VIRULENCE FACTORS OF *LEPTOSPIRA POMONA*

By

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AN ABSTRACT

Submitted to the School for Advanced Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

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ABSTRACT

The information available regarding the factors which contribute to the virulence of leptospirae is both meager and conflicting.

To gain some insight into the mechanisms of L. pomona infections an investigation was undertaken to demonstrate a factor or factors responsible for the toxic effects of the organism. During the studies the opportunity also arose to examine leptospiral extracts for enzymes of the citric acid cycle.

Leptospirae were separated from cultures by centrifugation and washed and resuspended to 1/100 the original volume of culture. The cells were disrupted by sonic oscillation and the cell-free extract tested for toxic and enzymatic activity. No toxic activity could be demonstrated following intradermal inoculation into hamsters or guinea pigs. No change in hematological values could be observed in hamsters following intracardial inoculation of the extracts. Hamsters which received L. pomona cells showed increased bilirubin and urea levels and reduced hemoglobin and hematocrit levels.

Extracts of L. pomona were tested for succinic dehydrogenase, malic dehydrogenase, isocitric dehydrogenase, fumarase and aconitase. Varying degrees of activity were demonstrable for all of these enzymes.

Further investigations on the mechanism of virulence of L. pomona were performed using the hemolysin found in supernatant fluids of leptospiral cultures. The hemolysin was found to be precipitated by 35% saturation with ammonium

sulfate. Separation and concentration of the hemolysin in this manner resulted in yields of about 30 per cent.

The toxicity of the concentrated hemolysin preparations was examined by intravenous inoculation of young lambs. Animals receiving large doses of hemolysin showed a 60 per cent loss of hemoglobin 30 hours after inoculation. The lambs were weak, icteric and hemoglobinuria was evident. Smaller doses of hemolysin resulted in a 30 to 50 per cent loss of hemoglobin, with the lambs appearing weak and pale. Lambs receiving intravenous injections of washed L. pomona cells developed an acute hemolytic anemia, evidenced by a 65 per cent drop in hemoglobin and hematocrit levels. Jaundice, weakness and hemoglobinuria were evident.

Gross and microscopic examination of the tissues of the lambs showed a generalized icterus, copper colored livers and petechial hemorrhages on the kidney. Areas of degeneration and necrosis in many areas of the liver were noted. The distal and proximal convoluted tubules of the kidney showed areas of degeneration and some tubules contained a hemogenous, dark red staining material. Animals receiving hemolysin or whole cells revealed similar lesions except in the latter group more extensive renal damage was present.

Another group of lambs received a series of small doses of hemolysin. They were then challenged with live organisms or hemolysin. No evidence of hemolytic anemia was observed in any of the animals. However, the results were difficult to interpret due to the development of agglutinating antibody after receiving hemolysin preparations.

Concentrated culture supernates of a nonhemolytic strain also stimulated the production of agglutinating antibody. Although antiserum inhibited the hemolysin no evidence was obtained to indicate that the hemolysin stimulated antibody production.

An assay procedure was developed for determining hemolytic activity which was rapid and relatively accurate.

By means of this procedure, *in vitro* properties of the hemolysin were investigated. The results indicated that the rate of hemolysis was greatly increased in incubation of the tests at 37°C was followed by a 4°C incubation. Further, it was demonstrated that adsorption of the hemolysin to erythrocytes occurs, which is probably a physical rather than chemical reaction. Antibody inhibited hemolysis if in contact with the hemolysin before the addition of erythrocytes. The addition of antiserum with or after the erythrocytes resulted in diminished inhibition.

On the basis of the results obtained, a mechanism is proposed for the development of hemolytic anemia associated with leptospirosis. It is also concluded that the hemolysin is not the only toxin of leptospirae which can produce an icteric condition and that it represents only one of the virulence factors of leptospirae.

The fact that five enzymes of the citric acid cycle are demonstrable indicates that this metabolic pathway is probably operating in leptospirae.

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INTRODUCTION

The properties of bacteria which contribute to their virulence may be separated into two broad categories. First, there are the characteristics associated with overcoming the host defenses and enabling the parasite to establish infection. Under this grouping may be included hyaluronidase, coagulase, fibrinolysin, leukocidin, and antiphagocytic substances. The second group of virulence factors are those which produce the lesions in the host's tissues, the so called exotoxins and endotoxins. The relative importance of these two groups in the disease process is extremely variable. The classical examples are of anthrax and botulism. Bacillus anthracis invades and multiplies in great numbers in the host tissues. However, an animal needs only to ingest food in which Clostridium botulinum has grown and produced toxin to die of botulism and only rarely is the organism recovered from the affected individual.

Information with regard to these virulence factors among the leptospirae is both meager and conflicting. As a basis for the treatment and control of an infectious disease, all phases of the host-parasite relationship should be considered. Therefore, for the purpose of gaining some insight into the mechanisms of Leptospira pomona infections an investigation was undertaken to attempt to demonstrate a substance or substances responsible for the toxic effects of the organism. During the course of the investigations the opportunity arose to examine cell free extracts from

L. pomona for enzymes of the citric acid cycle. Because the basis of the pathogenic effect of bacteria is chemical, the more information available regarding the chemical activities of a pathogen the greater is the opportunity for understanding the basis of its virulence.

LITERATURE REVIEW

A. Virulence Studies

Gsell (24) has described the host response to Leptospirae pomona infections as occurring in two phases. The first phase is characterized by the presence of the spirochetes in the circulatory system. The second phase is a period of tissue damage involving primarily the liver, kidneys, meninges and erythrocytes. This stage terminates usually with the appearance of antibody. Icterus may or may not be present. In man the disease is most commonly associated with muscular pains and meningitis but icterus is rarely observed (2). Pigs do not as a rule show overt signs of illness other than a slight temperature elevation and loss of appetite. However, in pregnant sows abortion is frequently associated with leptospirosis (7). The disease in cattle is variable. Young animals are more severely affected showing jaundice and hemoglobinuria (60). Adult animals appear icteric less frequently, but abortion and reduced milk flow are a frequent result of infection (53,55). Bryan (8) studied the symptoms of leptospirosis in 125 herds of cattle. Abortion occurred in 58 per cent, reduced milk flow in 47 per cent, hemoglobinuira in 30 per cent and anaemia and jaundice in 15 to 19 per cent.

L. pomona infections in sheep also seem to vary in severity depending on the age of the animals (37). A severe outbreak in lambs in New Zealand was reported by Hartley (25) in which icterus and hemoglobinuria were the prominent clinical

signs. Webster and Reynolds (69) described two outbreaks of leptospirosis in ewes and lambs. A severe hemolytic anemia with hemoglobinuria and jaundice were again prominent. Abortion and death of ewes has been recorded by Beamer and coworkers (5).

Experimental ovine infections were studied by Morse et al. (47). A hemolytic anemia was observed in 5 of 8 experimental animals with accompanying hemoglobinuria. A mild jaundice was noted in 1 lamb.

Several investigators have suggested the possible causes of the various lesions observed in leptospiral infections. Gsell (24) stated that the severity of the intoxication and the intensity of the septicemia determine the differences observed in the clinical picture. He described the disease picture of leptospirosis as a general intoxication in which a leptospiral toxin causes the typical lesions. It was admitted however, that any interpretation of the disease as being due to a toxin was hypothetical as long as no such agent was yet demonstrable. Ferguson et al. (18) suggested that the cause of bovine abortion in leptospirosis was a toxin, released from the leptospirae by lytic antibodies in the dam, which entered the fetus. Morse and coworkers (47) suggested a similar mechanism as the cause of the hemolytic anemia in sheep. In this case antibody was thought to release a hemolytic endotoxin from the spirochetes or that antibody would act with a hemolytic antigen and lyse erythrocytes in the presence of complement.

As early as two years after Inada and Ido (31) in

1915 incirminated L. icterohemmorhagiae as the etiological agent of Weil's disease, Matsuzaka (39) reported the toxic properties of heat killed leptospirae for guinea pigs. Later, Fukushima and Hosoya (19) and Higuchi (27) found that cultures of L. icterohemmorhagiae which had been maintained under anaerobic conditions or in vacuo at 37°C for 2 to 3 days, produced increased bilirubin levels in the blood of guinea pigs and hyperemia of the bulbar conjunctiva. Stavitsky (61) made extensive attempts to confirm these findings without success. This same worker tested leptospiral extracts for hyaluronidase, fibrinolysin, leukocidin and coagulase activity with negative findings (62). Subsequently, a report appeared (67) stating that hyaluronidase activity was demonstrable.

Faine (15) approached the study of leptospiral virulence by comparing the fate of virulent and avirulent L. icterohemmorrhagiae cells injected into young guinea pigs. Both strains were phagocytized by fixed phagocytes of the reticulocendothelial system. Leucocytic phagocytosis was not observed. The only difference between the strains was that virulent cells survived whereas avirulent did not. This same investigator (16) studied the relationship between the number of leptospirae present in guinea pig tissues and the appearance of lesions. A concentration of 10^6 leptospirae per ml of body fluids coincided with demonstable hemmorhagic lesions. Death occurred with cell concentrations of 10^8 per ml. Death could occur in the absence of high numbers of leptospirae. With animals infected with doses near the LD_{50}, death was observed 3 days after the appearance of serum antibody.

Such observations are confirmed by other studies (32, 46) on the effect of serum therapy during different stages of leptospirosis. Serum administered prior to or simultaneously with exposure to leptospirae afforded protection, but this effect diminished when serum was given after exposure.

In 1956 Alexander *et al.* (1) and Russell (57) reported the presence of a soluble hemolysin in culture supernates of some leptospiral serotypes. The hemolysin was found in greatest amounts in the supernates 1 to 3 days after maximum growth had occurred. Only a small amount of hemolytic activity was found in disrupted cell preparations. Activity was destroyed by heating at 56°C for 10 minutes. Maximum rates of hemolysis were achieved at 37°C, with no hemolysis observable at 0°C. The hemolysin was oxygen stable, non-dialysable and inhibited by leptospiral antiserum. Erythrocytes of sheep, cows and goats were reported to be most susceptible to the hemolysin (1). Rabbit and hamster erythrocytes were less susceptible and buinea pig RBC were unaffected (57, 4). Activity was observed with some strains of *L. canicola*, *L. benjamin*, *L. cynopteri*, *L. autumnalis*, *L. australis A*, *L. pomona*, *L. bebdomadis* and *L. bataviae*. The authors (1) suggested that the hemolytic activity of leptospirae was due to the presence of a toxin.

Bauer and Morse (4) compared the hemolytic activity of *L. pomona* cultures of varying degrees of virulence with their LD_{50} for hamsters. The lack of correlation was indicated by the fact that the culture with the highest hemolytic activity had a large LD_{50}.

Imamura and coworkers (30) reported in 1957 the demonstration of a heat stable substance in sonic extracts of L. icterohemmorrhagiae, which when injected intracutaneously into guinea pigs or rabbits produced an area of inflammation of 20 to 30 mm in diameter. The non-protein nature of the substance was indicated by its negative biuret reaction and positive results in the Molisch and Bial's orcin reactions.

In 1957 Kemenes (34) reported that by injecting lambs intravenously with 150 to 220 ml amounts of leptospiral culture supernates of high hemolytic activity, dyspnea, fever and hemoglobinuria were produced in 8 to 16 hours. Daily injections of smaller amounts of hemolysin, 20 to 50 ml, produced a hemolytic anemia in about 5 to 7 days. This in vivo hemolytic effect was observed with hemolysins of L. pomona, L. canicola and L. grippotyphosa. These workers observed that an inhibitor of the hemolysin was present in the rabbit serum used for cultivation of the leptospirae. Similar observations had been made by earlier investigators (1, 57).

A recent publication (56) has suggested that the hemolysin of L. pomona may be a phospholipase. This conclusion was based on results attained by the use of phospholipids as inhibitors of the hemolysin.

B. Citric Acid Cycle Enzymes.

Pathogenic leptospirae require blood serum for growth and all attempts to substitute defined nutrients for the serum have been unsuccessful (2). Fractionation of sera to ascertain the components necessary for growth and respiration

has been attempted by several workers. Schneiderman et al. (58) reported that serum albumin contained much of the nutrient value of whole serum. Albumin precipitated by 71 per cent ammonium sulphate was effective but alcohol precipitated albumin was not. The albumin could not be replaced by a mixture of amino acids which simulated the amino acid composition of the albumin as determined by microbiologic assay. Attempts by other workers to repeat these experiments have been without success (11).

Fulton and Spooner (20) tested various components of serum for respiratory activity as measured by manometric methods. Serum protein was found to be as effective as whole serum in stimulating respiratory activity of leptospirae. Attempts to show protein breakdown however, were not successful. Serum proteins were then extracted with ether to determine whether a non-protein factor was essential for respiration. The ether extracted proteins showed no reduction in their ability to stimulate respiration. Nevertheless the ether soluble substances did possess respiratory activity. Phospholipids were extracted from the ether soluble fraction by acetone precipitation. This fraction was as active as the protein fraction in stimulating respiration.

Helprin and Hiatt (26) also investigated the effect of lipids on the respiration of leptospirae. Human plasma faction V (albumin) was found to contain essentially all of the serum respiratory activity. However, after acetone extraction almost all of the activity was lost. Addition of the acetone precipitable lipids back to plasma fraction V

restored the original activity. Lipids alone inhibited respiration. A number of fatty acids were tested for respiratory activity with and without the protein fraction. In the absence of protein the fatty acids did not stimulate respiration but with protein all possessed activity. A correlation between the number of carbon atoms and the ability to stimulate respiration was observed, arachidic acid being the most active and butyric the least. Unsaturated acids were also highly stimulatory. The authors concluded from their results that the stimulatory effect of serum proteins on leptospiral respiration is due to both the contribution of fatty acids and a detoxifying effect of the albumin.

Recently Gerhardt and Ball (21) have reported that utilization of amino acids from rabbit serum by leptospirae can be demonstrated by chromatographic and microbiological assay procedures. It has been shown by several workers that leptospirae do not metabolize carbohydrates (20, 9).

Leptospirae require oxygen for growth. Czekalowski, McLeod and Rodican (13) observed the formation of narrow rings of leptospiral growth in tubes of semi-solid or solid agar containing 10 per cent rabbit serum. The bands of growth occurred 4 to 10 mm below the surface of the medium. Growth was minimal or absent in the clear areas above and below the dense areas of growth. From these results it was concluded that leptospirae require amounts of oxygen below that of the atmosphere and should be considered microaerophilic.

The generation time of leptospirae has been reported by several investigators as between 24 and 68 hours (20, 9).

These reports were based on results obtained by observing changes in turbidity in fluid cultures. Cox and Larson (12) developed a solid agar medium for growing leptospirae which permitted growth of the organisms to be determined by the plate count method. They obtained generation time values of from 6 to 24 hours depending on the culture medium (36). The discrepancy between these values and those of previous reports was suggested to be due to the possibility that turbidimetric evaluations could only be performed during the declining phase of growth, the number of leptospirae being too few during the logarithmic phase for turbidimetric evaluation.

The enzymes or enzyme systems operating in leptospirae have been subjected to only limited investigation. Czekalowski and coworkers (13) reported that catalase was not present and peroxide formation could not be observed. Tetramethyl para-phenylenediamine was added to areas of dense leptospiral growth in a semi solid medium. The formation of a dark blue color over the areas of growth, indicating the formation of indophenoloxidase, demonstrated the presence of bacterial oxidase. This same reaction is obtained with colonies of leptospirae (23). Leptospirae were found to be sensitive to cyanide and the authors concluded that a cytochrome system was probably present.

Fulton and Spooner (20) also noted the inhibitory effect of cyanide and also that of azide. Spectroscopic examination of suspensions of leptospirae showed adsorption bands at 550 mm and 525 mm corresponding to the bands of

cytochrome c. On shaking in air the bands disappeared but reappeared upon standing. M/10 cyanide prevented the disappearance of the adsorption bands indicating that cytochrome oxidase was operative. Other enzyme inhibitors which were active in inhibiting respiration of cell suspensions were arsenite, urethane and iodoacetate. Arsenate, pyrophosphate, malonate and fluoride were not inhibitory. On the basis of these studies it was suggested that enzymes containing sulfhydryl groups were active.

