To a suspension of crude toxin was added 10 per cent sodium acetate and one per cent acetic acid followed by a slow addition of absolute ethanol either at room temperature or at 4 C. Fractions were removed at one volume increments up to 5 volumes of ethanol. After each addition of ethanol the mixture was allowed to stand for a minimum of two hours at 4 C to allow the formation of a precipitate. If a precipitate formed, the mixture was allowed to stand overnight in the cold and the precipitate removed by centrifugation to be tested for activity.

Crude toxin was also fractionated by the cold ethanol procedure of Cohn (52). The ionic strength of the toxin suspension was unknown and was therefore disregarded. Fractionation was performed using reagents and techniques suggested by Cohn for plasma.

Acid precipitation - A suspension of crude toxin was adjusted from pH 7.0 to pH 4.0 in 0.5 pH unit steps and a precipitate was observed at the lower pH values. Either pH 4.0 sodium acetate-acetic acid buffer or normal acetic acid appeared to be effective. When the approximate range of precipitation was determined, titration at closer pH intervals was used to ascertain the exact range of activity.

Solvent extraction - The methanol, chloroform and acetone used in these studies were redistilled reagents from the commercially available C.P. grade solvents. The diethyl ether used was commercial anhydrous analytical reagent. Extraction by these solvents from a water suspension of the toxin was impossible; therefore, either lyophilized crude toxin or lyophilized acid precipitate of the toxin were used for extraction. Five hundred mg. to one gram samples of toxin were extracted with 150 ml. of solvent at room temperature for 12 hours with frequent agitation. The majority of the material was insoluble and could be filtered off at the completion of the extraction. After filtering the supernate, it was placed under vacuum from a water aspirator, kept at approximately 30 C and evaporated to one tenth volume. Ten ml. of distilled water were added and evaporation continued until the volume was 9 ml. and there no longer remained any odor of the solvent. An opaque white suspension remained which was stable to centrifugation. An aliquot was removed, placed in a tared watch glass and evaporated to dryness to determine weight of material present.

For successive extractions, the residue of the previous extraction was placed under vacuum until no odor of solvent remained and

the second solvent was added as before.

Combinations of solvents were tested for efficacy of extraction by adding two solvents in equal proportions to the dried toxin.

Serum fractionations - Blood was drawn from each of 8 normal rabbits and pooled. The clot was allowed to stand at 4 C overnight and the serum removed by centrifugation. This serum was either used immediately or stored at -25 C until used. Serum was not permitted to stand more than 24 hours in the cold nor was it thawed more than once.

Ammonium sulfate fractionation - Saturated ammonium sulfate was added dropwise with constant stirring either at room temperature or at 4 C. Fractions were removed at 0 - 33, 33 - 50 and greater than 50 per cent saturation of ammonium sulfate. Each fraction was removed by centrifugation, dialyzed and lyophilized as before.

Ethanol fractionation - The cold ethanol procedure of Nichol and Deutsch (53) was used to prepare fractions of serum. A flow sheet of this procedure is seen in appendix I. Serum was frozen, thawed and diluted 1/4 with distilled water. It was placed in a -10 C bath until ice crystals began to form. With constant stirring an equal volume of cold (-20 C) 50 per cent ethanol was added slowly. The precipitate was allowed to stand 15 to 20 minutes and then centrifuged at -5 C at 2000 R.P.M. for 40 minutes. The supernate containing albumin and alpha globulin was decanted, dialyzed against cold distilled water and lyophilized.

Ice crystals were added to the pellet and 100 ml. of cold distilled

were added. The pellet was put into solution and the pH adjusted to 5.1 with 0.05 M acetic acid. The solution was then cooled to -6 C and cold 50 per cent ethanol was added with constant stirring until the concentration reached 10 per cent. The precipitate was allowed to stand 30 minutes and centrifuged at -5 for 40 minutes at 2000 R.P.M. The supernate was decanted and the pellet was dissolved in distilled water, dialyzed and lyophilized. It contains the beta globulin.

The supernate was adjusted to pH 7.4 - 7.7 with 0.5 M sodium bicarbonate. The solution was cooled to -5 C and an equal volume of cold 50 per cent ethanol was added. After standing 4 hours the precipitate was centrifuged for 30 minutes at -5 C at 2000 R.P.M. The supernate was decanted and the pellet, containing the gamma globulin wad redissolved in distilled water, dialyzed and lyophilized.

By this procedure only the gamma globulin can be considered of very high purity, but a relatively high degree of purification is accomplished for each of the other fractions.

Also the cold ethanol procedure of Cohn (52) for the fractionation of human plasma was used to fractionate rabbit plasma. A diagram of this procedure is seen in appendix II. The following procedure was used for 100 ml. of plasma:

The plasma was cooled to 0 C in an ethanol-ice bath and 17.7 ml. of 50 per cent ethanol together with 0.1 ml. of 0.8 M sodium acetate buffer at pH 4.0 were added to give a final concentration of 8 per cent ethanol at pH 7.2 The resultant precipitate was centrifuged at 2000 R.P.M.

for 30 minutes at -5 C. The precipitate (Cohn fraction I) is almost entirely fibrinogen.

To the supernate was added 60.1 ml. of 50 per cent ethanol, 0.088 ml. of 10 M acetic acid, 0.044 ml. of 4 M sodium acetate and 0.23 ml. of 95 per cent ethanol. This resulted in a final ethanol concentration of 25 per cent at pH 6.9. The precipitate was centrifuged at 2000 R.P.M. for 30 minutes at -5 C. This gave a mixture of beta and gamma globulin in Cohn fraction II, III.

In two steps the supernate was adjusted to 18 per cent ethanol at pH 5.2 by the addition of 31.1 ml. of distilled water brought to -5 C and then 78 ml. of water adjusted to pH 5.2 with acetate buffer. This was stirred for one hour and allowed to stand for 6 - 8 hours. The precipitate is Cohn fraction IV_1 containing alpha globulin.

To the supernate was added 0.114 g sodium bicarbonate, 0.79 ml. of 4 M sodium acetate and sufficient water to bring the total to 77 ml. This was allowed to stand one half hour and 80.5 ml. of 95 per cent ethanol were added. This brought the concentration to 40 per cent ethanol at pH 5.8. The precipitate was removed by centrifugation as before. This resulted in Cohn fraction IV_4 containing alpha and beta globulins.

Fianlly the supernate was adjusted to pH 4.8 by the addition of 0.5 ml. of 10 M acetic acid, 0.025 ml. of 4 M sodium acetate, 0.105 ml. of 95 per cent ethanol and enough water to make 25 ml. This stood 3 hours at -5 C and was centrifuged as before. The resulting

precipitate was Cohn fraction V containing albumin.

The remaining supernate was Cohn fraction VI which contains only the inorganic materials from the plasma.

Preparation of erythrocytes - Blood was taken by cardiac puncture into syringes containing sufficient 10 per cent sodium citrate to give a final concentration of 1 per cent. The cells were immediately centrifuged and washed 3 times with physiological sodium chloride solution. After the final washing the cells were suspended according to cell count or per cent volume of packed cells by comparison to a standard curve of hemoglobin.

The standard curves of erythrocyte count and per cent concentration were prepared as follows: A suspension of cells was prepared containing 4 per cent erythrocytes by volume. Dilutions of this were made in 0.9 per cent sodium chloride, referred to throughout the thesis as "saline," and an aliquot of each was lysed with distilled water and measured colorimetrically on a Klett Summerson Photoelectric Colorimeter. Simultaneously cell counts were made of each dilution so that for any given hemoglobin content both cell count and cell concentration could be determined from the standard curves.

The cells were stored at 4 C until used but never more than 24 hours nor were they used if any evidence of hemolysis appeared in the supernate. It was found that storage for more than 24 hours produced cells which were fragile and not uniformly responsive to treatment with toxin.

Hemolytic tests - For qualitative studies, 0.2 ml. of a 1 per cent erythrocyte suspension was mixed with 0.2 ml. of a given toxin concentration. The tubes were shaken thoroughly and placed in a 37 C water bath. The tubes were shaken periodically for 2.5 hours at which time readings were made visually by the 0 to 4 + system.

For quantitative results, 1 ml. of a 1 per cent erythrocyte suspension was mixed with 1 ml. of a given toxin concentration. The tubes were shaken thoroughly and kept at 37 C as before. After 2.5 hours the tubes were removed, centrifuged in the cold for 3 minutes and the supernate decanted. The supernate was diluted 1/5 in order to give a sufficient volume for colorimetry and the hemoglobin determined on the Klett colorimeter at 540 millimicra.

Neutralization tests - Neutralization tests were performed similarly to the hemolytic tests except that serum was added to neutralize the toxin. For qualitative tests, 0.2 ml. of a toxin concentration was mixed with 0.1 ml. of 4 times the final concentration of serum desired and allowed to incubate 20 minutes at room temperature. Then 0.1 ml. of a 2 per cent erythrocyte suspension was added and the tubes incubated and results read as before.

For quantitative serum protection tests, 1 ml. of a toxin concentration was mixed with 0.5 ml. of 4 times the final concentration of serum desired. The tubes were incubated 20 minutes at room temperature and 0.5 ml. of a 2 per cent erythrocyte suspension was added.

