THE EFFECT OF SULFONAMIDES ON GUINEA PIG COMPLEMENT.

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INTRODUCTION

During the winter months of 1950-1951 a pneumococcal infection, type 19, broke out among our guinea pigs. Sulfonamide therapy was started immediately; all animals, healthy or sick, receiving one-half grain per pound body weight of sulfamethazine every day in their drinking water.

Sera, to be used as complement for the serological laboratory, was always obtained from large healthy guinea pigs. During the pneumococcal outbreak, greater care than ever was taken to see that only healthy animals were used, but in spite of the precautions taken, it soon became evident that the complement was unsatisfactory.

In the light of this, it was suggested that an investigation into complement activity during sulfonamide therapy would be of value.

HISTORICAL REVIEW

The literature published on complement and complement-fixation in the last sixty years is too extensive to be reviewed here in its entirety. The historical chapter of this thesis will be confined to a very brief summary of the discovery of complement, and will then deal with the nature of complement and some of the physical and chemical factors which effect it.

A. DISCOVERY.

According to Osborn (1937), it was von Grohmann in 1884 who was the first to observe the effect of plasma on micro-organisms. Von Grohmann noted that plasma retarded the development of moulds, and that clotting plasma reduced the virulence of the anthrax bacillus.

In 1888 Nuttall demonstrated that blood destroyed anthrax bacilli in vitro, and further, he observed that this destruction took place independently of the leucocytes. In the following year Buchner (1889) found cell-free serum to be destructive to the typhoid bacillus.

Both Nuttall and Buchner were aware that the bactericidal element in plasma or serum was destroyed by heating at 55°C. for thirty minutes. It was also recognized that the activity disappeared from serum which had been standing for a few days. Buchner named this bactericidal element "alexin".

Bordet (1895) reported that serum from an immunized animal, inactivated by standing or by heat, could be reactivated

or restored to its former bactericidal activity by the addition of a small volume of fresh normal serum. He therefore suggested the bactericidal action of serum was due to two factors. One of these, the "preventive substance", was relatively stable and remained unchanged in serum for a great length of time, but was incapable of killing bacteria in the absence of the second factor, although it could produce "clumping"of the organisms. The second factor, for which he continued to use the name "alexin", was destroyed by heat or standing, and was responsible for the actual lysis of the bacteria. The "preventive substance" (later called "sensitizer" and now more commonly called "antibody" or "amboceptor") was specific, and was increased in the process of immunization. The "alexin" (now called complement) was present in all normal sera and was not increased during immunization (Bordet, 1895); v. Dugern (1900); Bulloch (1901).

B. THE NATURE OF COMPLEMENT.

1. THE COMPONENTS OF COMPLEMENT.

It was known as far back as the time of Buchner (1893) that serum, diluted with or dialysed against distilled water, lost its complementary activity, and that this could be guarded against by dissolving an electrolyte in the diluting or dialysing fluid.

In 1907 Ferrata obtained the same inactivation by replacing the salts of serum with isotonic cane sugar. He suspected that the precipitation of globulin involved in this treatment had some connection with the inactivation of the serum.

By mixing the supernatant fluid, made isotonic with sodium chloride, and the precipitate, dissolved in 0.9 percent sodium chloride, he restored almost completely the full activity of the serum, although neither of these parts was active alone. The theory that complement was split into two parts, one resident in the globulin fraction and the other in the albumin fraction, was the accepted explanation of these results. It will be seen later that this theory is only partially correct. Sachs and Teruchi (1907) were not long in confirming Ferrata's work, in full.

Brand (1907) contributed the discovery that the globulin fraction must act before the albumin fraction. Having Ehrlichs side chain theory in mind, he pictured some constituent of the globulin fraction acting as a link between the antigen-antibody complex and the albumin fraction.

Other methods have since been evolved for the division of complement into the two same fractions. Complement has been split by diluting with distilled water, addition of hydrochloric acid, bubbling carbon dioxide through the serum and dialysis against phosphate buffers of known pH and ionic strength. The latter two methods are the ones used most frequently today.

Von Dugern in 1900 was the first to describe a phenomenon whereby complement is inactivated by treatment with yeast cells. His observation was confirmed by Ehrlich and Sachs (1902), and Coca (1914) further showed that the inactivation was due to the removal of a relatively heat stable component, and not to the addition of an inhibiting factor. He found that yeast treated

complement could be reactivated by the addition of normal guinea pig serum which had been inactivated by heating at 56°C. for one half hour. Coca also concluded that a similar inactivation of complement by means of cobra venom, observed by Braun (1911), Ormakov (1911) and Ritz (1912a), was due, not to the adsorption of the heat stable component as in the case of yeast, but to the inhibition of the function of this factor. He observed that cobra-venom-inactivated serum could at times supply the heat-stable factor to yeast inactivated serum.

Browning and Mackie (1914), working with cobra-venom-inactivated serum, found that when complement was split into midpiece and endpiece, the third component was found in the midpiece fraction. Jonas (1913) worked with pig serum, and found the serum of this animal to be extremely rich in C'3. He reported the third component to be associated with both the midpiece and endpiece fractions.