Recently the iron requirements of leptospirae have been studied (17). Growth did not occur in the absence of iron. When $FeCl_3$ or hematin was added growth occurred, but at a slower rate than when hemoglobin or hemoglobin derivatives were added. It was concluded that iron was needed to form essential porphyrin-containg compounds. The addition of hemoglobin to leptospiral culture media as a source of prophyrin compounds had been suggested by earlier workers (14).

MATERIALS AND METHODS

A. Virulence Studies

Four strains of L. pomona were used in these studies. Strain W was originally isolated from the urine of an infected cow and had been maintained in a virulent state by continuous passage in young guinea pigs. This strain was infective for sheep (47), swine (44), cattle (43), goats (45) and dogs (10). It was infective but not lethal for hamsters (4). Strain LW was a variant of strain W. The variations occurred when strain W was inocculated into lambs and cultured from these animals during leptospiremia. After 3 to 4 transfers of the organisms in culture media they became lethal for hamsters (4). Since its isolation two years ago, this strain has been maintained in culture with a passage through hamsters after every 10 to 15 culture transfers. No change in its lethal properties for hamsters has been observed. Strain LB was a hamster lethal variant of a strain of L. pomona isolated from cattle. This variant was produced in the same manner as strain LW. Strain J was a non pathogenic strain which has been maintained for 5 years in culture.

Large volumes of leptospiral cultures were obtained in the following manner. To one liter screw cap bottles was added 450 ml of sterile Stuart's medium (63) containing 10 per cent sterile rabbit serum. The inoculum consisted of 2 ml of a 3 to 5 day old culture. The cultures were incubated at 29°C and observed daily for evidence of turbidity.

Leptospirae were collected from the cultures when maximum turbidity had been attained. The organisms were removed from the cultures by centrifugation in a Lourdes model AX centrifuge at 12,000 RPM for 30 minutes. The cells were then washed three times and resuspended to the desired volume in saline solution.

To obtain large volumes of leptospiral hemolysin the cultures were incubated for 2 to 3 days after maximum turbidity had occurred. They were then placed in the refrigerator or frozen until ready for use. At this time they were filtered in a Seitz filter fitted with a Hercules ST-1 sterilizing filter disc. The filtrates were stored at 4°C or frozen.

Following incubation all cultures were tested for sterility by microscopic examination and inoculation of thioglycollate broth (Difco).

Titrations of hemolysin were made by two fold serial dilutions of culture supernates in saline solution, with a final volume of 0.5 ml. An equal volume of a 2 per cent suspension of washed sheep red blood cell was added and the tests incubated for 3 hours at 37°C and 12 to 15 hours at 4°C. The reciprocal of the highest dilution of hemolysin producing observable hemolysis was designated the number of units of hemolysin (HU) per ml.

Red blood cells (RBC) were obtained from sheep, cows and guinea pigs and placed in an equal volume of Alserver's solution (40). Such preparations were stored in the refrigerator for periods of up to 10 days, after which they were discarded. RBC were prepared for hemolytic tests by

washing three times and resuspending in saline solution or phosphate buffered saline solution pH 7.3 to make a 2 per cent suspension. After 4 days these preparations were discarded.

The animals used for experimental work were 4 to 6 week old hamsters, 4 to 7 week old guinea pigs and lambs from birth to 3 months of age. All animals were known to be free of leptospiral serum antibodies unless otherwise stated. The presence of serum antibodies was determined by a microscropic agglutination test previously described (47) in which 10 fold serial dilutions of serum were made in saline solution in a total volume of 0.1 ml. A 0.1 ml volume of a 3 to 5 day culture of strain J was added to each tube. After incubation for 2 hours at 37°C, the contents of each tube were deposited on a slide and the amount of agglutination determined by microscopic examination.

Hemolysin inhibition tests were performed by making 2 fold serial dilutions of serum in saline solution in a final volume of 0.5 ml to which was added 0.5 ml of a dilution of hemolysin containing approximately 50 units of hemolysin per ml. The mixtures were incubated at 37°C for 30 minutes after which 0.5 ml of a 2 per cent suspension of sheep RBC was added. The tests were then incubated at 37°C for 3 hours and 12 to 15 hours at 4°C. The highest dilution of inhibitor which prevented hemolysis was designated the inhibition titer.

Hemoglobin values were obtained by the Cyanmethemoglobin method (59). Hematocrit determinations were made in the

standard manner (59. Blood chemistry determinations of hamsters were made using micro-methods as outlined by Natelson (49). Differential leucocyte counts were determined using standard hematological procedures (59). The number of leptospirae in the plasma of hamsters and lambs was estimated by microscopic examination. Protein determinations were performed by the method of Lowry (38).

B. Citric Acid Cycle Enzymes

Determining if citric acid cycle enzymes were present was suggested by the fact that leptospirae utilize oxygen and possess a cytochrome system, plus the evidence that phospholipids are metabolized and the possible identity of the hemolysin with a phospholipase. Such a study of the terminal respiration of leptospirae was made by the following procedure.

The method used for obtaining cultures of leptospirae in large volumes has been described in Section A of Materials and Methods. The medium was inoculated with 2 ml of an actively growing culture of L. pomona strain LW. After incubation at 30°C for 5 to 8 days the cultures were tested for contamination by inoculation of thioglycollate broth (Difco) and by microscopic examination. Any results obtained using cultures, subsequently shown to be contaminated, were discarded. Leptospirae were collected from the cultures by centrifugation at 12,000 RPM for 30 minutes. Throughout centrifugation and subsequent procedures the organisms were maintained at 0 to 4°C. The packed cells were washed three

times and resuspended in diluent to 1/100 the original culture volume. The diluent used for determinations of activity of dehydrogenases was 0.15M NaCl. For assaying aconitase and fumarase the diluent was a buffered solution of citrate or fumarate, respectively. The concentrated cell suspensions were treated for 15 minutes in a 10 Kc Raytheon sonic oscillator and kept at 0 to 4°C until ready for use. This disrupted cell suspension constituted the enzyme preparation.

Isocitric dehydrogenase activity was assayed by the method of Horecker and Kornberg (29). The reaction mixture contained 0.2 ml of 0.5M Phosphate buffer pH 7.0, 0.1 ml of 0.1M $MgCl_2$, 0.02 ml of 0.025M TPN, 0.1 ml of 0.005 M d-l isocitrate, 0.1 ml enzyme and H_2O to a volume of 3.0 ml. The reduction of TPN was followed in a Beckman DU Spectrophotometer at 340 mu.

Malic dehydrogenase was assayed by the method described by Mehler et al. (41). The conversion of oxalacetate to malate was followed by the oxidation of DPNH to DPN at 340 mu. The cuvettes contained 0.3 ml of 0.25 M glycylglycine buffer, pH 7.4, 0.1 ml of 0.0015 M DPNH, 0.1 ml of 0.0076 M oxalacetate, pH 7.4, 0.1 ml of enzyme and 2.4 ml H_2O.

Succinic dehydrogenase activity was measured by the reduction of the dye, dichlorophenolindophenol. The reaction was followed by observing the change in optical density measured at 600 mu. Inhibition of the hydrogen transport system was achieved by the addition of cyanide. The reaction vessel contained 0.2 ml of 0.2M sodium succinate, 0.3 ml of 0.1 M histidine buffer, pH 6.5, 0.25 ml of 0.1 M KCN, 0.1 ml of

0.05 M $MgSO_4$, 0.2 ml of enzyme, 0.2 ml of dichlorophenolindophenol and H_2O to 3 ml.

Measurement of fumarase activity was accomplished by the decreased optical density at 300 mu occurring as malate is formed from fumarate (52). One tenth ml of .017M sodium fumarate, 0.1 ml of phosphate buffer, pH 7.3, 0.5 ml of enzyme and 0.5 ml of H_2O were added to the cuvette.

A similar procedure described by Racker (52) was employed for assaying aconitase activity. The increase in optical density at 240 mu was measured as aconitate was formed from citrate. The reaction mixture contained 1.0 ml of 0.3M sodium citrate, 1.0 ml of .05M phosphate buffer, pH 7.4, 0.5 ml of enzyme and 0.5 ml of H_2O.

Protein was estimated by the ratio of optical density at 280 mu to that at 260 mu as described by Warburg and Christian (68).

RESULTS

A. Virulence Studies

The first experiments were designed to determine if a toxic substance could be obtained from concentrated cell suspensions of L. pomona. Washed cells, strains LW and LB, were resuspended to 1/100 the original culture volume. They were then disrupted by treatment for 15 minutes in a Raytheon 10kc water-cooled, sonic oscillator. Intraperitoneal inoculation of hamsters with up to 2 ml of this sonic extract (SE) produced no observable signs of illness. The animals were sacrificed and observed for gross pathological changes. No lesions were observed.

SE preparations of strain LW were then tested for their ability to produce a skin reaction following intradernal inoculation. Shaved areas on 3 hamsters and 3 guinea pigs were injected intradermally with 0.1, 0.2 and 0.3 ml of SE. No reaction was observed over a 72 hour period in either hamsters or guinea pigs.

The next approach to the problem was to compare the hematological changes in hamsters infected with whole cells with those receiving cell extracts. Two groups of hamsters were employed in this experiment. Group I consisted of 25 animals, 17 of which were inoculated intraperitoneally with 1.0 ml of a 7 day old culture of strain LW. Eight animals served as controls and received 1.0 ml of sterile culture medium. At 28 and 48 hours after inoculation 3 hamsters

were sacrificed and a blood sample obtained. At each of these times one control hamster was also sacrificed. At 72 hours 5 animals were moribund and were sacrificed with 2 control hamsters. Ninety six hours after inoculation blood samples were obtained from all remaining hamsters.

Group II contained 9 hamsters, 6 receiving an intracardial inoculation of 1.0 ml of SE and 3 given 1.0 ml of sterile saline by the same route. Four hours later 3 hamsters and 1 control were sacrificed and a blood sample obtained. The remaining animals were sacrificed at 18 hours. No overt signs of illness were observed in this group. Twelve apparently normal hamsters were used to determine normal hematological values.

The changes observed in the blood chemistry values and differential leukocyte determinations are presented in Table 1. Infected hamsters remained normal throughout the first 48 hours after inoculation. At 72 hours the obvious illness of the animals was reflected in the blood picture. Urea values were increased as much as 15 fold, bilirubin levels were increased, while a decrease in hemoglobin and hematocrit readings was observed. Differential leukocyte determinations indicated an increase in the percentage of polymorphonuclear leukocytes (PMN) and a corresponding decrease in lymphocytes. At 96 hours the blood picture was similar except that bilirubin levels were increased while urea values, although abnormal, had decreased. Hematological values of control animals remained within normal limits during the experiment.

The blood chemistry values of Group II hamsters remained essentially normal. Differential leukocyte determinations demonstrated increased numbers of PMN and fewer lymphocytes. Control animals in this group remained normal.

No indication having been found that a toxic substance was present in extracts of L. pomona, the possibility that an aggressive agent was present in the extract was investigated. An experiment was performed to determine if extracts of the hamster lethal strain LW could effect the pathogenicity of strain W. Two hamsters were inoculated intraperitoneally with 1.0 ml of a culture of strain W. Two animals were similarly injected with 1.0 ml of SE of strain LW and 2 hamsters received 1.0 ml of a mixture of equal volumes of strain W culture and SE of strain LW. The animals were observed for 2 weeks without any appearance of illness. No lesions were observed in any of the 6 animals when sacrificed.

Failing to find any evidence that extracts of L. pomona contained a toxin or aggressive agent, attention was turned to the hemolysin found in the supernates of leptospiral cultures.

To ascertain what role the hemolysin might play in leptospirosis it seemed advisable to first find a method by which this substance could be separated from culture supernates and then concentrated. The thermal instability and non-dialysability of the hemolysin suggested that it might of of a protein nature. Therefore ammonium sulfate from a weighed container was added to 30 ml of a culture supernate of strain LW. When a precipitate formed, the amount of salt added was determined by the difference in weight of the salt container.

After standing 15 minutes at 4°C the precipitate was removed by centrifugation at 10,000 RPM for 15 minutes. The supernatent fluid was removed for further extraction and the sediment resuspended to the original volume in saline. This procedure was continued until 100 per cent saturation of ammonium sulfate was reached. The 12 resulting fractions were tested for hemolytic activity against sheep RBC. As a control of the isotonicity of the fractions, they were also titrated against guinea pig RBC. In table 2 the results of the tests are presented. All of the hemolytic activity was present in the precipitates formed at 31 and 35 per cent saturation. No hemolysis in the control tubes was observed.

This procedure was then applied to large volumes of culture filtrates as follows: culture filtrates were brought to 36 per cent saturation with ammonium sulfate and left standing at 4°C for 6 to 12 hours. The precipitate was removed by centrifugation at 10,000 RPM for 15 minutes. The precipitate was resuspended to about 1/20th the original volume in saline or buffered saline pH 7.3. The resulting pale yellow solution was then dialysed with distilled water for 2 hours and against saline of buffered saline for 12 hours. The dialysate was examined for the presence of sulfate ions by the addition of a few drops of a 10 per cent solution of barium chloride, the formation of a white precipitate of barium sulfate indicating incomplete dialysis. Following complete dialysis the preparation was stored either at 4°C or frozen at -20°C.

In the first experiment to determine the toxicity of

the hemolysin, 2 lambs of about 2 weeks of age and weighing 8 to 9 kgm, were used. Lamb 771 received an intravenous inoculation of 20 ml of hemolysin containing a total of 80,000 HU. Lamb 800 was injected with 20 ml of sterile saline. The effect of the hemolysin can be ascertained from the results presented in Table 3. Hemoglobin values and hematocrit readings of lamb 771 dropped rapidly after 10 hours. During the first 20 hours after inoculation the animal showed no overt signs of illness. At 26 hours the lamb was unable to stand, excreted a dark red colored urine and displayed a marked icterus of the mucous membranes. The animal was comitose at 29 hours and died 30 hours after inoculation. The hemolytic anemia was further manifested by the red tinged appearance of the plasma from 10 hours until death. Control lamb 800 remained essentially normal throughout the experiment.

A more extensive appraisal of the <u>in vivo</u> action of the hemolysin was undertaken in the next experiment. Six lambs of 2 to 3 weeks of age were selected for study. Their weights ranged from 10 to 15 kgms. All lambs were free of leptospiral antibodies except lamb 32 which had an antibody titer of 10^{-5} due to passive immunization from colostrum of its dam. Lamb 24 served as a control and received 30 ml of a concentrated culture supernate of strain J. No hemolytic activity was detectable in this preparation. Lamb 20 received 12 ml of washed cells strain LW. Lambs 25, 28, 29 and 32 received various dosages of hemolysin. All injections were made intravenously. Table 4 summarizes the data from this study.

Animal 25 received the largest dose of hemolysin and developed an acute hemolytic anemia evidenced by a drop in hemoglobin of about 60 per cent in 31 hours. Weakness, hemoglobinuria and icterus were observed. The animal was unconscious at 31 hours and was sacrificed. Lambs 28 and 29 received smaller doses of hemolysin and the extent of the resulting anemia was correspondingly less severe. Hemoglobin and hematocrit values decreased up to 73 hours after inoculation. Hemoglobin losses of from 30 to 50 per cent were noted. The animals were pale, their plasma was a dark yellow color and anorexia and dyspnea were noted. Lamb 32 received a lethal dose of hemolysin but except for a 6 per cent decrease in hemoglobin and a reduced hematocrit reading at 24 hours, no evidence of anemia was observed. Control lamb 24 showed a slight drop in hemoglobin and hematocrit levels but plasma samples were not icteric nor did the animal show observable signs of illness. Lamb 20 received washed leptospirae and after an incubation period of 4 days became weak and icteric. Hemoglobin and hematocrit levels decreased 65 per cent. Washed RBC from this animal lysed rapidly at 37°C. After 6 days the animal was comitose and the temperature was sub-normal. The animal was sacrificed at this time. The relationship between the course of the anemia, the number of organisms in the plasma samples and the appearance of antibody in lamb 20 is shown in Figure 1. Hemoglobin values decreased from the second day after inoculation through the fourth day at a relatively constant rate. During this same time the number of leptospirae in the plasma increased to 10^4 per ml. Antibody

could be detected on the third day and increased to a titer of 10^{-4} on day 5. At $4\frac{1}{2}$ days the number of leptospirae decreased and the rate of hemolysis was slightly increased.