Following this, the tubes were incubated and results read colorimetrically as before.

Properties of the toxin - To determine the effect of temperature on the rate of reaction, a given amount of toxin was mixed with a 1 per cent erythrocyte suspension. Aliquots of this were then incubated at various temperatures and samples removed at given intervals. These were immediately centrifuged, the supernate decanted, diluted 1:5 and read colorimetrically as before. Erythrocyte controls were treated similarly at each temperature.

In order to determine the stability of the toxin to heat and pH variations, a slightly different procedure was necessary. The toxin was suspended at a concentration of 10 mg. per ml. in buffers of various pH values: pH 3.01 (0.2 M acid potassium phthalate), pH 6.93 (0.2 M monobasic potassium phosphate + 0.2 N sodium hydroxide) and pH 11.08 (0.1 M dibasic potassium phosphate + 0.1 N sodium hydroxide). Aliquots of each of these were kept at room temperature, 65 C and 100 C for 2 hours. Aliquots of 0.5 ml. were removed periodically and diluted in pH 6.93 phosphate buffer for titration. Buffer controls were run simultaneously. Because of the pH of the solutions being used, the erythrocytes were also suspended in pH 6.93 buffer instead of saline. This was shown to have no noticeable effect on the cells.

Leucocytic tests - The procedure of Weld (29) was followed

for the test. Two hundred to three hundred g. guinea-pigs were inoculated intraperitoneally with 125 ml. of sterile saline. After 10 hours the guinea-pigs were sacrificed by exsanguination and the peritoneal fluid removed. The fluid was centrifuged slowly for 3 minutes causing the cells to settle but not pack. The upper 0.9 of the fluid was removed and the lower 0.1 was used. This was titrated with 0.1 ml. of methylene blue to determine the minimum amount necessary to give reduction in 20 minutes. This amount was then placed in a serological tube (13 x 70 mm) with a known concentration of toxin and the volume made up to 0.5 ml. The mixture was incubated at 37 C for 1 hour. 0.1 ml. of a 1/5000 methylene blue solution was added, the tubes were overlaid with mineral oil and incubated another hour at 37 C. Lack of reduction would indicate toxic activity.

Dermonecrotic activity - 0.1 ml. of a suspension of whole cells or fractions therefrom was injected introdermally into rabbits, guinea-pigs and rats. The suspending medium was either saline or 5 per cent hog gastric mucin. The animals were inspected periodically for 96 hours to observe erythema, induration and necrosis. Each animal was inoculated with a control of the suspending medium alone. Rabbits were shaved and inoculated on the dorsal portions while rats and guinea-pigs were shaved and inoculated on the ventral surfaces.

Lethal activity - Whole cells and cell fractions were tested for lethal activity in mice. Whole cells were inoculated intraperitoneally

in 0.5 ml. of saline or 0.5 ml of 5 per cent hog gastric mucin. Albino mice three weeks old of either the Swiss Webster or NIH strains were used. Mice were obtained from the Rawley Animal Farm in Plymouth, Michigan.

More purified products were tested both intraperitoneally and intravenously. A given weight of toxin dissolved in 0.1 ml. of saline was inoculated intravenously into the lateral tail vein or 0.5 ml. was inoculated in saline or mucin intraperitoneally.

RESULTS

Fractions of the cultural supernate were prepared by ethanol precipitation, ammonium sulfate precipitation and zinc acetate precipitation. These, together with crude supernate, were tested for hemolytic activity and were found to be inactive.

Crude toxin was readily obtained by grinding formalin killed yeast phase cells with sand and marbles in a ball mill and washing the ground cells with water or saline. This resulted in an opaque suspension which was stable to ordinary centrifugation and would not dialyze. It would pass through a fine sintered glass filter but not through a Seitz sterilizing pad. Since experiments using live cells and heat killed cells gave identical results to those using formalin killed cells, the latter were used for safety and convenience for the major portion of this investigation.

Purification of the hemolytic toxin was one of the more challenging of the problems faced in this investigation. Ammonium sulfate fractionation was not satisfactory, whether performed at room temperature or in the cold. Yields were small and activity was scattered throughout the spectrum of fractions. Those fractions which showed activity, showed no greater, and usually less activity, than the parent toxin. From this it was deduced that the active principle was probably being extracted by nonspecific precipitation or merely by adsorption to the materials being precipitated at each concentration of salt.

Precipitation by zinc acetate was likewise fruitless. Unlike some of the other heavy metal precipitants, precipitates form and may be removed at various concentrations of salt. Fractions were obtained at 0.05 M intervals from 0.05 M to 0.45 M zinc acetate. Each of these fractions was active. Obtaining the fractions by adding the given concentration of salt to an aliquot or increasing the concentration of the supernate of the previous precipitation seemed to make no difference. None of the fractions were as active as the parent toxin. Again this would indicate either non-specific precipitation or sorption of the toxic factor by the zinc salt precipitate.

Copper acetate precipitation was somewhat more effective. By addition of the proper amounts of saturated cupric acetate, a granular precipitate formed which, when the copper was removed, yielded an opaque white suspension having 4 times the original toxic activity.

Either of two methods was used for the removal of copper from the precipitate. Electrodialysis was effective but proved to be too slow and time-consuming. It was necessary to continue electrodialysis for a minimum of 4 days, after which the material was further dialyzed against running tap water to remove the electrolyte.

The toxic material migrated toward the positive electrode as evidenced by the fact that the insoluble white material packed against the diaphragm between the positive chamber and the central chamber. No activity could be detected in the electrode vessels after completion

of electrodialysis. This was expected since it was already known that the toxin would not dialyze.

The use of a cation exchange resin, Amberlite IRC-50, for the removal of copper from the precipitate was highly successful. Usually only 4 changes of resin were necessary for complete removal of the copper. This then provided a simple and convenient method for the preparation of toxin but the final product was still far from the purity of toxin desired.

Ethanol precipitation as used for the preparation of polysaccharides was attempted. After addition of 10 per cent sodium acetate and 1 per cent acetic acid, ethanol was added with constant stirring. Additions were made in 1 volume amounts up to 5 volumes.

Precipitation occurred at 2 volumes of ethanol only. This was removed and the concentration increased to 5 volumes of ethanol. Since no further precipitation occurred, the whole solution was dialyzed and tested. There was no activity in the 2 volume precipitate and no increase in activity of the supernate. This would indicate, since some material had been removed at 2 volumes, that there may have been a partial inactivation of the toxin.

Ethanol precipitation was performed at 0 C and without the addition of sodium acetate and acetic acid. Identical results were obtained.

The cold ethanol procedure of Cohn for the fractionation of

plasma was attempted using the crude toxin. Since the ionic strength of the crude toxin was unknown, the volumes of solutions proportional to those used for plasma fractionation were used. All of the toxic activity was obtained in Cohn fraction I. However, it was found that the addition of the pH 4 buffer had dropped the pH to 4.5 instead of 7.2 as in the case of plasma. Therefore, fraction I was obtained with 8 per cent ethanol at pH 4.5 instead of 8 per cent at pH 7.2. This precipitate showed an increase of 8 times that of the original material on a dry weight basis. Part of this fraction was completely insoluble and would settle out of suspension. The suspension of this fraction was therefore centrifuged and the activity of both supernate and precipitate were tested. Both were active but the precipitate was much less active and in fact was even less active than the crude toxin. However, the activity of the supernate was not noticeably increased by this removal of insoluble material.

It was then found that a product of equal activity could be obtained simply by lowering the pH. A considerable flocculent precipitate occurred between pH 4.5 and 4.9. This was enhanced somewhat by the presence of sodium ions but these were not necessary for complete precipitation of the active principle. The activity was apparently equal to that obtained by the addition of the ethanol and acetate. Also it was not necessary to perform the precipitation in the cold. Precipitation at room temperature resulted in good yields and the activity was not diminished.

Because the opaque crude toxin and the acid precipitated toxin were obviously not true solutions, a separation by centrifugation was attempted. Centrifugation on a Spinco ultracentrifuge at 40,000 R.P.M. (105,000 g) resulted in the formation of 3 layers in each tube, an upper "milky" layer, central clear zone and a pellet. Each was tested for activity. The precipitate was active as expected, but more significantly, the upper "milky" layer was active to about the same extent. The central zone was completely inactive.

Because of the physical appearance of the upper layer, it was suggested that perhaps the toxic factor was lipid in nature. Therefore, extraction of the crude toxin was attempted with various lipid solvents. It soon became apparent that methanol was by far the best solvent for this extraction. However, again though most of the crude material was insoluble, the activity of the toxin was not greatly increased.

A combination of acid precipitation and methanol extraction * of this precipitate have yielded the best product available to date. The crude suspension of toxin was first precipitated by the addition of N acetic acid to give a pH of 4.75 and the precipitate removed by centrifugation. The precipitate was then extracted with 200 ml. of methanol for 4 hours and the methanol removed by evaporation in vacuo at room temperature and the toxin suspended in saline. By this procedure it has been possible to obtain a product 100 times more active than the

* The author is indebted to Professor R. L. Garner for suggesting this method.

crude material. Five mcg. are sufficient to lyse 0.4 ml. of a 0.5 per cent erythrocyte suspension. This is the material referred to as "purified" toxin throughout this report.