Moore (1919), Coca (1920) and Hyde (1923) reported the discovery of a race of guinea pigs whose complement was deficient in C'3. The serum from these guinea pigs showed practically no activity, but could be activated by the addition of small amounts of heated serum in the same manner as yeast treated serum.

During a study of the action of pancreatic extracts on complement, Gordon, Whitehead and Wormall (1926a) became interested in the possible action of lipase, thinking it might throw some light on the part played by fats and lipids in complement action. The chief difficulty was to obtain lipase preparations free from

proteoclastic enzymes, for trypsin preparations have a destructive action on both the first and second components of complement.

The preparation they used consisted of a suspension of purified lipase in an ammoniated phosphate solution containing glycerol, and the results obtained by its action on complement indicated that the destruction of a hitherto unknown factor had occurred. A control experiment, however, showed that the boiled enzyme solution had the same function, and by a process of elimination it was found that the active agent was really the ammonia.

In effect, the addition of ammonia to complement and incubation at 37°C. for one to two hours destroyed the power of the complement to hemolyse sensitized red blood cells. The activity of the serum could be restored by the addition of complement which had been heated at 56°C. and which was itself inactive. The ammonia thus appeared to have destroyed some relatively heat-stable component of complement. When complement was split by carbon dioxide or dilute acids into the two protein fractions, the ammonia inactivated fraction remained chiefly with the end-piece. This component became known as the fourth component, C'4.

a. Nomenclature.

The components of complement have been given a variety of names by the different authors. Some of these names were coined to fit the theory of the time, and although the theory may have been disproved at a later date, the nomenclature is still in use. Other authors favour the German nomenclature, while yet another group will call the components by the name of the buffer used in

purifying three of the components of guinea pig complement, which in this paper they termed midpiece, endpiece and C'4. To guide this work electrophoretic diagrams were prepared of midpiece and endpiece (prepared by the carbon dioxide method) as well as of zymin and ammonia treated serum. The results indicate that the so called "globulin fraction" or midpiece contains at least four distinct proteins, two of which have electrophoretic mobilities faster than those originally present in whole serum; while the so called "albumin fraction" or endpiece also contains at least four distinct proteins, one of which appears to carry nearly all the gamma globulin originally present in whole serum. No significant difference was detected electrophoretically between normal serum and serum deprived of its C'4 by treatment with ammonia. Serum deprived of C'3 by treatment with zymin showed a disturbance of the alpha globulins, accompanied by a slight increase of the mobilities of the remaining serum proteins. It is evident therefore, that the terms "albumin fraction" and "globulin fraction" are unsatisfactory and misleading as applied here.

The separation of C'1, C'2 and C'4 was carried out under rigidly controlled conditions by fractional precipitation with ammonium sulfate, accompanied by various extractions and dialysis procedures.

The midpiece was found to be a euglobulin with an apparent isoelectric point of 5.2, and electrophoretic mobility of 2.9×10^{-5} in phosphate buffer of ionic strength 0.2 at pH 7.7, and a sedimentation constant of $6.4\ 10^{-13}$ in potassium chloride of ionic strength 0.2. The activity of this fraction was destroyed

by heating at 50°C. for thirty minutes, by hydrogen ion concentrations alkaline to its isoelectric point, and also in dilute solutions. This fraction was inhibited by protein concentrations exceeding 0.02 percent, while full activity was observed at protein concentrations between 0.002 and 0.02 percent. The final yield of this protein from 1070 ml. of serum was 400 milligrams or 0.6 percent of the total serum proteins.

C'2 and C'4 were present in the same serum fraction. After final purification the protein was identified as a mucoeuglobulin, 98 percent of which had an electrophoretic mobility of 4.2×10^{-5}. The final yield was 0.18 percent of the total serum proteins. Its apparent isoelectric point was 6.3-6.4. It contained 10.3 percent carbohydrate, and had a specific optical rotation of -192.5°. Treatment at 50°C. for thirty minutes destroyed all the C'2 activity while C'4 was not totally destroyed until heated at 66°C. for thirty minutes. This fraction therefore contained two components, one a relatively heat-stable constituent and the other a relatively heat-labile constituent. The hypothesis was offered that C'2 and C'4 are a carbohydrate-protein complex, C'2 being the protein carrier and C'4 the carbohydrate.

The unusually high carbohydrate content of the mucoeuglobulin, and its ability to reduce Schiff's reagent, is not inconsistent with this hypothesis.

These authors did not succeed in the separation and purification of C'3. They found this component to be present in nearly every fraction of serum.