Lambs 771, 24, 25, 29 and 20 were submitted to gross and microscopic pathological examination. In lambs receiving hemolysin the gross changes were reported as consisting of generalized icterus with the liver, mucous membranes and skin being most markedly affected. The livers were friable and swollen. Approximately 10 petechial hemorrhages were observed on each kidney. The urinary bladder of lambs 771 and 25 was filled with dark red urine.

Microscopic lesions consisted of areas of centrilobular necrosis in many parts of the liver. The hepatic cords were disrupted in the peripheral portions of the affected areas. In the kidney there were numerous pyknotic nuclei in the proximal and distal convoluted tubules. Many tubules contained a homogenous red staining material. The spleen was markedly congested. No lesions were observed in the brain, heart, adrenal gland or skeletal muscle of any of these animals.

The lamb killed during the acute stage of the leptospiral infection showed grossly a slight icterus with the liver appearing copper colored and slightly friable. The kidneys were markedly pale with numerous petechial hemorrhages. The spleen was swollen and congested.

Microscopically the lesions were similar to those observed in the lambs given hemolysin with the following exceptions. The affected areas of the liver were not as

extensive and there appeared to be an increased number of lymphocytes in the intertubular areas in the kidney.

No gross or microscopic lesions were observed in the control lamb.

To further ascertain the role played by the hemolysin in leptospiral infections and to determine if the hemolysin was immunogenic, 5 lambs weighing 15 to 25 kgm were given 3 sub-lethal doses of hemolysin over an 18 day period. One lamb, number 29, had recovered from a sub-lethal dose of hemolysin in the experiment previously described.

The injection schedule was as follows: 5,000 HU on day 1, 1,000 HU on day 5, and 20,000 HU on day 18. After the first injection a mild anaphylactic reaction was observed in lamb 29 but not after any of the subsequent inoculations. Thirty to 40 hours after the last injection one of the lambs died. Examination of the cadaver revealed extensive icterus of the liver, skin and mucous membranes and petechial hemorrhages on the kidney.

Serum samples taken from the lambs during the series of hemolysin injections were tested for hemolysin inhibition titers (HI) and agglutinating antibody titers (AB). No increase was observed in HI titers nor were AB titers observed except in lamb 29. This animal demonstrated an increase of HI titer of from 8 to 64 and an AB titer of 10^{-1} following the last injection.

Three of the 4 lambs which had received hemolysin injections were challenged with strain LW. A control lamb 23 which had not received hemolysin inoculations was also

inoculated with this strain. The challenging dose consisted of an intravenous inoculation of 10 ml of washed cells. About 10^8 organisms per ml were contained in the inoculum. The remaining lamb which had received small doses of hemolysin was challenged with 10,000 HU/kgm of hemolysin.

Following challenge the hemoglobin values, temperature, and serum antibody titers were determined at periodic intervals. These values are summarized in Table 5. Lamb 29 which had an AB titer at the time of challenge showed little change in hemoglobin values. Lambs 31 and 40 demonstrated a decrease in hemoglobin levels which terminated with the appearance of antibody. No organisms could be observed in the plasma of these animals at 41 hours after inoculation or at any time thereafter. Lamb 22 which was challenged with hemolysin developed only a minimal loss of hemoglobin which terminated with the presence of antibody.

Lamb 23 developed an acute case of leptospirosis. An abbreviated incubation period of about $1\frac{1}{2}$ days with a persistent febrile response was noted. A loss of 70 per cent of the hemoglobin occurred during a period of 5 days. Hemoglobinuria was observed at 96 through 120 hours after inoculation and the animal was weak and icteric. A gradual recovery started on the sixth day. The hemoglobin changes, the number of organisms in the plasma and the appearance of antibody in this lamb are shown in Figure 2.

Because lambs 29 and 22 developed an AB titer after receiving only hemolysin injections, it was of interest to determine if the hemolysin or another leptospiral antigen in

the preparation was the antigenic stimulus. Therefore a 3 week-old lamb was given a series of intravenous inoculations of 10 ml of concentrated culture filtrates of strain J. No hemolytic activity could be detected in this material. After 3 injections at 7 day intervals this animal had an AB titer of 10^{-3}. The lamb was then challenged with 15,000 HU/kgm of hemolysin. After 5 hours the hemoglobin level had decreased from 9.0 to 8.2. Twelve hours later the animal was found dead. Examination of the cadaver revealed no evidence of icterus. The tissues were unsuitable for post mortem examination. The cause of death was not determined but the lamb had had a diarhea and appeared bloated prior to challenge.

The next investigation was concenred with the appearance of an inhibitor of the hemolysin in normal sera. Lamb 771 was observed at birth and a blood sample was obtained before the lamb was allowed to nurse. Lamb 800 was bled within 30 minutes after receiving colostrum. The HI titers and AB titers of both lambs were then determined for a 48 hour period. The results are presented in Table 6. The serum of neither lamb was inhibitory at birth but the HI titers increased to 16 after 7 to 24 hours. No agglutinating antibody could be detected in either lamb.

In a similar experiment, summarized in Table 6, twin lambs 974 and 975 were taken at birth and a blood sample obtained. Lamb 975 was then allowed to nurse whereas lamb 974 was kept from feeding for 2 hours before receiving colostrum. It can be seen from the table that animal 974

had an increase in HI titer during the 2 hour fasting period. After receiving colostrum which contained agglutinating antibody both lambs developed increased HI and AB titers. It should also be noted that a consistent correlation between HI and AB titers was not present.

Before any further studies could be carried out on the nature and properties of the hemolysin it was necessary to develop an assay procedure which was more accurate and rapid than the titration method. Previous observations had indicated that with low dilutions of hemolysin some hemolysis took place after 30 minutes at 37°C. A series of dilutions of hemolysin were then prepared in a total volume of 6 ml and after thermal equilibration, an equal volume of a 2 per cent suspension of sheep RBC was added. After incubation at 37°C for 30 minutes the tests were placed in the refrigerator (4°C). Three ml samples were withdrawn from the mixtures at intervals and placed in matched cuvettes. The RBC were sedimented by centrifugation and the optical density of the supernate measured at 540 mu in a Bausch and Lomb Spectronic 20 colorimeter. The rates of hemolysis are shown in Figure 3. After 60 minutes at 4°C the rates appeared to be essentially linear and this time was selected for the assay procedure. The protocol for the procedure was as follows: The hemolysin to be assayed was diluted 1:10 and from this master dilution a series of 4 to 6 dilutions from 1:15 to 1:100 were made in a total volume of 1.5 cc in optically matched test tubes. The dilutions and a 2 per cent suspension of sheep RBC were placed in a 37°C waterbath for 5 minutes to allow for thermal

equilibration. A volume of 1.5 ml of the RBC suspension was then added to each tube and mixed well. The tests were incubated at 37°C for 30 minutes with agitation at about 10 minute intervals. The tubes were then placed at 4°C for 60 minutes. Following incubation the tubes were centrifuged for 5 minutes at 1,500 RPM to sediment the intact RBC and stroma. The optical density of the supernates was recorded at 540 mu. With each test was included one tube containing 1.5 ml RBC suspension and 1.5 ml of a saponin solution and one tube with 1.5 ml saline and an equal volume of RBC suspension. The optical density of the tube containing saponin was designated 100 per cent hemolysis. The other control tube served as a blank. The optical density readings were converted to per cent hemolysis and plotted against dilutions of hemolysin. The reciprocal of the dilution of hemolysin producing hemolysis of 50 per cent of the erythrocytes was designated as the number of hemolytic units (HU) per ml of hemolysin.

To determine if the procedure was truly quantitative, varying amounts of hemolysin were placed in tubes and brought to a volume of 1.0 ml. Each of these samples was then assayed for hemolytic activity. The results seen in Figure 4 indicated that the method was a true indication of the amount of hemolysin present. The reproducibility of the procedure as measured over a 10 day period with a concentrated culture filtrate is summarized in Table 7. A comparison of the values obtained with the titration procedure and the new method (assay procedure II) is shown in Table 8.

The ratios of the titration method and procedure II range from about 18 to 38, giving some indication of the error in the titration procedure. The average ratio between the two methods was 28 which is a useful factor for converting results obtained by one method to that of the other. All subsequent studies employed procedure II for determining hemolytic activity.

An accurate and comparitively rapid assay procedure now available, the degree of purification of the hemolysin achieved by ammonium sulfate precipitation was examined. Further purification was also attempted by selective adsorption and elution from calcium phosphate gel (33). A large loss in activity resulted with little or no observed purification. The highest degree of purification was achieved by dialysing the precipitated hemolysin against distilled water. A precipitate formed which was soluble in saline. The water insoluble fraction had a specific activity $2\frac{1}{2}$ times that of the water soluble portion, and $7\frac{1}{2}$ times that of the original culture filtrate. The purification can be followed by the data presented in Table 9. The average yields of hemolysin obtained by salt precipitation were 30 to 35 per cent. Further purification resulted in approximately a 15 per cent yield.

An attempt was made to remove the hemolysin inhibitor present in the rabbit serum used for growing the cells. Because phospholipids and cholesterol are normal hemolysin-inhibiting constituents of serum, chloroform and ether extractions were performed. Culture filtrates were shaken for

10 minutes with 2 volumes of extractant and then the extracted phase removed in a seperatory funnel. Ammonium sulfate was added to 36 per cent saturation and the precipitate resuspended and dialysed overnight against saline. Dilutions of the preparations were made and after addition of an equal volume of a 2 per cent RBC suspension, the tests were incubated for 30 minutes at 37°C. The per cent hemolysis was then determined as previously described. The results are presented in Figure 5. Only a slight removal of inhibitor was accomplished with ether and chloroform while no inhibitor was removed by ammonium sulfate precipitation.

Although it has been reported that following incubation at 37°C, incubation at low temperatures is necessary for complete hemolysis (57), no report has been made of the effect of low temperatures on the rate of hemolysis. Therefore the rates of hemolysis were examined during various intervals of incubation at 37°C and 4°C. A 1:10 and a 1:100 dilution of a hemolysin preparation which contained 60 HU/ml were used. Examination of Figure 6 shows that following incubation at 37°C, a lower temperature increases considerably the hemolytic rate. At the higher dilution the length of incubation at 37°C seems to effect the degree of hemolysis which will occur.

The next experiment concerned the effect of leptospiral antisera on the course of hemolysis. L. pomona antiserum (sheep) with an AB titer of 10^{-3} was added to hemolysin at 37°C, 5 minutes before the addition of RBC suspension, simultaneously with the RBC suspension and 5 minutes after the RBC suspension. The final reaction vessels contained 5.0 ml of

hemolysin, 1.0 ml of antiserum and 5.0 ml of RBC suspension. Samples were removed at 15 minute intervals for determinations of per cent hemolysis. The results as seen in Figure 7 indicate that the longer the antiserum was in contact with the hemolysin before addition of erythrocytes, the greater the inhibition of hemolysis. Although not presented in the graph it should be noted that normal serum shows a similar inhibition but to a lesser degree.

The next study was made to ascertain whether the hemolysin is adsorbed by the erythrocytes and if so, the specificity of this reaction. Varying amounts of packed sheep and guinea pig erythrocytes were added to 2.0 ml of a 1:8 dilution of hemolysin containing 58 HU/ml. The mixtures were agitated for 5 minutes in a 37°C waterbath. The RBC and hemolysin were separated by centrifugation and the hemolytic activity determined. The erythrocytes were washed twice and resuspended to 1.0 ml in saline and incubated at 37°C for 1 hour and 2 hours at 4°C. The results, summarized in Table 10, indicated that both species of RBC removed equal amounts of hemolysin although only the sheep RBC were lysed. There was no apparent difference in the amount of hemolysin removed by the 3 different volumes of erythrocytes, indicating that under the conditions of the experiment even 0.2 ml was an excess of RBC.

Because guinea pig RBC removed amounts of hemolysin equivalent to the reactive sheep RBC, the possibility that the adsorption was a physical one rather than a chemical combination was tested by using polystyrene latex spheres (Dow Chemical Co.) as the adsorbent. The spheres were

examined microscopically and ranged in size from a diameter of about 5 to 12 μ with an average diameter of 6 to 9 μ. They were, therefore, approximately the same size as erythrocytes. Hemolysin (2 ml) and 0.5 ml and 1.0 ml of spheres were incubated at 37°C for 5 minutes. After removing the spheres by centrifugation the hemolytic activity of the supernates was determined. Table 10 shows that 1.0 ml of spheres which represented a larger surface area than any of the amounts of erythrocytes used, removed slightly less hemolysin than the RBC. The 0.5 ml volume of spheres was about one half as effective as erythrocytes in removing hemolysin.

The possibility that the hemolysin might be a phospholipase has been suggested (56). If true, the hemolysin should react with a saline extract of egg yolk to produce a turbid suspension, the lecithovitellin reaction (48). A 5 per cent suspension of egg yolk in saline was prepared and clarified and sterilized by passing through a Seitz filter. Dilutions of hemolysin in a final volume of 2.0 ml were added to an equal volume of the egg yolk extract and incubated at 37°C. A control tube contained saline in place of hemolysin. Optical density readings were made at 640 mu and are recorded in Table 11. It can be seen that no detectable increase in turbidity could be observed.

B. Citric acid cycle enzymes.

To further ascertain the biological activity of extracts of L. pomona, SE from strain LW were tested for the presence of five enzymes of the citric acid cycle. Although

several determinations were made on most of the enzymes, the results obtained with the most active preparations are presented here.

Succinic dehydrogenase activity of cell free extracts from L. pomona is shown in Figure 8. The rate of oxidation was calculated using the specific absorption coefficient of $1.6 \times 10^4 cm^2$/ mole for dichlorophenolindophenol. The oxidation rate of leptospiral succinic dehydrogenase was 7.4 μM O_2/mgm Protein/hr. The effect of malonate on the rate of oxidation is evident from the graph. An inhibition of about 50 per cent was obtained in this experiment.

Isocitric dehydrogenase activity was demonstrable as seen in Figure 9. The rate of reduction of TPN to TPNH was 0.72 μM TPN/mgm Protein/hr. This value is about 25 per cent that reported for yeast isocitric dehydrogenase (29).

The oxidation of DPNH to DPN during the conversion of oxalacetate to malate was evidence for the presence of malic dehydrogenase (Figure 10). A rate of 9.42 μM DPNH/mgm Protein/hr. was obtained in this study.

Considerable difficulty was experienced in demonstrating fumarase and aconitase activity. Preparations made using a buffered diluent were consistently inactive. When the specific substrates, fumarate or citrate, were incorporated into the suspending medium the cell extracts demonstrated low activities as seen in Figures 11 and 12. Because of the high endogenous rates obtained due to the presence of substrate in the diluent, endogenous values were subtracted from the readings obtained after addition of more substrate.

During the course of these studies the sonic oscillated cell preparations were examined by darkfield microscopy to determine the efficiency of the breaking procedure. It was noted that enzyme activity was demonstrable only in preparations in which few or no intact leptospirae were observed.

Enzymatic activity also diminshed rapidly after disruption of the cells. Preparations lost almost all activity after 5 hours either when kept at 4°C or frozen at -20°C.