Qualitative and semiquantitative chemical determinations of the purified toxin were made. The biuret test for protein and the molish test for carbohydrates were both negative. The folin-ciocalteau determination of tyrosine (or phenols) was slightly positive indicating small amounts of protein or free amino acids of the phenol type. Kjeldahl nitrogen determinations indicated only 1 per cent nitrogen.

Qualitative chromatograms were run in each of two solvent systems: phenol buffered at pH 11 or acetone-acetic acid. Whether the toxin had been hydrolyzed or not, 5 amino acids were demonstrated. These were not identified.

A partial solvent spectrum was established for the acid precipitated toxin. Preliminary investigation had suggested the solubility spectrum but indicated either that the material was only slightly soluble in some solvents or was partially inactivated by them. Table 1 shows the spectrum tested and the results when each extraction was followed by an extraction with methanol. Because of the high degree of activity of methanol extracts, it was assumed that methanol did not inactivate the toxin. It can be seen in Table 1 that the toxin is soluble in methanol, chloroform and acetone, but not in diethyl ether. The extent to which it is soluble in each is seen by the fact that when activity

TABLE I

EFFICACY OF VARIOUS LIPID SOLVENTS
FOR EXTRACTING TOXIC ACTIVITY FROM CRUDE TOXIN

Solvent	Hemolytic titer*		
	Solvent control	Solvent extract of crude toxin	Methanolic extract of residue
None		(0.25)	
Methanol	--	0.008	--
Acetone	--	0.016	0.125
Chloroform	--	0.032	0.063
Diethyl ether	--	0.25	0.032

* Minimum weight in mg. of fraction necessary to completely lyse 0.4 ml. 0.5 per cent Rabbit Red Blood Cells.

of the first solvent is less than the original methanol extract activity, additional activity could be extracted from the residue with methanol. Repeated extraction with the first solvent did not yield more toxic activity.

Crude toxin was used for preliminary investigation of the hemolysin produced while purification studies were being conducted. The susceptibility of erythrocytes from various animal species is shown in Table 2. It will be noticed that most erythrocytes tested are equally susceptible with the exception of those from the sheep and chicken. It will also be noted that at the lower temperature of incubation, activity is much decreased.

Figure 1 and Figure 2 show the effect of temperature on the activity of purified toxin. Figure 1 is a graph showing the rate of reaction at 37 C, 22 C, and 0 C. By preliminary experiment it was determined that 37.5 mcg of toxin per ml. was sufficient to give complete lysis of a 0.5 per cent suspension of red blood cells in 1 hour at 37 C. It will be seen that when this concentration of toxin is incubated at 22 C or at 0 C the rate is greatly diminished. By 2 hours those cells incubated at 22 C had lysed about 89 per cent while those incubated at 0 C showed only 54 per cent lysis. Also the lag period before initiation of lysis was markedly different. At 37 C lysis became apparent at 15 minutes while at 22 C and 0 C the lysis did not appear until 45 and 60 minutes respectively.

TABLE 2

SUSCEPTIBILITY OF VARIOUS ANIMAL SPECIES
RED BLOOD CELLS TO CRUDE TOXIN

Species	Hemolytic Titer*	
	37 C	5 C
Rabbit	0.32	2.0
Rat	1.0	2.0
Guinea pig	0.32	2.0
Sheep	1.0	2.0
Chicken	1.0	2.0
Human (o)	0.32	2.0

* Mg of crude toxin necessary for complete lysis of 0.4 ml. of 0.5 per cent red blood cells.

FIGURE I

EFFECT OF TEMPERATURE ON RATE
OF REACTION OF PURIFIED TOXIN

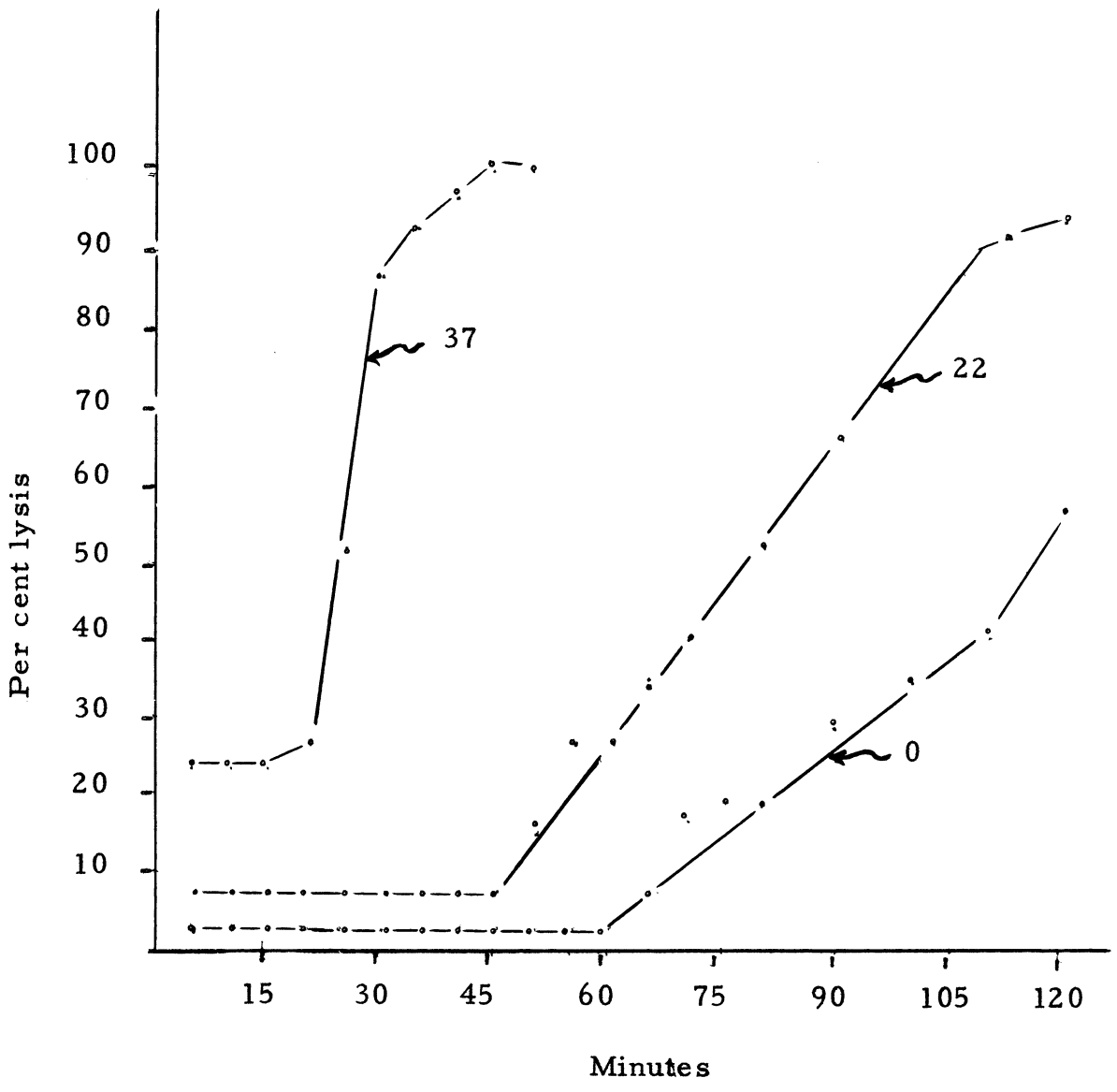
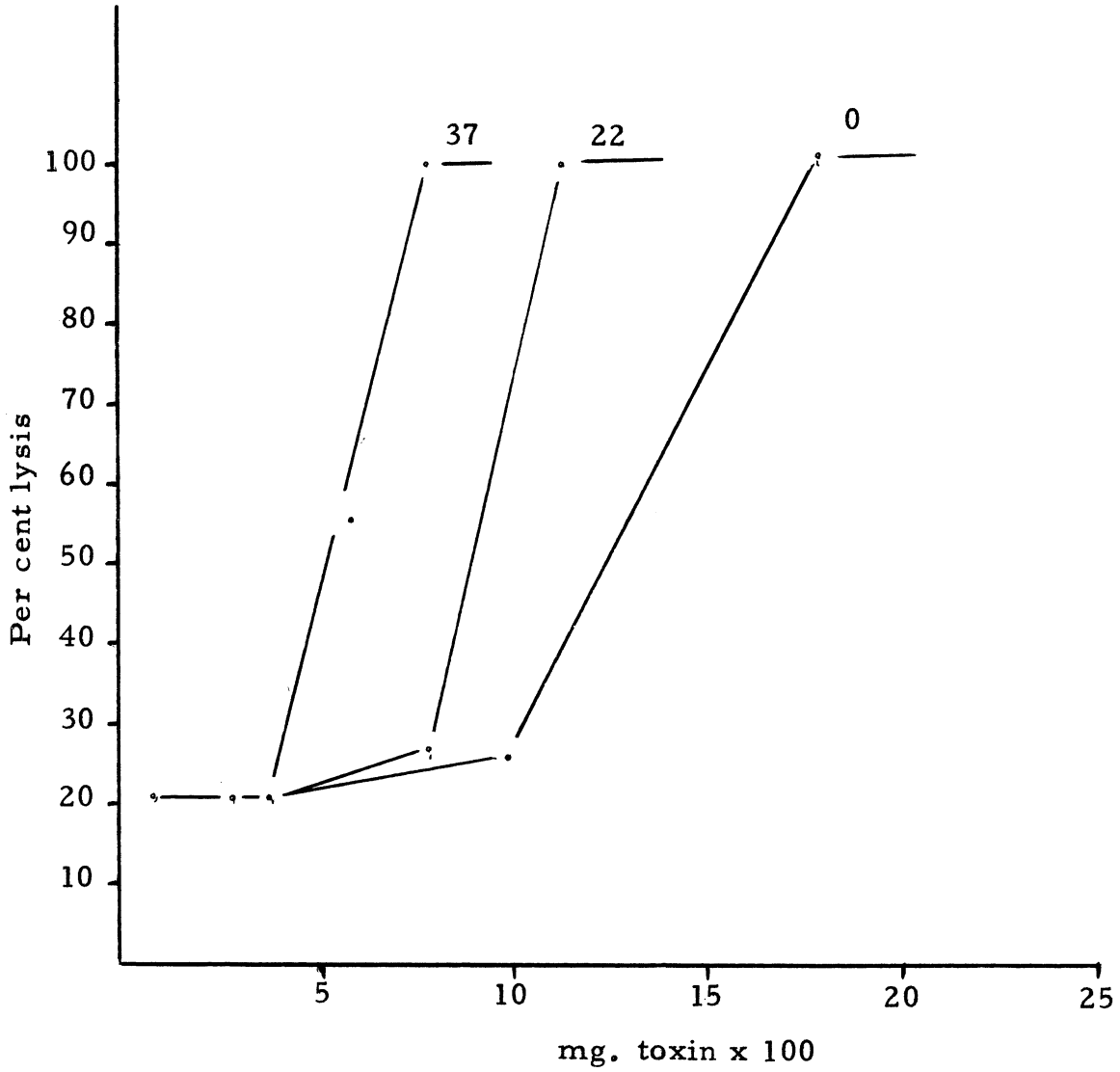


FIGURE 2

EFFECT OF TEMPERATURE ON RATE
OF ACTIVITY OF PURIFIED TOXIN



It can be seen from Figure 2, which is a graph of the effect of temperature on the activity of the toxin, that if the concentration of toxin is great enough, complete lysis will occur in 2 hours at each temperature. At 37 C this concentration need only be 40 mcg. per ml. while at 22 C it must be 60 mcg. and at 0 C it must be 90 mcg.

In order to test the effect of oxidizing or reducing agents on the hemolytic reaction, the test was performed in the presence of hydrogen peroxide or sodium thioglycollate. Each was first titrated with red blood cells to determine the maximum amount which would not lyse the cells. 0.12 per cent hydrogen peroxide and normal sodium thioglycollate could be tolerated by the cells. Results are shown in Table 3. At least to the extent the potential was altered by these agents, there was no noticeable effect. In each case the cells became a little more sensitive but not markedly so.

That the toxin obtained is stable to temperature and pH variation is shown in Table 4. Aliquots of toxin were suspended in buffers ranging from pH 3 to pH 11. Aliquots of each of these were heated at room temperature, 65 C, 100 C and 120 C (in the autoclave) for periods extending to 10 hours. Hydrolysis and charring took place at pH 3 when heated to 100 C or higher but activity was not eliminated. Although about 90 per cent of the activity was gone after 10 hours autoclaving, the activity was not totally destroyed even at this extreme temperature and pH. Other pH and temperature variations had limited effect which

TABLE 3

EFFECT OF OXIDIZING AND REDUCING SUBSTANCES ON REACTIVITY OF CRUDE TOXIN

Treatment	Hemolytic* titer	Control
--	0.5	--
0.12 % H_2O_2	0.25	--
0.06 % H_2O_2	0.25	--
N sodium thioglycollate	0.25	--
0.5 N sodium thioglycollate	0.25	--

* Mg. crude toxin necessary for complete lysis of 0.4 ml. of 0.5 per cent rabbit red blood cells.

TABLE 4

EFFECT OF HEAT AND PH VARIATIONS
ON TOXICITY OF CRUDE TOXIN

	pH				
Temp.	3	5	7	9	11
R.T.	0.25*	0.063	0.063	0.125	0.125
60 C	0.25	0.125	0.125	0.125	0.125
100 C	0.25	0.125	0.125	0.125	0.125
120 C**	0.50				

* Mg. crude toxin necessary for lysis.
** Heated in autoclave 10 hours. Other titrations made after 1.5 hours.

could probably be attributed to experimental error. These effects held true for both the crude and the purified toxin.

The effect of concentration of red blood cells on the amount of toxin necessary for lysis is shown in Figure 3. It can be seen that as the concentration of red blood cells is increased, the amount of toxin required for lysis is likewise increased. It can be seen that at a concentration of 32 per cent red blood cells, which is approaching the *in vivo* concentration of cells, there is still complete hemolysis of the cells when as little as 0.5 mg. of toxin are present in 0.4 ml.

That this lysis is a surface phenomenon is shown by the fact that if the toxin is first incubated with cell stroma, it then loses its ability to lyse cells. Red cells were lysed with water and the stroma were centrifuged out. The supernate was decanted and the suspension of toxin was added directly to the packed cell stroma. After mixing and allowing to stand for 10 minutes at room temperature, the red blood cell suspension was added and incubated at 37 C for 2 hours. At the end of this time there was no lysis in the tube containing stroma while the control tube had completely lysed.

In an attempt to protect the red cells from the toxin, immune serum was tested. It was found that not only was immune serum capable of neutralizing the hemolytic toxin, but also normal rabbit serum possessed a toxin neutralizing factor. Attempts were then made to determine what factor in normal serum is responsible for the neutralization.