DISCUSSION

There are three predominant activities which leptospirae initiate in a susceptible host. They are kidney damage, abortion and icterus. The prevalence with which each of these is found varies with the species of infecting leptospirae and the host species. For example L. icterohemorrhagiae infections of humans are typified by an acute nephritis and icterus. In contrast, human cases of L. pomona infections rarely show jaundice but renal damage is prevalent. Swine infected with L. pomona will frequently abort and develop varying degrees of nephritis whereas sheep infected with the same organism will often show all three manifestations.

For a complete understanding of the virulence of leptospirae the factor or factors responsible for the kidney alterations, abortion and icterus must be elucidated. The studies reported in this thesis have been confined to the possible mechanisms by which L. pomona produces its effect on renal tissue and to the basis of the development of jaundice.

Our failure to find any toxic activity in sonic extracts of L. pomona is not in accord with the reported findings using extracts of L. icterohemorrhagiae (30). It is possible that the toxic system is extremely labile and its effects, therefore, difficult to reproduce. The discrepancy in the findings may also be a reflection of a difference in the virulence mechanisms of the two leptospiral species. It seems unfortunate that the extract of L. icterhemorrhagiae

was not examined for its effect on other than cutaneous tissues since skin lesions in leptospirosis are relatively infrequent.

The hematological changes occurring in hamsters infected with L. pomona give some indication of the possible cause of death. On the third day after inoculation all the hamsters examined were moribund and death would probably have occurred in 2 to 6 hours. Although hemoglobin levels were reduced about 50 per cent the approximate 10 fold increase in the blood urea values indicate that the deaths would probably be due to uraemia. L. icterhemorrhagiae infections in humans and guinea pigs and L. canicola infections in humans and dogs also produce death from uraemia (2).

The observation that the leptospiral hemolysin could be precipitated from culture supernates by ammonium sulfate, afforded the opportunity to study the toxicity of this agent using comparatively small volumes. The capacity of the preparation to produce hemolysis in vivo was indicated by the reduced hemoglobin levels and hematocrit readings in lambs 771, 25, 28 and 29. That the hemolytic activity of the inoculum was of leptospiral origin was evident from the protective effect of leptospiral antiserum (lambs 32 and 22). The amount of hemolysin necessary to produce a fatal result seemed to be somewhat variable. Lamb 771 succumbed to a dose of 10,000 HU/kgm whereas lambs 28 and 29 recovered from similar dosages. Animal 25 received 15,000 HU/kgm and developed a hemolytic anemia which would probably have been fatal. Probably these discrepancies are due in large part

to the fact that the hemolytic activities of the inocula were determined by the relatively inaccurate titration procedure. The approximate two-fold error in this method was indicated by subsequent findings (Table 6). However, this is not an adequate explanation for the death occurring in a lamb after receiving three "sub-lethal" hemolytic doses. The final inoculation of about 1,000 HU/kgm was received 2 weeks after a dose of 200 HU/kgm. It is doubtful whether after this length of time an accumulative effect could be responsible. Because three other lambs received a similar series of injections without observable effect, it can only be concluded that this animal was uniquely susceptible to the hemolysin.

The gross and microscopic changes in the tissues of lambs receiving hemolysin were similar to those found in lambs receiving leptospirae. The liver seemed to be most severly affected whereas kidney damage was slight. It is difficult to determine to what extent the lesions are due to the primary effect of the hemolysin or to secondary effects of the existing anemia. There is no apparent reason why the cytotoxic action of the hemolysin should be confined to erythrocytes. However, in view of the extent and relatively localized nature of the tissue damage, it would seem wise at this time to assume that the cause of the cellular changes is anoxia. The pathological changes observed in the lambs receiving hemolysin are similar to those reported by Hartley (25) in a severe outbreak of leptospirosis in sheep.

The results of the experiments concerning the immunogenicity of the hemolysin were obscured by an apparent

contaminating leptospiral antigen in the hemolysin preparation. This conclusion is based on the fact that preparations from culture filtrates of both strain LW and the non-hemolytic strain J initiated the appearance of leptospiral agglutinating antibodies. It is possible that strain J contained undetectable amounts of hemolysin and that antibodies to the hemolysin are capable of acting on the surface of leptospiral cells to produce agglutination. An attempt was made to clarify the serological properties of the hemolysin by producing antibody to the antigen of the strain J preparation and then challenging this animal with hemolysin. If the antigen in the preparation was not the hemolysin then the animal should have developed hemolytic anemia. Unfortunately the animal died shortly after challenge of an apparently non-hemolytic condition of undetermined origin. Attempts to demonstrate an antibody to the hemolysin in leptospiral antisera by means of a tube precipitin test have been unsuccessful (3). Application of the agar diffusion technique (50) to this system might be rewarding. The use of the hemolysin inhibition test as a means of demonstrating antibody to the hemolysin is complicated by the natural inhibitor present in normal sera. The data in Table 6 indicate that hemolysin inhibition titers cannot be correlated with agglutinating antibody titers. Lambs 771 and 800 had HI titers of 16 without any demonstrable agglutinating antibody. The same HI titer was observed in lamb 974 with an agglutinating titer of 10^{-3}. Although there is little doubt that leptospiral antiserum inhibits the hemolysin the question as to whether the hemolysin is antigenic can not be answered at this time.

At birth lambs have little or no antihemolytic substance in their sera. This property increases in a few hours after birth evidently without the influence of colostrum. The absence of hemolytic inhibition may be a factor in abortion associated with leptospirosis. If the death of the foetus is due to hemolytic anemia, as has been proposed (18), the foetus would seem to be highly susceptible to the action of leptospiral hemolysin. Whether the hemolysin can pass through the placental membranes, however, has yet to be ascertained.

Alston and Broom (2) have objected to describing the hemolysin as a toxin. Their criticism was based on the first report of the presence of hemolysin in culture supernates and not on any study of the in vivo effects of this substance. They reasoned that if guinea pig and human erythrocytes were not lysed by the hemolysin and if L. icterohemorrhagiae cultures were not hemolytic, the hemolysin could not be the cause of the icterus and hemorrhage associated with classical Weil's disease. This is obviously a valid criticism although it should be mentioned that subsequent reports have shown that some strains of L. icterohemorrhagiae do produce small amounts of hemolysin and that human RBC are moderately susceptible to hemolysin.

Some resolution of the problem seems possible when it is recognized that the icterus associated with acute leptospirosis originates from two separate causes. One is the so called toxic jaundice in which extensive damage to hepatic tissue prevents the normal conversion of hemobilirubin to cholebilirubin, and hemobilirubin is therefore present in

large amounts in the blood, eventually staining the tissues. Toxic jaundice is characteristic of L. icterohemorrhagiae infections in humans and guinea pigs. The second type of icterus associated with leptospirosis is hemolytic icterus. Rapid lysis of large numbers of erythrocytes results in the formation of excessive amounts of hemobilirubin. Although the liver tissue is functioning normally it cannot cope with such large amounts and again hemobilirubin is present in the circulation, resulting in icterus. Hemolytic icterus is produced in L. pomona infections of cattle (54) and sheep (47) and to a lesser extent in infections due to L. canicola and L. grippotyphosa (2).

Therefore, it would seem best to classify the hemolysin as a toxin produced by some strains of leptospirae which is responsible for the icterus seen primarily in ruminant animals. The cause of the hepatic damage resulting in icterus which is observed in other leptospiral infections must be assigned to another leptospiral toxin. Further evidence that other leptosiral toxins must be active, is the fact that the characteristic kidney lesions of leptospirosis cannot be produced by injections of hemolysin.

In view of the mechanisms proposed for the hemolytic anemia in sheep (47) it is interesting to examine the data in Figures 1 and 2. The hypothesis is that lytic antibody destroys the leptospirae in the blood stream resulting in the release of a hemolytic endotoxin or hemolytic antigen. In both lambs, 20 and 23, the initiation of hemolysis seems to be closely associated with the presence of leptospirae in

the blood stream. With the appearance of antibody, the organisms disappear and the rate of hemolysis seems to be increased. This latter observation would be in accord with the hypothesis that lysis of leptospirae releases the hemolysin. However, the results of other experiments do not seem to be in agreement with this hypothesis. Lambs 32 and 22 were protected from the hemolysin by antibody, and the inhibitory effect of antiserum on *in vitro* hemolysis is evident from Figure 7. Therefore, if antibody is lysing the leptospirae it should neutralize or inhibit the released hemolysin. Further evidence that antibody is not associated with the hemolytic effect is afforded by the fact that hemolysis proceeds before antibody is detectable, and that in culture, the hemolysin is present in greatest amounts 1 to 3 days after maximum leptospiral growth has occurred but does not increase upon aging and autolysis of the leptospirae.

Although these results do not show conclusively that hemolysis does not result from the lysis and release of hemolysim by antibody it seems worthwhile in view of the results obtained to propose an alternative sequence of events which result in a hemolytic anemia. After establishing themselves in the liver and lymphatic tissue the leptospirae multiply and release hemolysin into the circulatory system. As the leptospirae increase in number they appear in the blood and hemolysin production reaches a maximum. The hemolysin is adsorbed to the erythrocytes (Table 10) and hemolysis proceeds. One to 2 days after the appearance of leptospirae in the circulation, antibody can be detected and the multiplication

of leptospirae is halted. Hemolysin which is adsorbed to the erythrocyts is not effected by the antibody (Figure 7 lines 2 and 3), and hemolysis continues. The apparent increase in the hemolytic rate about 24 hours after the leptospirae have been destroyed by antibody can be explained by examining the data in Tables 3 and 4. When lambs are injected with hemolysin, maximum rates of hemolysis do not occur until about 24 hours after inoculation. Some of this inhibition is probably due to the hemolysis inhibitors present in normal sera. Therefore, the increased hemolytic rate would be due to the fact that leptospirae were in greatest number and therefore producing maximum amounts of hemolysin 24 hours before the effect of this hemolysin was manifested.

It may be argued that the large number of leptospirae inoculated into lambs 20 and 23 do not compare with the inoculum in a natural exposure to leptospirae, and that the results therefore, do not reflect a true picture of the relationship between leptospiremia, hemolysis and appearance of antibody. This is probably true to some extent in lamb 23 in which the incubation period was less than 2 days. However, previous work (14) has indicated that the cultures used for the inocula were at a stage of <u>in vitro</u> cultivation such that their LD_{50} for hamsters was about 10^4 organisms. Furthermore the number of leptospirae required to initiate infection in a hamster was found to be approximately one. After washing and resuspension the inocula for the lambs contained a total of about 10^8 leptospirae per ml. If only 1 virulent organism was present for every 10^4 total cells,

then about 10^4 virulent leptospirae per ml were present in the inocula. Ten to 12 ml of suspension was injected so that the total number of virulent organisms was approximately 10^5. This is not an unreasonable number when it is recognized that swine urine, a frequent source of infection, may contain approximately 10^7 leptospirae per ml (44). It is the author's opinion that the results obtained from lambs 20 and 23 are applicable, for the most part, to natural infections.

The assay procedure developed for determining the hemolytic activity of hemolysin preparations seems to be relatively accurate and rapid. Other investigators (1) have employed spectrophotometric methods for assaying leptospiral hemolysin but incubation was performed at 37°C. The results shown in Figure 6 indicate that with high dulutions of hemolysin incubation at 37°C should be followed by a period at lower temperatures for maximum hemolysis to take place. This so called hot-cold hemolysis has also been observed with the alpha toxin of Clostriduim welchii (65) and the beta toxin of staphylococci (22).

The attempts to remove the inhibitor of the hemolysin present in rabbit serum were not successful. Ponder (51) has described four hemolysin inhibitors in normal sera: lecithin, cholesterol, albumin and globulin. Chloroform and ether extraction removed only small amounts of inhibitor (Figure 5), indicating that lecithin and cholesterol were not the principal cause of the inhibition. Thirty-five per cent saturation with ammonium sulfate did not remove inhibitor

which would indicate that albumin was probably not responsible. Whether serum globulin is the principal inhibitor of leptospiral hemolysin cannot be ascertained at this time. The fact that the hemolysin is precipitated by 35 per cent saturation with ammonium sulfate indicates that it has similar properties to a globulin or that it is adsorbed to the globulin molecules. When the precipitated hemolysin is dissolved and dialysed with distilled water a precipitate is formed (Table 9). Euglobulin is insoluble in distilled water and probably much of the precipitate is composed of this substance. Pseudoglobulin is probably one of the major components of the water soluble supernate. The hemolysin is essentially equally distributed between these two fractions which may indicate that the hemolysin is adsorbed to the globulin. However, when hemolysin preparations are diluted the inhibitor can not be detected. If globulin is the inhibitory component of serum then it does not seem likely that hemolysin is adsorbed to globulin. In contrast if hemolysin is adsorbed to globulin it is unlikely that globulin is the inhibitor. The answers to these questions will be possible when further purification of the hemolysin has been achieved. The numerous methods now available for separation and purification of proteins should be applied to purification of the hemolysin. The problem of the identity of the hemolytic inhibitor can also be approached by fractionation of serum and testing for the inhibitory component.

The adsorption of hemolysin to erythrocytes does not seem to be a specific union of "enzyme" with "substrate"

(Table 10). Guinea pig RBC, which either do not have the reactive site for the hemolysin or the site is not available to the hemolysin, seem to adsorb hemolysin to the same degree as the reactive sheep cells. Polystyrene latex spheres also adsorb the hemolysin but to a somewhat lesser degree. On the basis of the available data it appears that the adsorption of hemolysin to erythrocytes is non-specific and probably of a physical nature.

The inhibitory effect of antiserum on hemolysis, as seen in Figure 7, is similar to that of other toxin-antitoxin systems (6, 70). If antiserum and hemolysin are in contact before the addition of erythrocytes, hemolysis is inhibited. When the antiserum and hemolysin are in contact in the presence of RBC the inhibition is diminished. These results indicate that once hemolysin and erythrocyte have combined antisera has no effect. The possible significance of these findings in relation to <u>in vivo</u> hemolysis has already been discussed.

The failure to find any action of the hemolysin on lecithovitellin is not in agreement with the suggested phospholipase activity of the hemolysin (56). This suggestion was based on the fact that lecithin and other phospholipids inhibit the hemolysin of leptospirae. The fact that lecithin is an inhibitor of many types of hemolysins such as saponin and digitonin has been described by Ponder (51). Therefore, the observation that lecithin inhibits leptospiral hemolysin does not seem to indicate necessarily that lecithin is the substrate for the hemolysin. However, the failure of the

hemolysin to produce a positive egg yolk reaction does not necessarily preclude that phospholipase activity is absent. For instance snake venom lecithinase does not cause turbidity of egg yolk suspensions (66). The mode of action of the hemolysin should be investigated by examining the products of the reaction of hemolysin with erythrocytes and various possible substrates. Such procedures have been employed in determining the action of the hemolysin of mumps virus (42, 60), and would be a useful guide to the investigation of other hemolysin reactions.

B. Citric Acid Cycle Enzymes

The rates of enzymatic activity observed with extracts of L. pomona were low in comparison to enzymes of other microorganisms. However, a low metabolic rate would not be unexpected when one considers the fact that the generation time of leptospirae is about 8 hours as compared to 30 to 40 minutes for many bacteria. Probably the rates observed for fumarase and aconitase are not a true indication of the activity of these enzymes. Their evident instability probably resulted in considerable loss of activity during the preparation of the cell extracts.

Fulton and Spooner (20) reported that malonate did not inhibit the respiration of resting cell preparations of leptospirae. In the studies reported in this thesis, malonate inhibition of the succinic dehydrogenase system was readily detectable. The fact that the earlier work used whole cells in contrast to the cell extracts employed in this study may

indicate that leptospirae are impermeable to malonate. Impermeability of resting bacterial cells to various substrates and inhibitors has frequently led to erroneous conclusions regarding their role in metabolism (35).

Although not all of the enzymes of the citric acid cycle were investigated, the presence of succinic, malic and isocitric dehydrogenases, fumarase and aconitase is evidence that the citric acid cycle is probably operating in leptospirae. However, it has not been ascertained whether other metabolic pathways may also be functioning. The glyoxylate cycle (28), involving the breakdown of isocitrate to glyoxylate and succinate with subsequent combination of acetate and glyoxylate to form malate, may be a second method by which leptospirae metabolize nutrients.