FIGURE 3

EFFECT OF CONCENTRATION OF RED BLOOD CELLS ON TITER OF HEMOLYTIC TOXIN

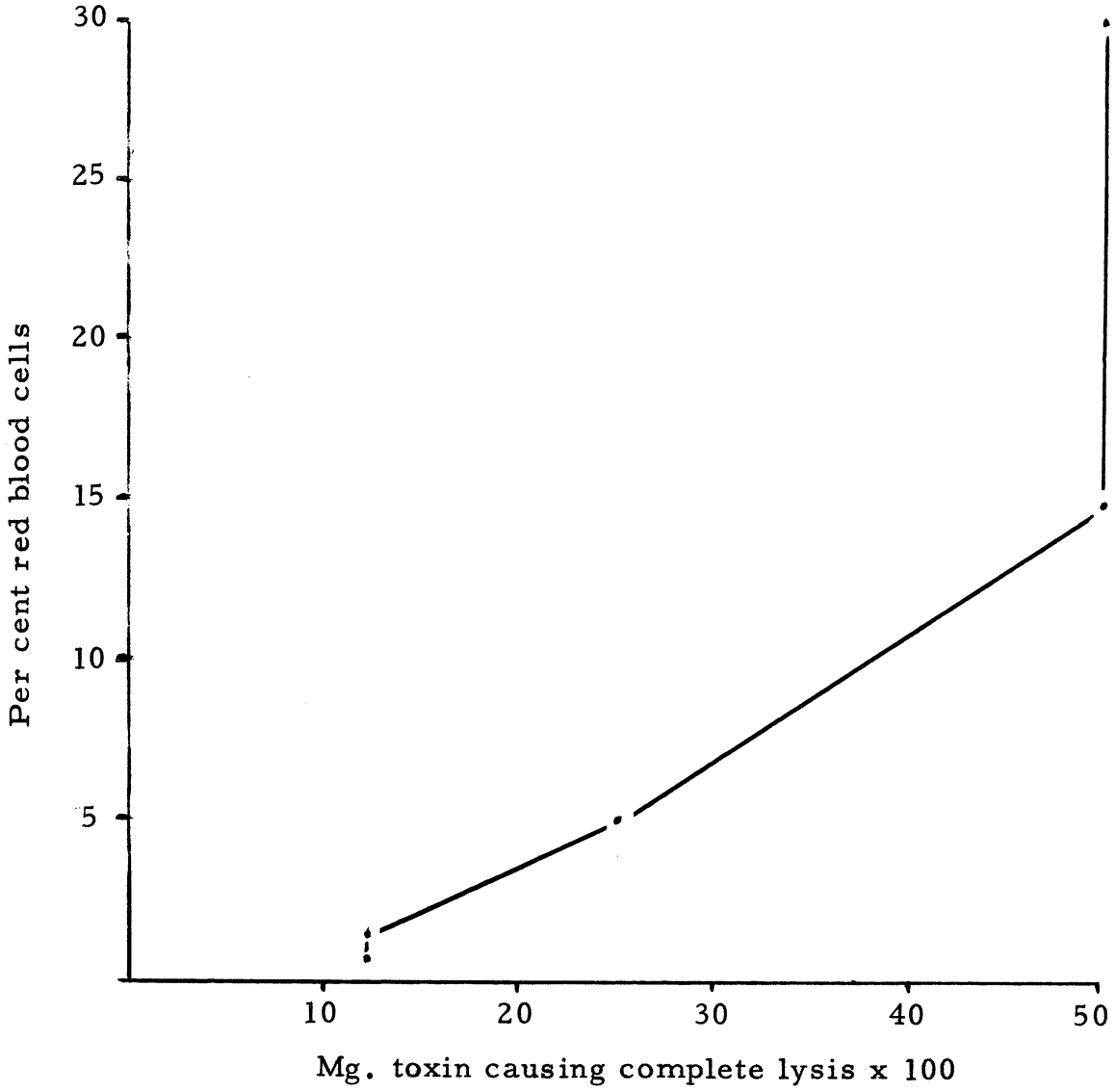


Table 5 shows typical results when normal rabbit serum is titrated against various concentrations of toxin. One unit of toxin is the minimum amount of toxin which will lyse 0.4 ml. of a 0.5 per cent red blood cell suspension. There is a direct relationship between the amount of toxin present and the amount of serum necessary to neutralize that amount of toxin. A 1:2 dilution of serum was not capable of neutralizing the hemolytic activity of 32 units of pure toxin.

The role of complement and its fractions against crude toxin was determined as shown in Table 6. The usual methods of inactivating complement and its fractions were employed with no noticeable effect. Also Table 6 shows that dialysis of the serum had no effect, indicating that the protective property is not due to small molecular substances. Heating at 65 C had only slight effect which is illustrated in Figure 4. The protective effects are lessened but only to an extent which becomes apparent at high dilutions. At low dilutions the serum is approximately as active as unheated serum.

Ammonium sulfate fractionation of serum was performed and typical results are shown in Table 7. Fractions were removed at 0-33 per cent, 33 - 50 per cent and greater than 50 per cent saturation with ammonium sulfate. Whole lyophilized serum contained 5.2 protecting units per mg. The 0 - 33 per cent and the 33 - 50 per cent fractions each contained about 1/4 that amount or 1.33 units per mg. One unit of protective factor is that amount which completely protects

TABLE 5

PROTECTIVE ABILITY OF SERUM AGAINST VARIOUS CONCENTRATIONS OF TOXIN

Units of hemolysin	Reciprocal of final serum dilution*
0.5	256
1.0	256
2.0	128
4.0	32
8.0	16
16.0	4
32.0	2

* Minimum amount of serum completely inhibiting lysis.

TABLE 6

EFFECT OF VARIOUS TREATMENTS ON THE PROTECTIVE PROPERTIES OF SERUM

Treatment	Final dilution of serum		
	1:2	1:20	1:50
None	5*	0.63	0.63
Dialyzed	5	0.63	0.63
56 C 30'	5	2.5	1.25
56 C 60'	5		
65 C 60'	2.5		
NH_4OH	5	2.5	1.25

* Mg. crude toxin necessary to lyse 0.4 ml. of 0.5 per cent red blood cells

FIGURE 4

PROTECTIVE PROPERTIES OF
SERUM AFTER HEATING

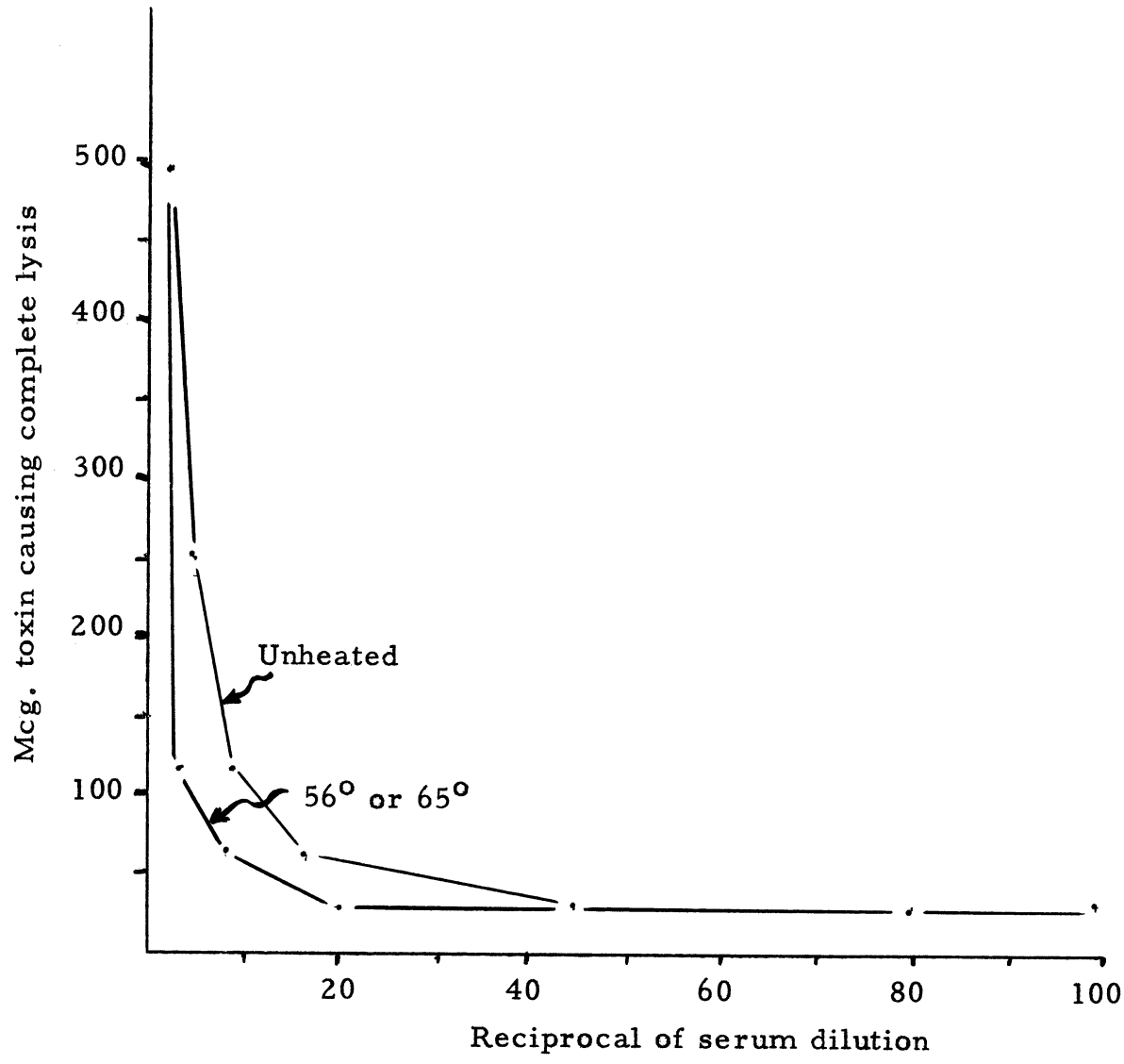


TABLE 7

PROTECTIVE PROPERTIES OF AMMONIUM SULFATE FRACTIONS OF RABBIT SERUM AGAINST CRUDE TOXIN

Fraction	mg. protecting	units/mg.
Whole serum	0.75*	5.2
0 - 33% sat.	3.0	1.33
33 - 50% sat.	3.0	1.33
above 50% sat.	0.19	21.3

* Mg. fraction protecting against 4 units of toxin.

against 1 unit of toxin. At approximately 50 per cent saturation of ammonium sulfate, the albumin and the last of the globulins will precipitate. Therefore, in this fraction one would expect both albumin and alpha globulin.

The cold ethanol procedure of Nichol and Deutsch for the fractionation of rabbit serum was used with very encouraging results. These are summarized in Table 8. Whole lyophilized serum, whether dialyzed or not contains 5 protecting units per mg. Beta and gamma globulin as prepared by this procedure are practically inactive. Albumin on the other hand contains 10 units per mg. or twice that of whole serum. However, by this technique, the albumin fraction also contains practically all of the alpha globulin of the serum. It is important to note, that in several species serum tested, this same picture results: practically no activity in beta and gamma globulin and high activity in the albumin - alpha globulin fraction.

The Cohn cold ethanol fractionation of human plasma is not as effective for rabbit plasma as might be hoped, but it is still possible to obtain similar fractions to those described by Cohn for human plasma. Table 9 shows that as with the Nichol technique, Fraction I (fibrinogen) and Fraction II, III (beta and gamma globulin) contain very little activity, while Fraction IV_1 (alpha globulin) and V (albumin) contain considerable activity. The albumin fraction and Fraction IV show twice the activity of whole serum. It is reasonable to assume

TABLE 8

PROTECTIVE PROPERTIES OF NICHOL FRACTIONS OF RABBIT SERUM AGAINST HEMOLYTIC TOXIN

Fraction	Mg. fraction			units/mg
	2	0.2	0.02	
Whole serum	1*	0.25	0.125	5
Whole serum dialyzed	1	0.25	0.125	5
Albumin-alpha globulin	2	0.5	0.125	10
Beta globulin	lytic	0.125	0.125	--
Gamma globulin	0.25	0.125	0.125	--

*Mg. toxin necessary to lyse 0.4 ml. of 0.5 per cent red blood cells in the presence of serum fraction.