Phospholipids and amino acids seem to be utilized by leptospirae. Both substrates would be eventually metabolized in the citric acid cycle, either by the breakdown of fatty acids to acetyl coenzyme A or in the case of amino acids by the appropriate transaminase forming intermediates of the cycle.

If leptospirae possess a phospholipase for phospholipid metabolism, its possible identity with hemolysin provides a common area of investigation for virulence and metabolism studies. Attempts to correlate the various virulence factors of pathogens with their metabolic enzymes or products would contribute much to our concepts of parasitism.

SUMMARY AND CONCLUSIONS

Washed cell suspensions of L. pomona were subjected to sonic oscillation and the cell-free extracts tested for toxic activity. Neither hamsters or guinea pigs showed evidence of an inflammatory reaction following intradermal inoculation of 0.1 to 0.5 ml of extract. Intracardial injection of 1.0 ml of extract in hamsters did not produce significant alterations in the hematology, whereas whole cells produced increased urea and bilirubin levels and decreased hemoglobin and hematocrit values.

The hemolysin produced by leptospirae was precipitated by 35 per cent saturation with ammonium sulfate. Concentrated hemolysin preparations were inoculated into lambs with a resulting loss of hemoglobin of 20 to 60 per cent. Weakness and jaundice were noted in lambs receiving large doses of hemolysin. Gross and microscopic examination of tissues of the lambs revelaed areas of degeneration and necrosis in the liver and petechial hemorrhages on the kidney with small areas of degeneration of the renal tubules. A generalized jaundice was prominent in all animals receiving large doses of hemolysin. The gross appearance and lesions of lambs receiving washed cells of L. pomona were similar except for more extensive renal damage. Lambs which had demonstrable serum agglutinating antibody were protected from lethal doses of hemolysin. Attempts to immunize lambs with small doses of hemolysin were hampered by the development of agglutinating

antibody. Concentrated culture filtrates of a non-hemolytic strain also produced agglutinating antibody. Antibody to the hemolysin could not be demonstrated by use of hemolysin inhibition tests. Although hemolysin is inhibited by leptospiral antiserum no evidence could be obtained that the hemolysin was antigenic.

In vitro studies on the hemolysin demonstrated that ammonium sulfate precipitation gave yields of hemolysin of about 30 per cent. Maximum purification was achieved by precipitating concentrated hemolysin preparations by dialysis with distilled water. A 7 to 8 fold increase in purity resulted from this procedure.

The effect of temperature on the rate of hemolysis was indicated by the demonstration of increased rates if the hemolysin tests were incubated at 4°C after initial incubation at 37°C. Adsorption of hemolysin to erythrocytes was demonstrated. RBC of both guinea pigs and sheep adsorbed the hemolysin but only sheep cells were lysed. The non-specificity of the adsorption was further shown by the adsorption of the hemolysin to polystyrene spheres.

The effect of antiserum on hemolysis was shown to be dependent on the sequence in which hemolysin, antiserum and RBC were mixed. If hemolysin and antiserum were in contact before addition of RBC, hemolysis was inhibited. When antiserum was added after or with the addition of RBC to the hemolysin the inhibitory effect was diminished.

Attempts to demonstrate phospholipase activity with hemolysin preparations by means of the lecithovittelin reaction were negative.

The results of *in vitro* studies of the adsorption properties and effect of antiserum on the hemolysin were used to attempt to describe the *in vivo* processes which produce a hemolytic anemia in leptospirosis.

With the demonstration of the toxicity of leptospiral hemolysin, our understanding of the hemolytic anemia associated with leptospirosis has been greatly clarified. Although the hemolysin is probably not of major significance in all leptospiral infections, its effects probably contribute significantly to the economic losses suffered by the livestock industry from leptospirosis. The evidence that once the hemolysin has initiated its effect, halting multiplication of the leptospirae does not arrest hemolysis, emphasizes the need for an adequate vaccination program for the control of leptospirosis.

Cell free extracts from *L. pomona* were tested for enzymes of the citric acid cycle. Various degrees of activity were demonstrated for succinic dehydrogenase, isocitric dehydrogenase, malic dehydrogenase, fumarase and aconitase. From this evidence it is concluded that the citric acid cycle is probably operating in leptospirae.

It is hoped that by use of some of the techniques described in this thesis for purification and assaying of the hemolysin and for demonstration of enzyme systems, further investigations will be undertaken concerning the hemolysin and enzymes of leptospirae. Because of the comparative ease with with leptospirae can be grown *in vitro* they are ideally suited for investigations of the pathogenic spirochetes. Our present lack of information concerning the biological properties of

this group of bacteria may well be corrected in the future by the results of investigations of the leptospirae.

TABLE I

HEMATOLOGICAL VALUES OF HAMSTERS INFECTED WITH *L. POMONA* OR INJECTED WITH CELL EXTRACTS

Normal Ranges		HGB g/100ml	Hem'crit %	Bilirubin mg/100 ml	Urea mg/100 ml	Differential WBC% Es	Stab	Pmn	Lymph	Mono
	High	16.8	55	0.68	28	0	10	25	80	2
	Mean	12.7	43	0.41	19	0	4	20	75	1
	Low	9.0	31	0.34	10	0	2	16	63	0
Group I Whole Cells Strain LW 28 hrs.	High	13.8	52	0.53	21	0	9	22	71	6
	Mean	13.5	51	0.47	18	0	7	19	68	3
	Low	13.2	50	0.42	15	0	6	17	66	2
48 hrs.	High	14.2	49	0.56	18	1	5	44	87	2
	Mean	13.1	48	0.51	16	0.2	4	26	70	1
	Low	12.2	46	0.42	14	0	4	11	51	1
72 hrs.	High	10.0	35	1.02	325	0	16	48	87	6
	Mean	7.1	26	0.60	250	0	10	36	53	3
	Low	6.4	14	0.34	140	0	3	7	34	1
96 hrs.	High	12.0	44	7.70	166	0	20	66	52	4
	Mean	8.2	26	3.10	118	0	16	49	31	3
	Low	4.0	10	.91	77	0	8	36	14	0

Group II										
Cell	High	12.0	44	0.58	32	0	26	56	34	0
Extract	Mean	11.3	42	0.39	27	0	17	54	22	0
Strain LW	Low	11.0	38	0.34	20	0	13	53	16	0
4 hrs.										
	High	14.0	58	0.51	25	0	12	60	50	0
18 hrs.	Mean	12.0	44	0.38	18	0	10	52	38	0
	Low	9.4	32	0.34	14	0	6	38	28	0

TABLE 2

AMMONIUM SULFATE FRACTIONATION OF CULTURE SUPERNATES FOR ISOLATION OF HEMOLYSIN

Fraction	Original	1	2	3	4	5	6	7	8	9	10	11	12
% Saturation	0	31	35	39	43	47	50	55	61	66	71	75	100
Hemolytic Activity (Units/ml)	8	4	4	0	0	0	0	0	0	0	0	0	0

TABLE 3

TOXICITY OF HEMOLYSIN FOR LAMBS
EXPERIMENT I

	LAMB 777 10,000 HU/kgm*			LAMB 800 (control) 0 HU/kgm**		
Time	Temp.	Hgb g/100 cc	Hematocrit %	Temp	Hgb g/100 cc	Hematocrit %
Pre-Inoculation	--	9.4	35	--	10.4	39
Post Inoculation	102.2	9.0	40	104.3	10.0	38
2.5 hrs.	104.2	7.8	30	104.2	9.8	36
5.5 hrs.	102.2	7.4	28	103.7	9.4	35
10.0 hrs.	102.4	6.9	27	102.5	9.4	34
26.0 hrs.	--	3.0	7	--	9.8	35
30.0 hrs.	98.1	2.4	&***	--	9.6	34

*HU/kgm = hemolytic units per kilogram body weight. Lamb received 20 ml of hemolysin preparation.

**Lamb received 20 ml of sterile saline.

***Animal died.

TABLE 4

TOXICITY OF HEMOLYSIN FOR LAMBS. EXPERIMENT II

Time	Lamb 24 Control 0 HU/kgm*			Lamb 25 15,000 HU/kgm**			Lamb 28 10,000 HU/kgm		
	Temp.	Hgb. g/100 cc	Hem'crit %	Temp.	Hgb. g/100 cc	Hem'crit %	Temp.	Hgb. g/100 cc	Hem'crit %
0	102.8	6.5	30	103.8	7.9	34	102.6	9.0	34
6 hr.	--	6.4	24	--	7.0	26	--	9.8	35
18 hr.	103.1	5.8	26	105	4.5	18	102.3	8.6	38
24 hr.	103.7	5.8	25	103.9	3.5	12	102.8	8.1	27
31 hr.	--	--	--	--	3.2	12[tt]	--	--	--
42 hr.	102.7	5.4	27				101.6	6.8	30
52 hr.	102.0	5.8	27				101.8	7.0	26
67 hr.	102.6	5.4	21				101.1	6.6	29
73 hr.	102.8	5.4	25				103.5	6.5	26
90 hr.	103.5	5.6	28				--	--	--
114 hr.	--	6.0	27[t]				--	6.8	28[t]

*Animal received 30 ml of concentrated culture supernate of non-hemolytic strain J.

**HU/kgm = units of hemolysin per kilogram body weight.

[t]Animal sacrificed

[tt]Animal comitose and sacrificed

TABLE 4 (CONTINUED)

	Lamb 29 9,500 HU/kgm*			Lamb 32 15,000 HU/kgm Antibody titer 10-5**				Lamb 20 Whole organisms***		
Time	Temp.	Hgb g/100 cc	Hem'crit %	Temp.	Hgb g/100 cc	Hem'crit %	Time	Temp.	Hgb g/100 cc	Hem'crit %
0 hr.	103.2	9.0	40	102.8	9.4	42	0 days	102.4	7.3	--
6 hr.	--	9.2	32	--	9.0	35	1 days	104.0	7.5	34
18 hr.	102.9	8.0	33	10.30	8.8	38	2 days	101.1	7.7	--
24 hr.	102.2	7.4	31	102.7	8.8	29	3 days	102.3	7.2	35
42 hr.	101.6	6.0	22	102.4	9.6	40	4 days	103.0	6.2	24
52 hr.	101.0	5.6	21	102.6	9.3	37	4.5days	104.6	5.7	22
67 hr.	100.6	4.6	17	102.3	9.2	37	5 days	105.0	4.0	16
73 hr.	103.0	4.4	18	--	--	--	5.5days	102.6	3.4	13
90 hr.	102.6	5.8	23	--	--	--	6 days	100.0	2.8	12tt
114 hr.	--	6.3	25	--	9.0	31				

*HU/kgm = units of hemolysin per kilogram body weight.

**Animal developed agglutinating antibody titer from colostrum of immune dam.

***Animal received 12 ml of washed leptospirae strain LW, concentrated 1:8.

ttAnimal comitose and sacrificed.

TABLE 5

EFFECT OF SUBLETHAL DOSES OF HEMOLYSIN ON LAMBS CHALLENGED WITH L. POMONA, STRAIN LW AND WITH HEMOLYSIN

	Lamb 23 No previous hemolysin injections			Lamb 29 4 sub lethal doses of hemolysin		
Challenging Preparation	10 ml washed cells Strain LW			10 ml washed cells Strain LW		
Time After Challenge	Temp.	Hgb. g/100 cc	AB*	Temp.	Hgb g/100 cc	AB
0 hrs.	103.3	12.8	0	103.0	8.4	1
18 hrs.	106.6	--	0	102.8	--	1
41 hrs.	105.6	11.0	0	102.6	8.2	3
72 hrs.	105.6	9.0	1**	102.2	8.8	5
96 hrs.	105.8	6.4	3	102.8	8.3	5
112 hrs.	106.2	3.7	4	--	--	-
120 hrs.	105.2	3.6	-	102.3	7.8	5
196 hrs.	102.9	4.6	4	102.0	8.6	5

*AB = serum antibody

**Reciprocal of dilution of serum agglutinating 50 per cent of the antigen.

TABLE 5 (CONTINUED)

Lamb 31 3 sub lethal doses of hemolysin			Lamb 40 3 sub lethal doses of hemolysin			Lamb 22 3 sub lethal doses of hemolysin		
10 ml washed cells Strain LW			10 ml washed cells Strain LW			10,000 HU/kgm		
Temp.	Hgb. g/100 cc	AB	Temp.	Hgb. g/100 cc	AB	Temp.	Hgb. g/100 cc	AB
102.2	6.8	0	101.8	6.8	0	101.8	9.8	0
105.0	--	0	105.0	--	0	102.0	9.8	0
102.4	5.9	0	103.0	5.0	0	102.4	9.6	0
102.6	5.8	2	102.6	5.6	3	102.5	9.2	1
102.7	6.0	4	102.0	5.6	-	--	9.4	2
--	--	-	--	--	4			
103.0	6.8	4	102.5	5.5	4			
102.7	7.0	4	102.0	6.7	4			

TABLE 6

HEMOLYSIN INHIBITION TITERS AND AGGLUTININ TITERS
OF SERA OF NEWBORN LAMBS

Time After Birth	Lamb 771 HI*	Lamb 771 AB**	Lamb 800 HI	Lamb 800 AB	Time After Birth	Lamb 974 HI	Lamb 974 AB	Lamb 975 HI	Lamb 975 AB
0.5 hr.	0	0	-	-	0.25 hr.	2	0	2	0
	nursed		nursed					nursed	
1 hr.	-		0	0	2 hr.	4	0	4	0
						nursed			
3 hr.	4①	0	-		13 hr.	8	100②	64	10,000
4 hr.	-		8	0	37 hr.	16	1,000	32	10,000
7 hr.	16	0							
24 hr.	16	0	16	0					
48 hr.	16	0	16	0					

*HI = Hemolysin Inhibition Titer
**AB = Asslutinating Antibody Titer
① = reciprocal of highest dilution inhibiting hemolysis.
② = reciprocal of highese dilution of serum agglutinating 50 per cent of the antigen.

TABLE 7

REPRODUCIBILITY OF ASSAY PROCEDURE II

Determination	Days After First Determination	HU/ml
1	0	61
2	1	58
3	4	62
4	5	60
5	10	60
	Mean	60.2
	Standard Deviation	1.32

TABLE 8

COMPARISON OF TITRATION AND ASSAY PROCEDURE II
FOR DETERMINING HEMOLYTIC ACTIVITY

Hemolysin Preparation	Titration Method HU/ml	Procedure II HU/ml	Ratio of Titration Method to procedure II
150-O	512	25	20.5
150-S	2048	58	35.3
150-P	2048	56	36.6
149	1024	54	19.0
142	512	28	18.3
143	8192	213	37.8
		Avg.	28.0

TABLE 9

PREPARATION OF HEMOLYSIN

Fraction	Treatment	Volume (ml)	HU/ml	mgm Protein/ml	Specific Activity HU/mgmP	Yield (%)
1	Sterile Culture Filtrate	1680	25	8.05	3.1	100
	Precipitated with 36 per cent saturation $(NH_4)_2 SO_4$. Precipitate resuspended in saline and dialysed vs. distilled H_2O. Precipitate removed and resuspended.					
2	Fraction soluble in H_2O	110	58	6.36	9.1	15.2
3	Insoluble in H_2O	110	56	2.44	23.0	14.7

TABLE 10

ADSORPTION OF HEMOLYSIN TO ERYTHROCYTES
AFTER 5 MINUTES AT 37°C

	Adsorbent	ml Adsorbent	HU/ml after Adsorption	% Hemolysin Adsorbed	Hemolysis of Adsorbant RBC
1	Packed Sheep Erythrocytes	0	62	0	
2	"	0.2	14	77	+
3	"	0.4	14	77	+
4	"	0.6	15	76	+
5	Packed Guinea Pig Erythrocytes	0	62	0	
6	"	0.2	15	76	-
7	"	0.4	12	81	-
8	"	0.6	14	77	-
9	Packed Polystyrene Spheres	0	38	0	
10	"	0.5	23	40	
11	"	1.0	13	66	

TABLE II

NON-REACTIVITY OF HEMOLYSIN WITH LECITHOVITELLIN
AS DETERMINED BY CHANGE IN TURBIDITY

Tube	1 26HU 2 ml Lecithovitellin	2 58HU 2 ml Lecithovitellin	3 84HU 2 ml Lecithovitellin	4 0 HU 2 ml Lecithovitellin
Time				
0 min.	0.06*	0.05	0.06	0.06
10 min.	0.07	0.05	0.06	0.07
20 min.	0.07	0.05	0.06	0.06
30 min.	0.07	0.05	0.06	0.06
60 min.	0.08	0.06	0.08	0.08

*Optical Density at 650 mμ.

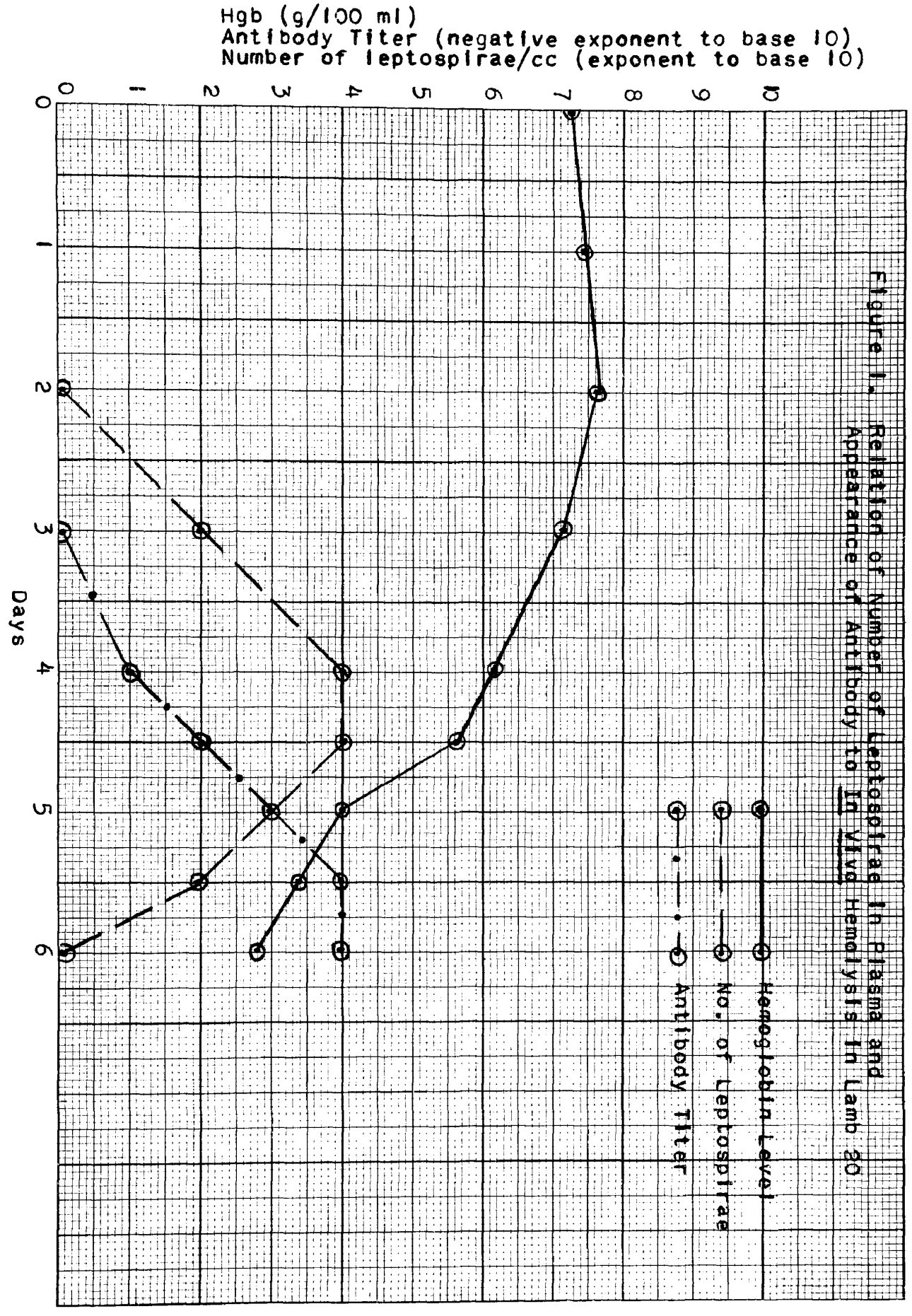


Figure 1. Relation of Number of Leptospirae in Plasma and Appearance of Antibody to In Vivo Hemolysis in Lamb 20

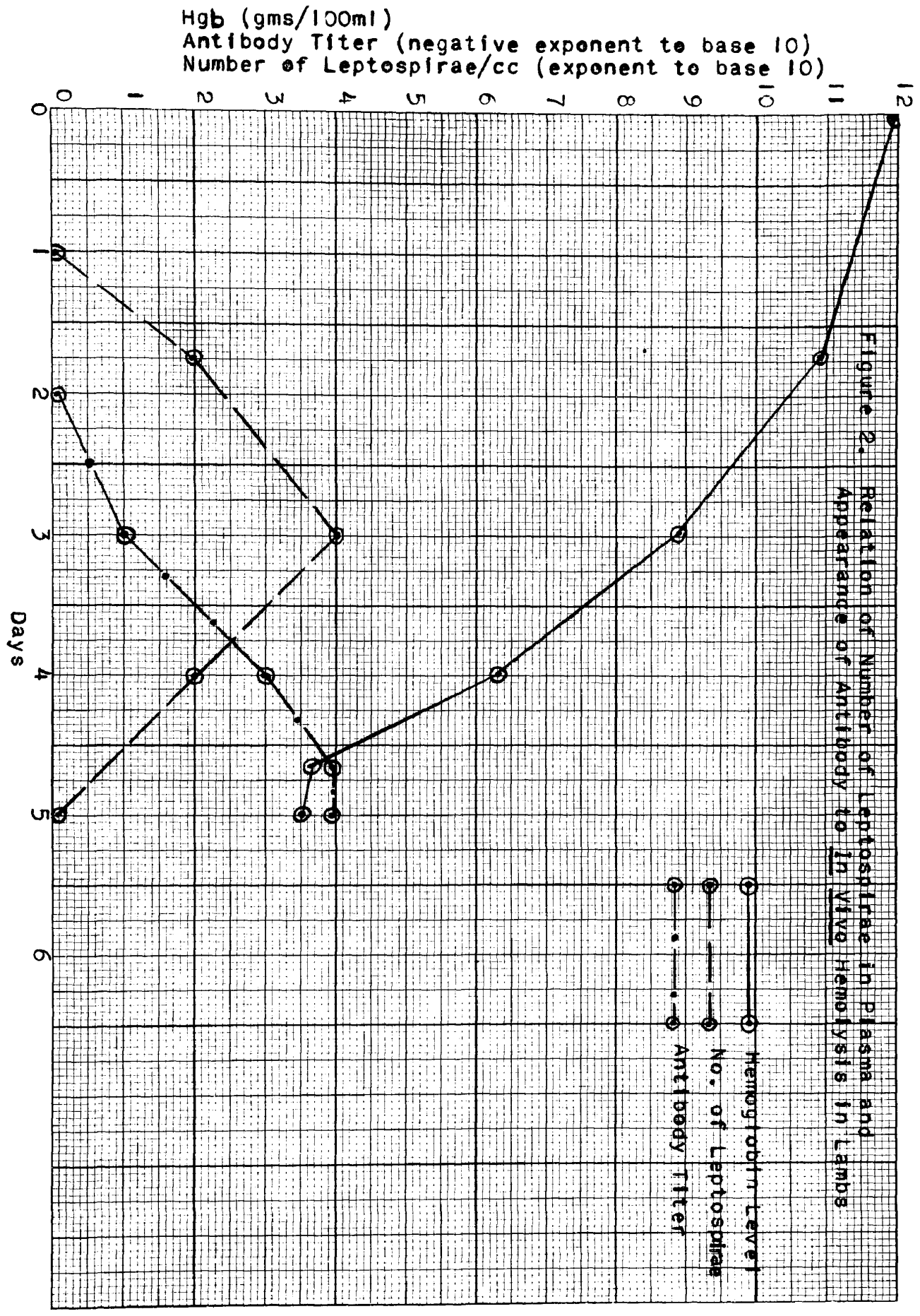


Figure 2. Relation of Number of Leptospirae in Plasma and Appearance of Antibody to *in Vivo* Hemolysis in Lambs