TABLE 9

PROTECTIVE PROPERTIES OF COHN FRACTIONS OF RABBIT SERUM AGAINST HEMOLYTIC TOXIN

Fraction	mg. protecting	units/mg.
Whole serum	0.75*	5.2
Whole serum dialyzed	0.75	5.2
I	1.5	3.0
II, III	1.5	3.0
IV_1	0.38	10.4
V	0.38	10.4

* Mg. necessary to protect against 4 units of toxin.

that Fraction V is at least partially contaminated by Fraction IV. Therefore, it would appear that practically all the activity is localized in the alpha globulin fraction of the plasma.

Experiments to show other toxen activities of this purified hemolytic toxic were negative. It is not dermonecrotic. Concentrations as high as 10 mg. when inoculated intravenously into mice or suspended in hog gastric mucin and injected intraperitoneally into mice showed no lethal properties.

Whole cells were shown to be both dermonecrotic and lethal. Dermonecrosis was evident but not as striking as might have been expected from the disease picture. Necrosis only occurred to a marked extent in the presence of 5 per cent hog gastric mucin; however, it was found that mucin itself would cause a marked erythema. In the absence of mucin, an induration and erythema would result in 24 - 48 hours but little if any visible necrosis would result.

Whole cells were lethal for albino mice as shown in Table 10. Both heat killed and formalin killed cells of Histoplasma capsulatum were tested. It can be seen that heat killed cells are approximately 4 times as toxic as are the formalin killed cells. Experiments have shown that the decrease in activity of the formalin killed cells does not occur until greater than 6 hours exposure to formalin. It was again impossible to obtain toxic activity without the use of hog gastric mucin. In this case, however there was no apparent detrimental effect on

TABLE 10

TOXICITY OF KILLED YEAST PHASE CELLS* OF H. CAPSULATUM

Quantity of cells (mg)	Heat killed	Formalin killed
20	46/51**	10/14
10		7/14
5		10/22
2.5		1/8
2.0	23/51	
1.0		1/8
0.5		0/8
0.2	12/59	
approximate LD50	2.0	7.8

* Injected intraperitoneally in 0.5 ml. of 5 per cent mucin.
** Deaths in 72 hours/total animals inoculated.

on the host by mucin alone. Other routes of inoculation were attemped and intracerebral inoculations of small amounts produced death, but this was considered too artificial a means for testing and was not pursued further.

DISCUSSION

For a complete understanding of any disease, a study of the toxins produced by the causative agent is of major importance. Anemia, which is a constant laboratory finding in histoplasmosis, suggests the production of a hemolysin by H. capsulatum. The isolation and characterization of such a toxin were successfully undertaken, although its possible role in the pathogenesis of the disease has not been studied.

The first assumption made was that the hemolysin was an exotoxin excreted into the blood stream. However, an in vitro exotoxin could not be demonstrated. Cultural supernates from several isolates of the organism were concentrated and fractionated. No activity was demonstrable from the whole supernates or any fraction therefrom.

The presence of a hemolytic endotoxin was readily demonstrated by preparing a suspension from ground yeast phase cells. This was an extremely stable suspension which could be separated at only very high speed centrifugation. It was apparently of large molecular size since it would not dialize or pass through a Seitz sterilizing pad. Microscopic examination revealed lipoidal globules of various sizes.

Results of fractionation were for some time confusing. Though fractions could be obtained, either all fractions contained the same

amount of toxic activity or those that showed activity showed no increase in activity over the crude material. It was concluded that the material containing activity was not being specifically precipitated but simply being non-specifically sorbed by the precipitate which did form. From later data, this conclusion became very plausible. The toxic material is lipid in nature and therefore might easily be sorbed by any protein precipitate formed in precipitation.

Ammonium sulfate precipitation yielded fractions which were of low reactivity and not reproduceable. Duplicate fractions may or may not have similar activity. That is, a fraction may be obtained one time at 20 per cent saturation showing high activity, but when another aliquot of the same toxin suspension is precipitated with 20 per cent saturation of ammonium sulfate, the fraction may not have any activity. This may be explained by the non-specific precipitation. Unknown factors may be involved which allow the toxin to be sorbed by the precipitate at one time and not another.

The non-specificity of precipitation was more strikingly demonstrated by zinc acetate precipitation. Results similar to those found with ammonium sulfate were obtained with this technique also. More interesting, however, was the fact that activity was never located in one or a few fractions but was usually associated to the same extent in all fractions.

Copper acetate precipitation, on the other hand, gave

reproduceable precipitation of a material with increased activity. The difference in this technique was that instead of precipitating at various concentrations of metal, only one concentration was used. The amount of saturated cupric acetate causing miximum precipitation when added to a suspension of toxin was used. This resulted in only one fraction. In experiments testing for precipitation with less cupric acetate, slight precipitation occurred but had about the same activity as that precipitate occurring at maximum precipitation. It was concluded that a specific precipitation had occurred. However, it is likely that results similar to those obtained with ammonium sulfate and zinc acetate would have occurred had fractional precipitation been performed to the same extent. Increase in activity of this fraction is probably due to the fact that with such a large precipitate forming, most of the toxin was sorbed and precipitated, leaving reasonably inactive material in the supernate.

Amberlite IRC-50, a weak cationic exchange resin, was particularly useful in removing the copper from the above precipitate. The particles were of large size which permitted easy separation from the suspension without centrifugation. This allowed rapid exchange of resin when desired.

Attempts to precipitate toxin with ethanol were completely fruitless. Apparently conditions were not right for the sorption of the toxin by the precipitate formed. The fact that a lipid solvent was

being used could contribute to the explanation. Precipitation did not occur until 50 per cent ethanol had been added which possibly caused the partial solution of the lipid-like toxin and thus prevented its sorption by the precipitate.

In attempts to precipitate an active fraction with ethanol, the Cohn plasma fractionation technique was used. This yielded a precipitate at Fraction I which was of considerably higher activity than anything previously obtained. However, it was found that the precipitation was due to the lowering of the pH and not to the addition of ethanol. A suspension of more purified toxin would not precipitate upon addition of acid. It was, therefore, assumed that the acid precipitation primarily involved some material other than the toxin although the toxin apparently coprecipitated.

Because of the physical properties of the toxic factor, lipid solvents were tested and found to be the best method available for extracting the toxin. The product of highest activity obtained was prepared by precipitation with acid and extraction of the precipitate with methanol. This yielded a material which possessed a hundred times the activity of crude toxin.

Chemical and physical characterizations have indicated that the toxin is probably a lipid of high molecular weight. The purified toxin is biuret and Molisch negative showing only 1 per cent nitrogen. This nitrogen content can be attributed to amino acids. Since these amino

acids may be detected chromatographically whether the material has been hydrolyzed or not, this would indicate that the amino acids are not firmly bound to the lipid.

The toxin is soluble in lipid solvents. A partial spectrum indicates it is soluble in methanol chloroform, and acetone but not in diethyl ether. This solvent spectrum coupled with preliminary infrared spectra indicate this is not of the sterioid type. The possible significance of the accompanying amino acids remains to be determined.

Erythrocytes from all common laboratory animals seem to be susceptible to this hemolysin. Chicken and sheep cells appeared to be less sensitive but were susceptible.

The hemolytic reaction is definitely temperature sensitive. Changing the temperature of incubation from 37 C to 22 C more than doubled the time necessary for complete hemolysis and changing the temperature to 0 C caused a greater than 4 fold decrease in rate. If large enough concentrations of toxin were used, hemolysis at the three temperatures would take place simultaneously. However, a proportional amount of toxin was necessary; about 1.5 times as much toxin at 22 C and 2.5 times as much at 0 C. These facts could simply indicate the necessity of contact for activity. With increased temperature there is increased molucular activity and thus more chance for reaction to occur. Since the mechanism of action is unknown, it is difficult to carry conclusions further.

The toxin is highly stable to heat and pH variations. Even when autoclaved at 120 C at pH 3 for 10 hours, activity was not completely destroyed. The opaque suspension had cleared and turned brown indicating charring and probably hydrolysis. This extended period at extreme conditions would certainly denature any protein and probably most peptides if such were present. If so, their activity should have been eliminated. Because activity remained, it was concluded that protein or peptide constituents were not present in agreement with the qualitative analyses.

The possible biological importance of this toxin is shown by the fact that 0.4 ml. of a 32 per cent erythrocyte suspension was completely lysed by as little as 0.5 mg. of toxin. Lysis was detected in this concentration of cells with as little as 0.05 mg. of toxin. This represents the toxin from approximately 100 ml. of culture or only 10^{10} cells. In advanced stages of infection there may be many times this number of cells present.

The protection against hemolytic toxins by normal serum has been reported by others (37, 38). However, there are disputes as to the active serum component. Therefore, it seemed important to identify this component, not only to help understand the resistance to histoplasmosis, but for its possible value in understanding of other infections.

There appears to be an almost direct relationship between the amount of toxin present and the amount of serum necessary to protect

against it. As long as the erythrocyte concentration is constant, the amount of serum necessary for neutralization is dependent upon the amount of toxin present. Therefore, one would conclude that the serum is acting directly on the toxin. It is not a "coating" of the cells but a neutralization of the toxin. If it were a coating of the cells then the amount of serum would vary with cell concentration and not with toxin concentration. A 1:2 dilution of serum did not protect against 32 units of purified toxin.

Neutralization by serum was not attributable to complement or its fractions. Serum treated in various ways to inactivate complement fractions showed little difference in reactivity from untreated serum.

Precipitation with ammonium sulfate or ethanol led to the conclusion that alpha globulin is the active principle of the serum. With ammonium sulfate it was found that fractions up to 50 per cent saturation had little activity while greater than 50 per cent saturation was highly active. It is known that at 50 per cent saturation there is still alpha globulin in solution which will precipitate upon higher concentration of ammonium sulfate.

The Nichol cold ethanol technique yielded similar data. Nichol reports that alpha globulin and albumin precipitated from solution together, and beta and gamma globulin each precipitate individually. Again, no activity was detected in beta and gamma globulin and high

activity was observed in the alpha globulin - albumin fraction.

The Cohn cold ethanol procedure gave the most conclusive data. All the activity was found in Fraction IV_1 and V. Fraction IV_1 is entirely alpha globulin while Fraction V is most of the albumin and the remainder of the globulins. This being true, IV_1 indicates the activity of alpha globulin and V may indicate the activity of the alpha globulin contaminating the albumin.

This indication that the alpha globulin fraction can neutralize this toxin is analogous to the results reported by Stollerman (41) and others (39).

SUMMARY

It has been possible to obtain a hemolytic toxin from yeast phase cells of H. capsulatum. A crude toxin was prepared by making a saline extract from a suspension of ground cells. One hundred fold increase in activity could be gained by precipitating the crude toxin with acid and extracting the precipitate with methanol.

Chemical data indicate the purified toxin is lipoidal in nature. It is soluble in lipid solvents and is negative to biuret and Molisch reactions. Free amino acids have also been detected in the lipoidal material, but their significance has not been determined.

Neutralization tests have shown that normal rabbit serum will inactivate the hemolytic properties of the toxin. This appears to be due to an action directly on the toxin and not a change in the erythrocytes. By several fractionation techniques, it was concluded that the protective component is probably in the alpha globulin fraction.

BIBLIOGRAPHY

1. Roux, E. and Yersin, A. 1888 Contribution a l'etude de la diphtherie. Ann. Inst. Past. 2: 629 - 661.

2. Kitasato, S. 1891 Experimentelle Untersuchungen uber das tetanusgift. Zeitsch. f. Hyg. 10: 267 - 305.

3. Glenny, A.T., Barr, M., Llewellyn-Jones, M., Dalling, T. and Ross, H.E. 1933 Multiple toxins produced by some organisms of the Clostridium welchii group. J. Path. Bact. 37: 53 - 74.

4. Nagler, F.P.O. 1939 Observations on a reaction between the lethal toxin of Clostridium welchii (type A) and human serum. Brit. J. Exper. Path. 20: 473 - 485.

5. Seiffert, G. 1939 Eine Reaktion menschlicher Sera mit Perfringenstoxin. Zeitschr. Immunitatsf. 96: 515 - 520.

6. MacFarlane, R.G., Oakley, C.L. and Anderson, C.G. 1941 Haemolysis and the production of opalescence in serum and lecithovitellin by the α-toxin of Clostridium welchii. J. Path. Bact. 52: 99 - 103.

7. Oakley, C.L. and Warrack, G.H. 1941 Factors affecting the activity of the α-toxin of Clostridium welchii. J. Path. Bact. 53: 355 - 370.

8. Zamecnik, P.C. and Lipmann, F. 1947 A study of the competition of lecithin and antitoxin for Clostridium welchii lecithinase. J. Exp. Med. 85: 395 - 403.

9. MacFarlane, M.G. and Knight, B.C.J.G. 1941 The biochemistry of bacterial toxins. I. The lecithinase activity of Clostridium welchii toxins. Biochem. J. 35: 884 - 902.

10. van Heyningen, W.E. 1941 The biochemistry of the gas gangrene toxins. II. Partial purification of the toxins of Cl. welchii, type A. Separation of α and θ toxins. Biochem. J. 35: 1257 - 1269

11. Ganley, O.H., Merchant, D.J., and Bohr, D.F. 1955 The relationship of toxic fractions of a filtrate of Clostridium perfringins type A to the pathogenesis of clostridial myonecrosis. J. Exper. Med. 101, 605 - 615.

12. Van der Velde, H. 1894 Etude sur le mecanisme de la virulence du staphylocoque pyogene. La Cellule 10: 403 - 460.

13. Denys, J. and Havet, J. 1898 La Cellule 10: 7.

14. Lingelsheim, W. von 1899 Beit. Exp. Ther., Heft. 1: 49.

15. Kraus, R. and Clairmont, P. 1900 Ueber Hamolysine und Antihamolysine. Wein. klin. Wschr., 13: 49 - 56.

16. Neisser, M. and Wechsberg, F. 1901 Uber das Staphylotoxin. Z. Hyg. Infektkr. 36: 299.

17. Schwabacher, H., Cunliffe, A.C., Williams, R.E.O. and Harper, G.J. 1945 Hyaluronidase production by staphylococci. Brit. J. Exper. Path. 26: 124 - 129.

18. Christie, R., North, E.A. and Parkin, B.J. 1946 Criteria of pathogenicity in staphylococci. Austral. J. Exp. Biol. Med. Sci. 24: 73 - 78.

19. Gillespie, Q.A. and Simpson, P.M. 1948 Pathogenic staphylococci. Detection of α-lysin production on rabbit and sheep-blood-agar plates. Brit. Med. J. 2: 902 - 905.

20. Elek, S.D. 1948 The recognition of toxigenic bacterial strains in vitro. Brit. Med. J. 1: 493 - 496.

21. Bigger, J.W., Boland, C.R. and O'Meara, R.A.Q. 1927 A new method of preparing staphylococcal haemolysin. J. Path. Bact. 30: 271 - 277.

22. Bryce, L.M. and Roundtree, P.M. 1936 The production of - toxin by staphylococci. J. Path. Bact. 43: 173 - 189.

23. Llewellyn-Smith, M. and Price, S.A. 1938 Staphylococcus - hemolysin. J. Path. Bact. 47: 361 - 377.

24. Neill, J.M. 1927 Studies on the oxidation and reduction of immunological substances. V. Production of antihemotoxin by immunization with oxidized pneumococcus hemotoxin. J. Exp. Med. 45: 105 - 113.

25. Neill, J.M. and Mallory, T.B. 1926 Studies on oxidation and reduction of immunological substances; streptolysin. J. Exp. Med. 44: 241 - 260.

26. Todd, E.W. 1934 A comparative serological study of streptolysins derived from human and from animal infections, with notes on pneumococcal haemolysin, tetanolysin and staphylococcal toxin. J. Path. Bact. 39: 299 - 321.

27. Todd, E.W. 1941 The oxygen-labile haemolysin or θ toxin of Clostridium welchii. Brit. J. Exp. Path. 22: 172 - 178.

28. Herbert, D., and Todd, E.W. 1941 Purification and properties of a haemolysin produced by group A haemolytic streptococci (streptolysin O). Biochem. J. 35: 1124 - 1139.

29. Cohen, B., Perkins, M.E. and Putterman, S. 1940 The reaction between hemolysin and cholesterol. J. Bact. 39: 59 - 60.

30. Weld, J.T. 1934 The toxic properties of serum extracts of hemolytic streptococci. J. Exp. Med. 59: 83 - 95.

31. Weld, J.T. 1935 Further studies with toxic serum extracts of hemolytic streptococci. J. Exp. Med. 61: 473 - 477.

32. Todd, E.W. 1938 The differentiation of two distinct serological varieties of streptolysin, streptolysin O and streptolysin S. J. Path. Bact. 47: 423 - 445.

33. Smith, F. 1937 The relation of peptone to production of haemolysin by streptococci. J. Bact. 34: 585 - 601.

34. Okamoto, H., 1940 Uber die hochgradige Steigerung des Hamolysinbildungsvermogens des Streptococcus haemolyticus durch Nukleinsaure. I. Mitteilung. Part 4, Pharmacology. Jap. P. Med. Sci. 12: 167 - 208.

35. Bernheimer, A.W. 1949 Formation of a bacterial toxin (streptolysin S) by resting cells. J. Exp. Med. 90: 373 - 392.

36. Bernheimer, A.W. and Rodbart, M. 1948 The effect of nucleic acids and of carbohydrates on the formation of streptolysin. J. Exp. Med. 88: 149 - 168.

37. Kerrin, J.C. 1930 Studies on the Haemolysin produced by atoxic strains of B.tetani. Brit. J. Exp. Path. 11: 153 - 157.