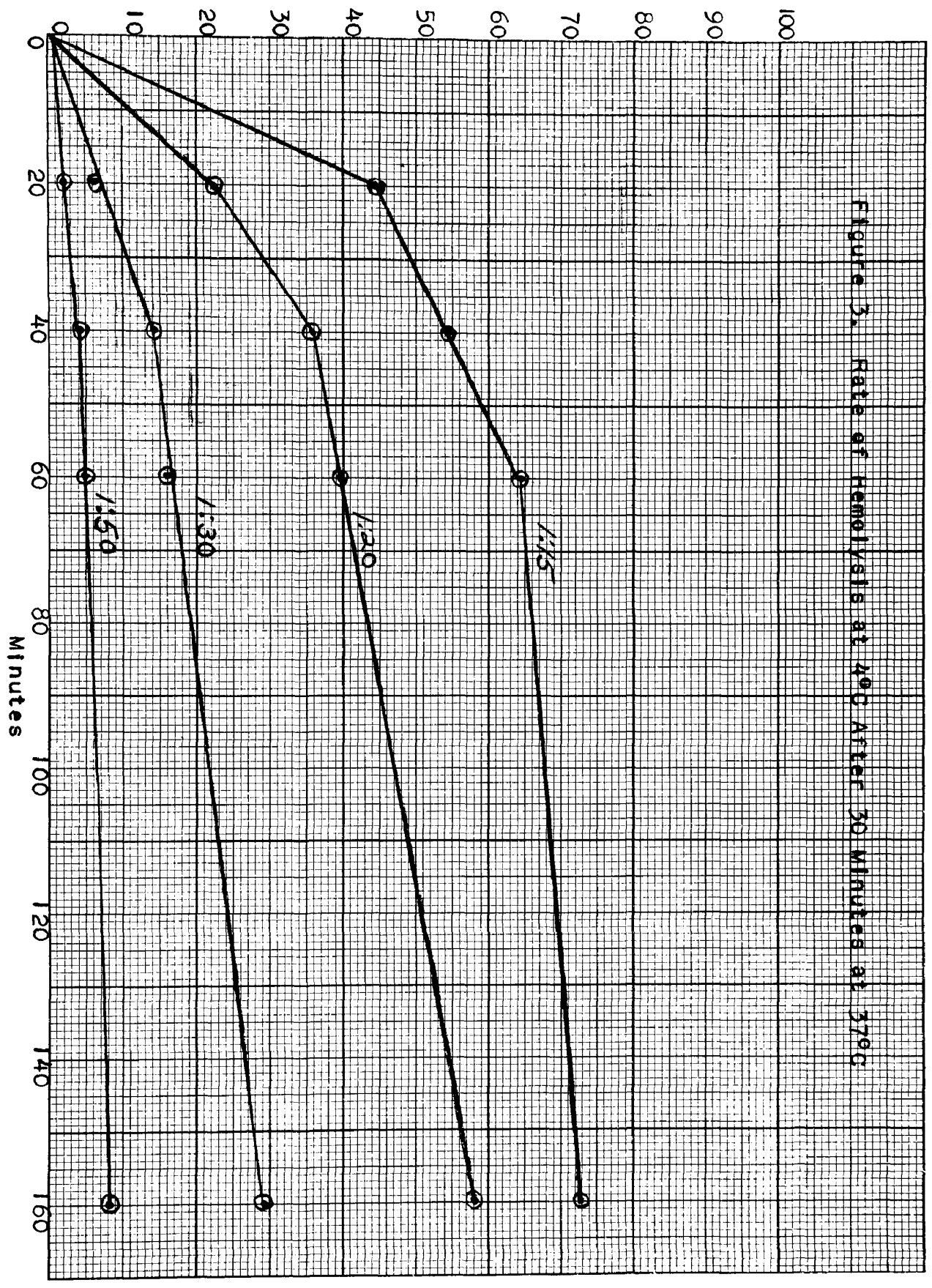


Figure 3. Rate of Hemolysis at 4°C After 30 Minutes at 37°C

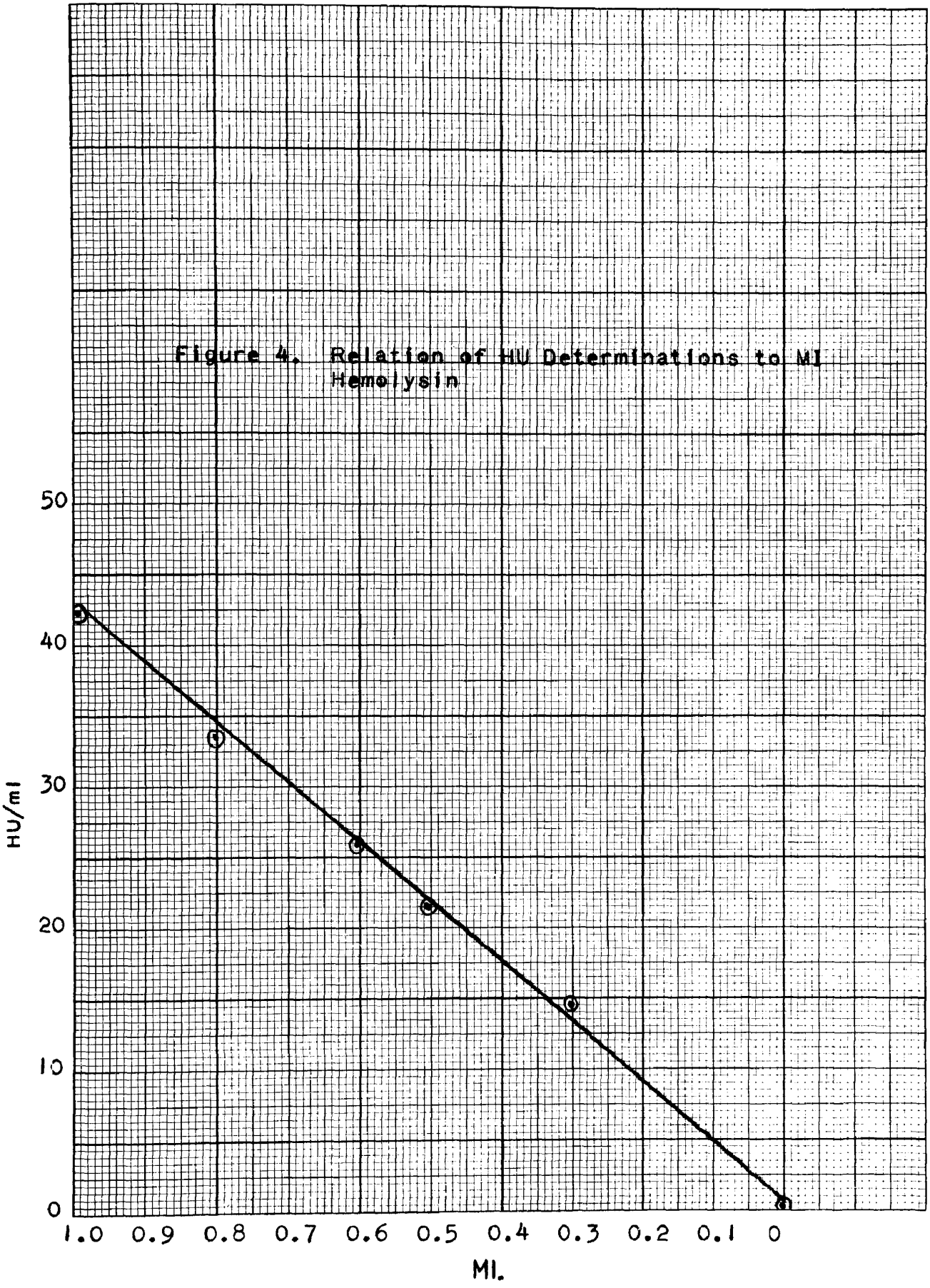


Figure 4. Relation of HU Determinations to MI Hemolysin

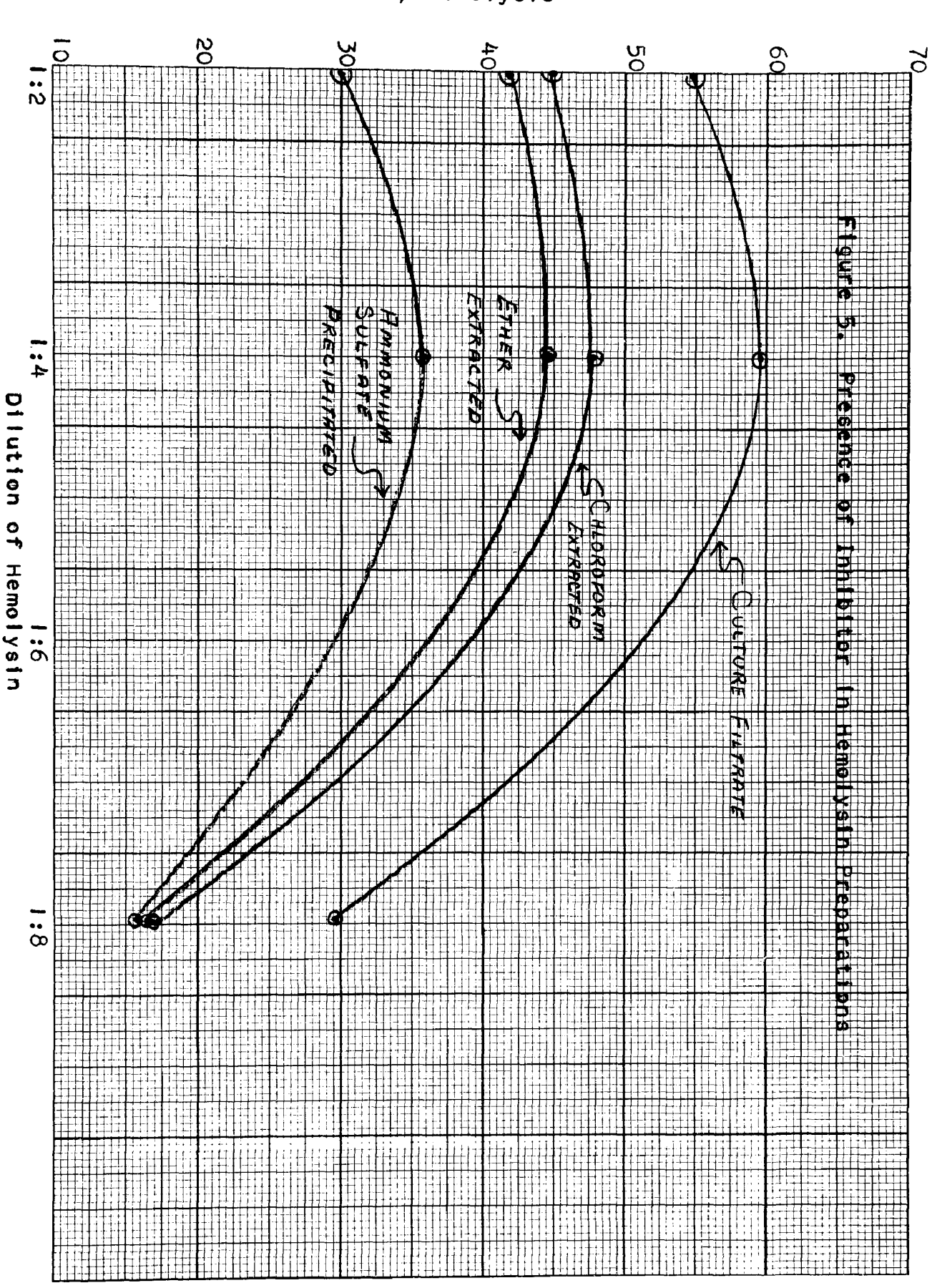


Figure 5. Presence of inhibitor in Hemolysin preparations

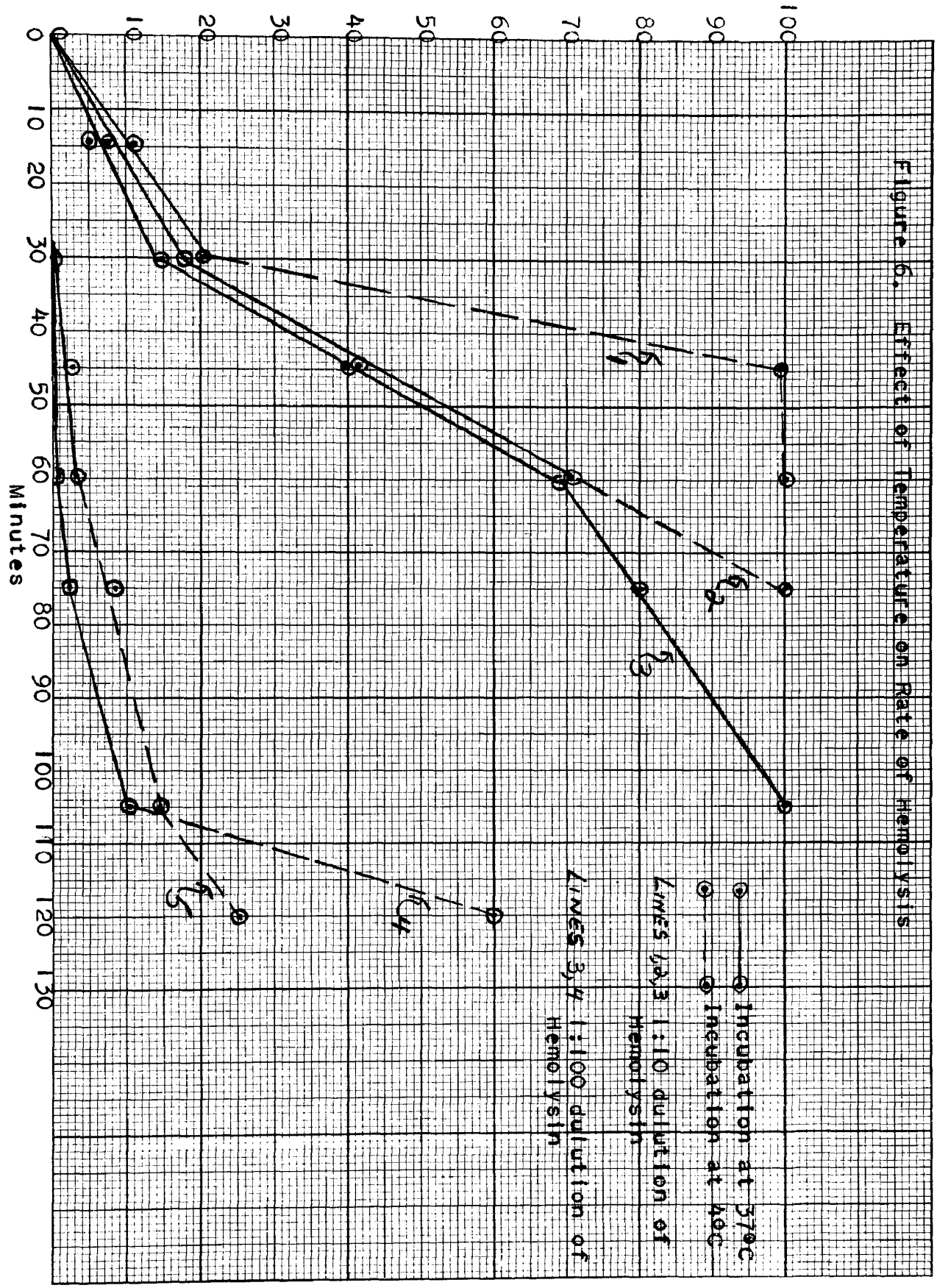


Figure 6. Effect of Temperature on Rate of Hemolysis

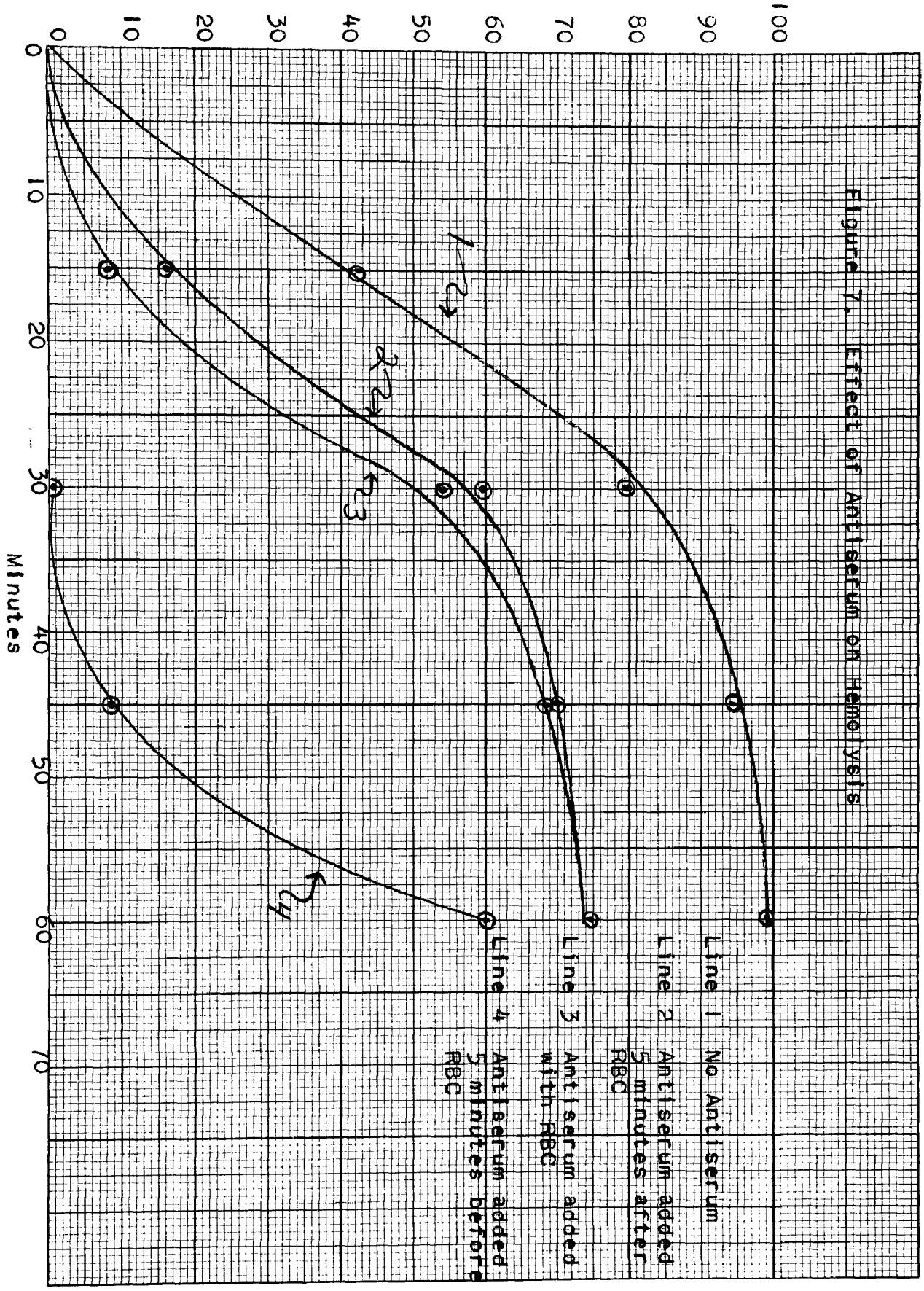


Figure 7. Effect of Antiserum on Hemolysis

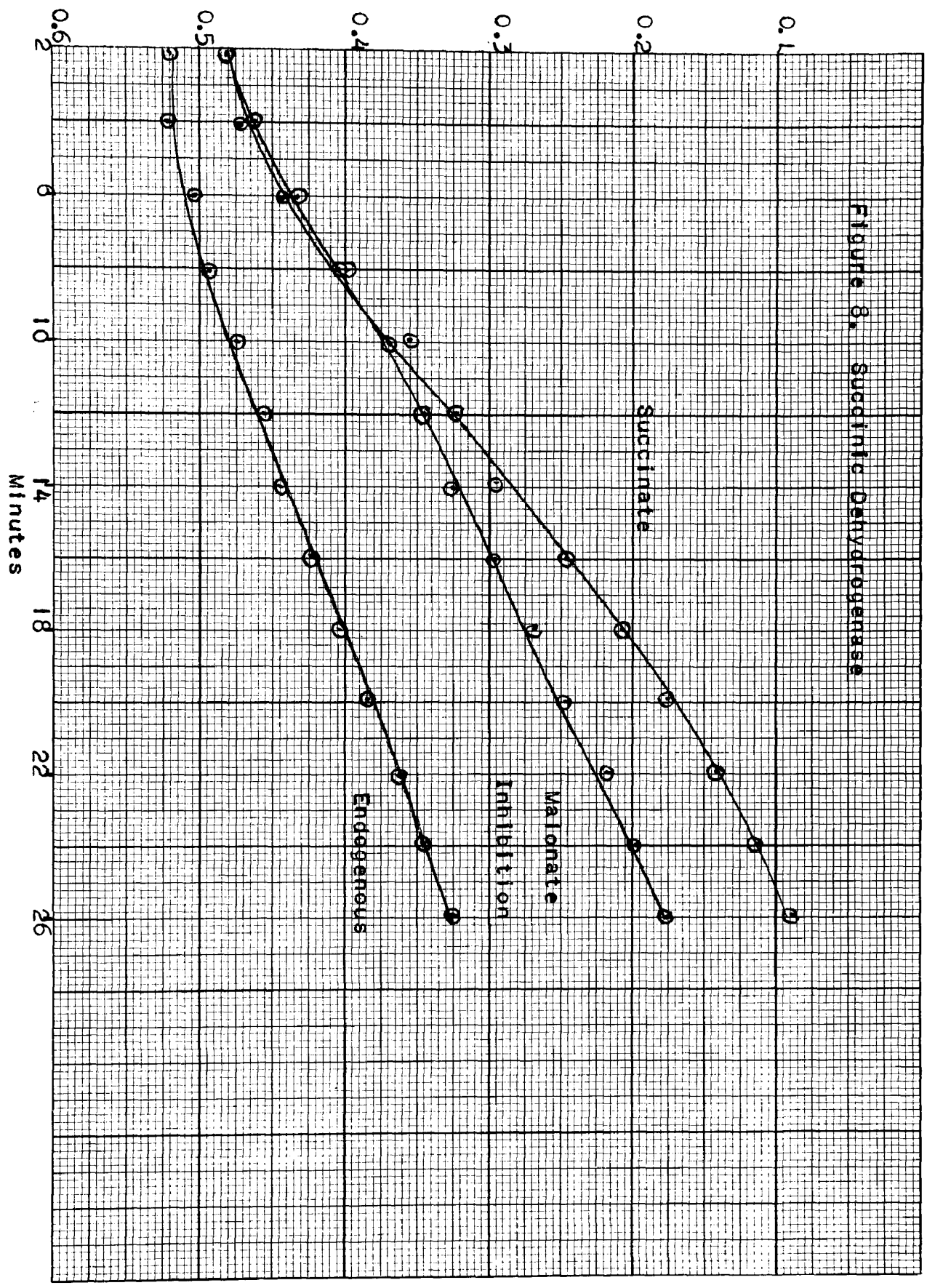