38. Todd, E.W. 1939 The streptolysin of various groups and types of hemolytic streptococci; a serological investigation. J. Hyg. 39: 1 - 11.

39. Humphrey, J.H. 1949 The nature of antistreptolysin S in the sera man and other species. The lipoprotein properties of anti-streptolysin S. Brit. J. Exp. Path. 30: 365 - 375.

40. Stollerman, G.H. and Bernheimer, A.W. 1950 The inhibition of streptolysin S by the serum of patients with rheumatic fever and acute streptococcal pharyngitis. J. Clin. Invest. 29: 1147.

41. Stollerman, G.H., Bernheimer, A.W. and MacLeod, C.M. 1950 The association of lipoproteins with the inhibition of streptolysin S by serum. J. Clin. Invest. 29: 1636.

42. Davis, D.B. and Dubos, R.J. 1947 The binding of fatty acids by serum albumin, a protective growth factor in bacteriological media. J. Exp. Med. 86: 215 - 228.

43. Hewitt, L.F. and Todd, E.W. 1939 The effect of cholesterol and of sera contaminated with bacteria on the hemolysins produced by hemolytic streptococci. J. Path. Bact. 49: 45 - 51.

44. Ceni, C. and Besta, C. 1902 Uber die toxine von Aspergillus fumigatus und Aspergillus flavescens und deren Beziehungen zur Pellagra. Centralbl. f. allgemein. Path. und Pathol. anat., 13: 930.

45. Bodin, E. and Gauthier, L. 1906 Researches sur les Poisons Produits par L'Aspergillus fumigatus. Ann. Inst. Past. 26: 371 - 380.

46. Henrici, A.T. 1939 An endotoxin for Aspergillus fumigatus. J. Immunol. 36: 319 - 338.

47. Gortner, R.A. and Blakeslee, A.F. 1914 Observations on the toxin of Rhizopus nigricans. Am. J. Physiol. 34: 353 - 367.

48. Abel, J.J. and Ford, W.W. 1907 On the poisons of Amanita phalloides. J. Biol. Chem. 2: 273 - 288.

49. Drouhet, E. and Segretain, G. 1951 Inhibition de la migration leucocytaire in vitro par un polyoside capsulaire de Torulopsis neoformans. Ann. Inst. Past. 81: 674 - 676.

50. Salvin, S.B. 1952 Endotoxin in pathogenic fungi. J. Immunol. 69: 89 - 99.

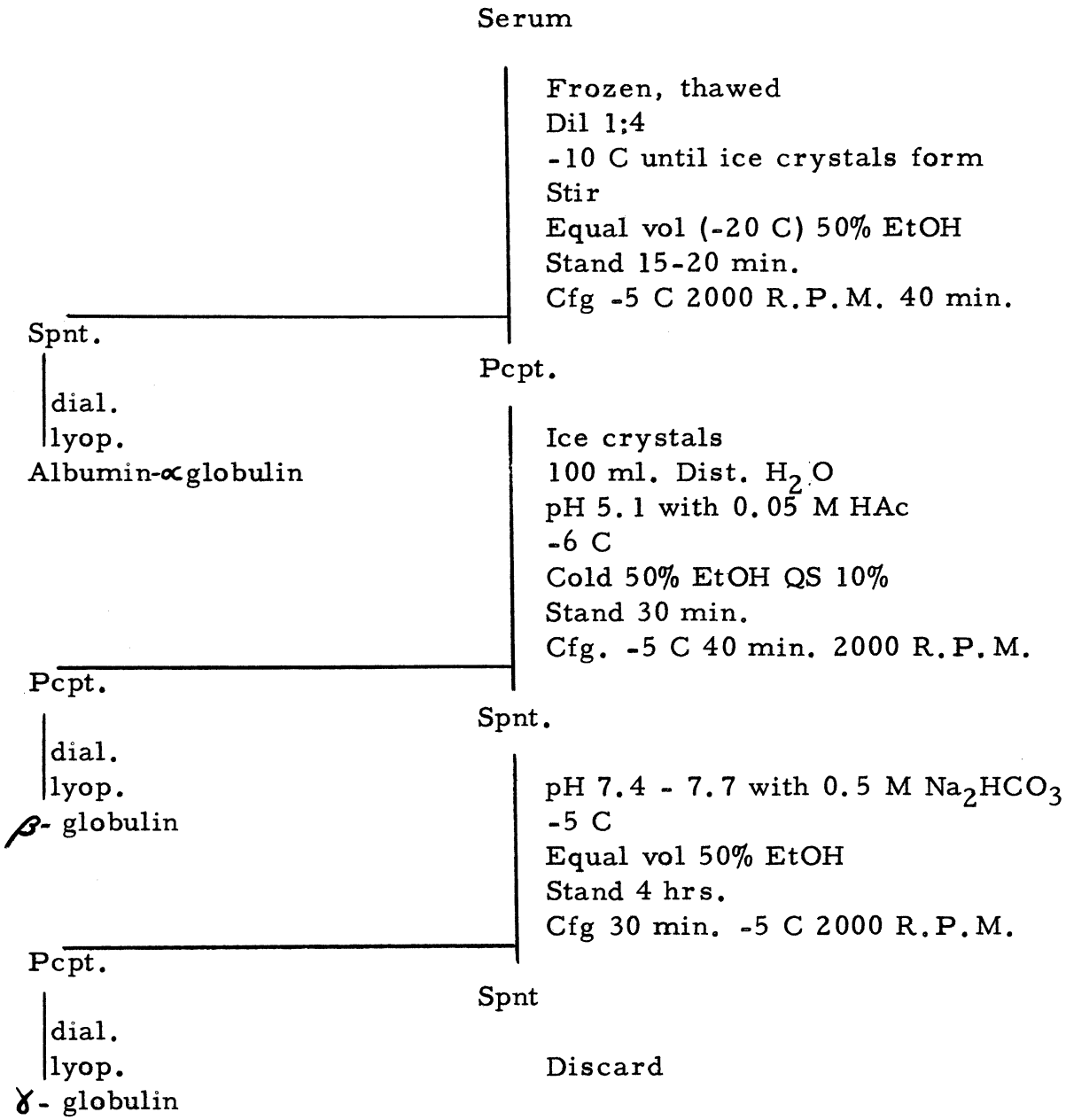
51. Salvin, S.B. 1951 Hemolysin from the yeast like phases of some pathogenic fungi. Proc. Soc. Exper, Biol. Med. 76: 852 - 854.

52. Evans, E.E. and Kessel, J.F. 1951 The antigenic composition of Cryptococcus neoformans. II. Serologic studies with the capsular polysaccharide. J. Immunol. 67: 109 - 114. See also, Dyson, J.E. 1954 A study of yeast phase antigens in the delayed skin reactions of experimental histoplasmosis and blastomycosis. Doctoral Thesis, University of Michigan.

53. Cohn, E.J., Strong, L.E., Hughes, W.L., Jr., Mulford, D.J., Ashworth, J., Melin, M., and Taylor, H.L. 1946 Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the proteins and lipoprotein components of biological tissues and fluids. J. Am. Chem. Soc. 68: 459.

54. Nichol, J.C. and Deutsch, H.F. 1948 Biophysical studies of blood plasma proteins. VII. Separation of gamma globulin from the sera of various animals. J. Am. Chem. Soc. 70: 80.

APPENDIX I. FLOW SHEET OF COLD ETHANOL FRACTIONATION OF RABBIT SERUM BY THE PROCEDURE OF NICHOL AND DEUTSCH (54)



APPENDIX II. FLOW SHEET OF COLD ETHANOL FRACTIONATION OF HUMAN PLASMA BY THE PROCEDURE OF COHN (53)

100 cc Plasma

Stir @ 0 C no ice
EtOH buffer 8% @ pH 7.2
17.7 cc 50% EtOH
0.1 cc 0.8M NaAc buffer at 4.0
Cfg -5^{o}

Pcpt. I

Spnt.

Fibrinogen

QS 25% EtOH @ pH 6.9
60.1 cc 50% EtOH
0.088 cc 10M HAc
0.044 cc 4 M NaAc
0.23 cc 95% EtOH
Cfg -5 C

Pcpt II + III

Spnt.

β - γ globulin

18% EtOH @ pH 5.2
(1) 31.1 cc H_2O @ -5
(2) 78 cc H_2O 0 C + Ac @ pH 5.2
Stir 1 hr., stand 6-8 hrs.
Cfg -5 C

Pcpt IV-1

Spnt

α globulin

40% EtOH (0.09M) @ pH 5.8
00.114g $NaHCO_3$
0.79cc 4M NaAc
H_2O QS 77cc
1/2 hr.
95% EtOH QS 40% (80.5cc)
Cfg -5 C

Pcpt IV-4

Spnt. (clarified by filt.)

$\alpha + \beta$ globulin

pH 4.8
0.5 cc 10M HAc
0.025 cc 4M NaAc
0.105 cc 95% EtOH
QS 2.5 cc H_2O
Stand 3 hrs -5 C
Cfg -5 C

Pcpt V

Spnt.

Almost all of Serum Albumin