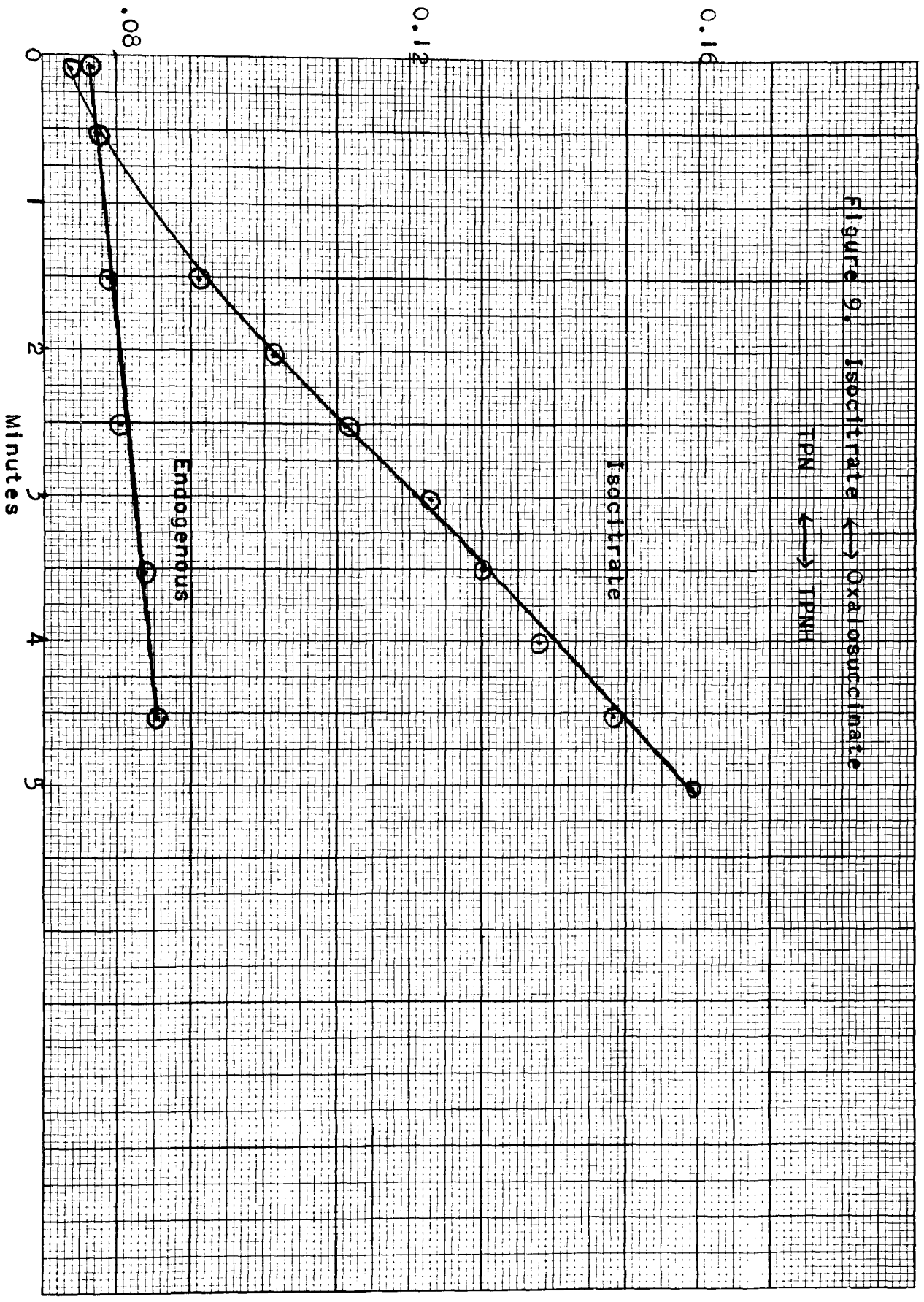
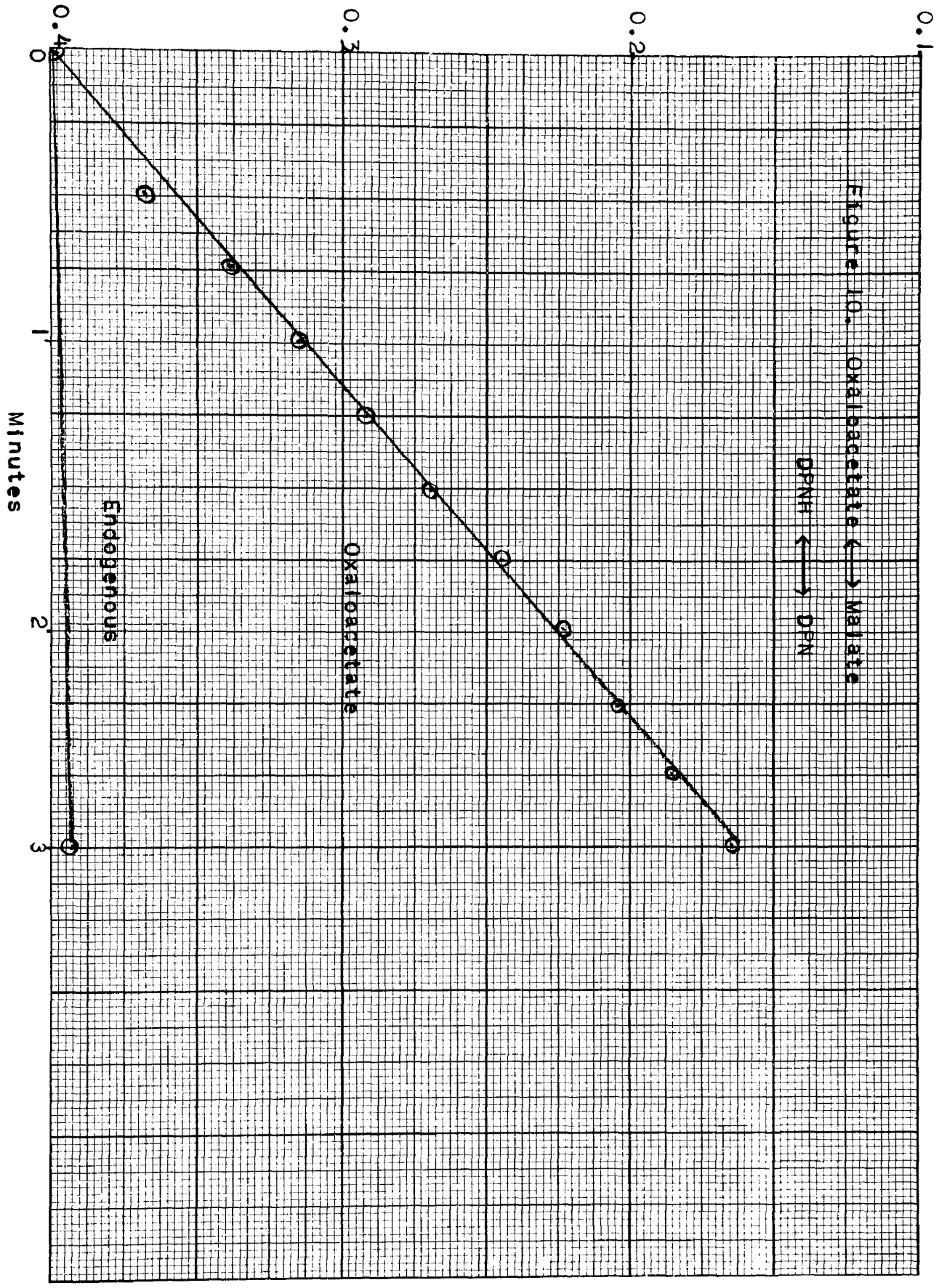


Figure 8. Succinic Dehydrogenase


Optical Density
.08
0.12
0.16
0
1
2
3
4
5
Minutes
Endogenous
Isocitrate
TPN ⟷ TPNH
Figure 9. Isocitrate ⟷ Oxalosuccinate


Optical Density
0.4
0.3
0.2
0.1
0
1
2
3
Minutes
Endogenous
Oxaloacetate
Figure 10. Oxaloacetate ⟷ Malate
OPNH ⟷ OPN

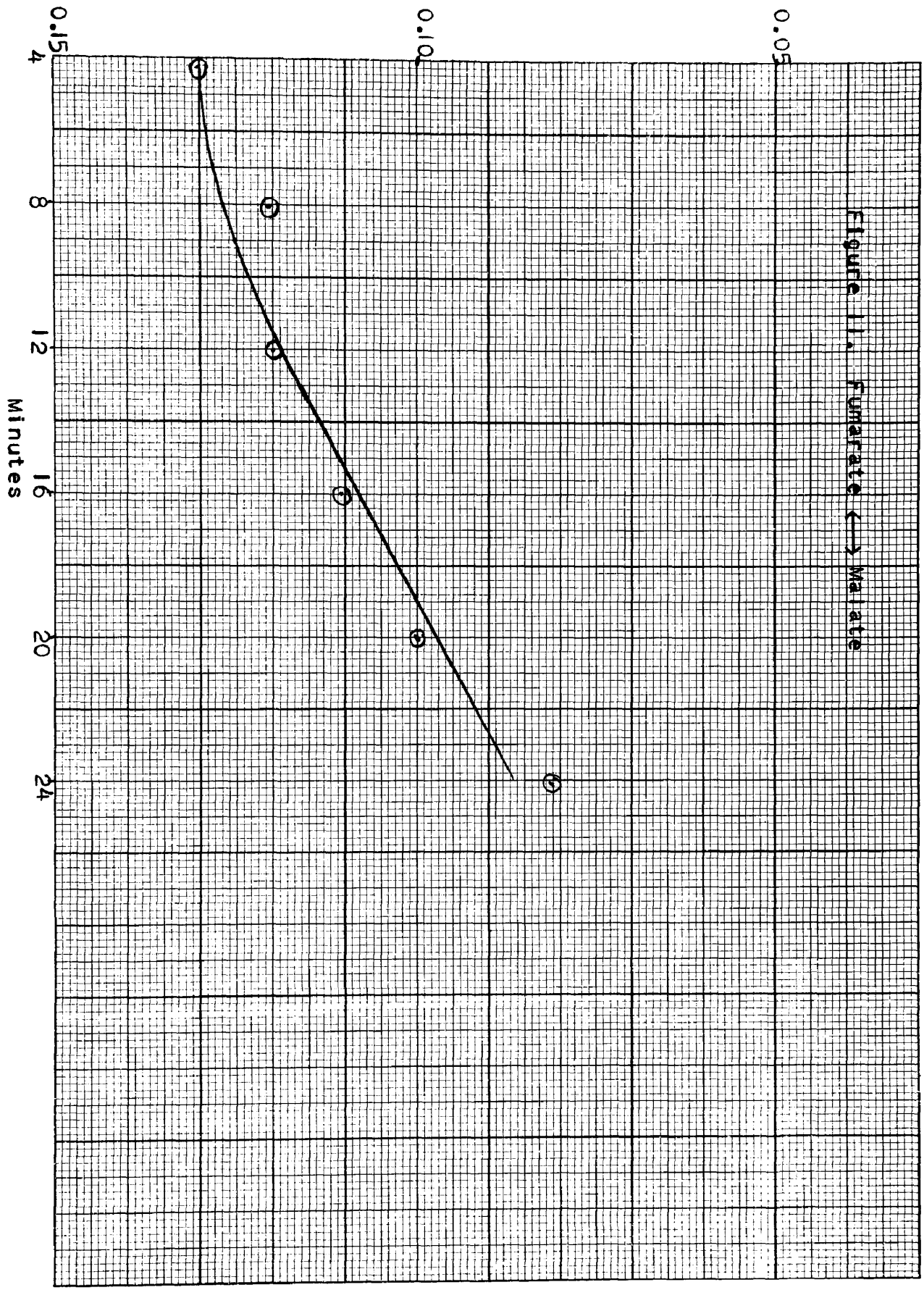


Figure 11. Fumarate ⟷ Malate

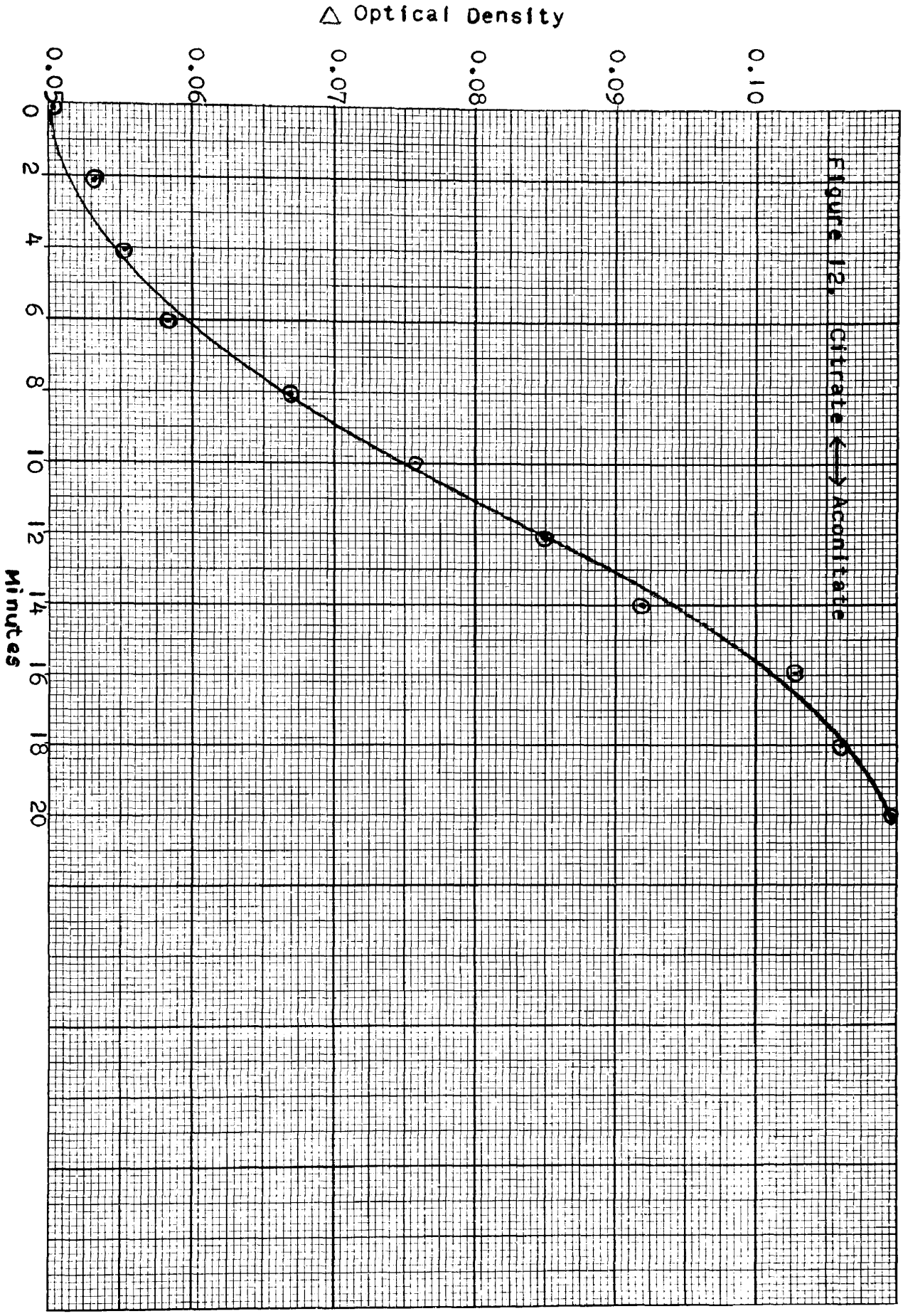


Figure 12. Citrate ⟷ Aconitate

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