As pig serum is ordinarily characterized by unusually high titers of C'3(Hegedus and Greiner, 1938), Jonsen et al (1951) used pig serum for their study of this component . While their present studies have not yet yielded pure C'3, they have shown that this component of pig complement occurs among the water insoluble globulins, more particularly in the non lipid portion of the beta globulins. Fractions rich in C'3 may be obtained by precipitation with ammonium sulfate or by precipitation with alcohol. Since some of these precipitates failed to show measurable titers of C'1, C'2 or C'4, and possessed only C'3 activity, it would appear that the third component in pig complement is actually an individual substance. Preparations assaying as high as 6000 C'3 units per ml. have been isolated.

c. Function in Immune Hemolysis.

Although the activity of complement is exhibited in bactericidal, bacteriolytic and hemolytic phenomena, the hemolysis of sensitized red blood cells is the most clearly defined property. In 1942 Pillemer and coworkers (1942b) determined the role of the various components of complement in immune hemolysis under the most favourable conditions.

Pillemer, Seifter and Ecker (1942a) had shown that with the exception of C'3, the other components, C'2 and C'4, and varying amounts of C'1, are fixed by sensitized red blood cells. Fixation of the components of complement in this work was determined by measuring the residual component activity in fresh guinea pig serum after fixation. This, however, did not disclose whether the adsorbed components were functionally active.

Pillemer's data contributes little one way or another to the enzymatic interpretation of C'1, C'2 and C'4, it appears to warrant the classification of C'3 as a catalyst, since this component is not fixed and is not used up in the process of hemolysis (Deisler(1932); Nathan (1913); Weil (1913); Thorsch (1914)).

2. COMPLEMENT IN DIFFERENT ANIMAL SPECIES.

Owing to the widespread use of fresh guinea pig serum in diagnostic complement fixation tests, more is known in regard to the constitution and general behavior of this complement than that of any other animal species. Our knowledge of the nature of different complements, and more particularly of the interchangeability of their components, is still very limited.

In the last ten years the complement of various species has undergone investigation. Ecker, Pillemer and Seifter (1943) have made a complete analysis of human complement. They found human serum to possess four components, analogous to the four in guinea pig serum, of which C'2 was present in the smallest amount. C'1 has been isolated and closely resembles guinea pig C'1; both had similar electrophoretic mobilities and sedimentation constants. The carbohydrate content of human C'1 was slightly higher, 3.2 - 3.7 percent, and so was the apparent isoelectric point, pH 6.0 - 6.4 as against pH 5.2 - 5.4. In human serum C'1 constituted about 0.6 - 0.8 percent of the total serum protein (Pillemer, Seifter, San Clemente and Ecker, 1943). A highly purified C'2 fraction was obtained by precipitation at pH 6.8 with 2.4 - 2.6

M ammonium sulphate that contained only 3 percent carbohydrate as compared to about 10 percent in guinea pig C'2. Human C'3 was destroyed by heating at 54 - 58°C., inactivation being considerable even at 46°C. Whereas in guinea pig complement the persistent association of C'4 with C'2 led to the view that C'4 was a prosthetic group attached to C'2, in the case of human complement C'4 has been found in both midpiece and end-piece fractions (Ecker, Seifter and Dozois, 1945)

Of the many mammalian complements studied, those of the guinea pig, hamster, rabbit, cat, rat, swine, dog, monkey and man produced hemolysis of sheep red cells sensitized with rabbit-anti-sheep serum (Rice and Crowson, 1950; Rice, 1950a). They differed widely however in hemolytic titer and stability.

In the majority of the investigations, evaluation of complement activity has been based on its capacity to lyse sheep red cells treated with antibody prepared in the rabbit. This is only one of the many characteristics of complement, and the failure of certain animal and avian sera to lyse sheep red cells sensitized with rabbit hemolysin, does not necessarily indicate a lack of complement, or one of its components. To be adequate, a study of the interchangeability of the complement components of different animal species must include investigations of other complement activities in addition to the hemolysis of the above mentioned "standard hemolytic system".

That fresh sera of certain animals may aggregate erythrocytes, treated with cow serum, was observed in 1906 by Bordet and Gay (1906) and by Muir and Browning (1906). The reaction

was termed "conglutination", to differentiate it from agglutination, which did not require the cooperation of complement. Rice (1950b) used this conglutination test to see if additional information could be gained about the complements of twelve mammalian and two avian species. Of the mammalian complements, horse, swine and cat were actively conglutinative; some samples of human, cow and deer sera were weakly so. Fresh sera of the guinea pig, rabbit, hamster, rat, and chicken, although hemolytic, were not conglutinative, while sheep and mouse complements were neither. Turkey complements behaved in an irregular manner.

C. FACTORS AFFECTING COMPLEMENT:

1. PHYSICAL FACTORS.

a. Temperature.

A temperature of 56°C. for one half hour is considered sufficient to inactivate most sera. This temperature does not destroy all the components of complement; in the guinea pig C'1 and C'2 are destroyed, while in human serum C'1, C'2 and C'3 are inactivated. C'3 and C'4 in the guinea pig are destroyed at temperatures of 63°C. and 66°C., respectively, for one half hour. The components of complement in various species differ to a certain extent, and may be more or less sensitive to heat. It is generally agreed, however, that of the four recognized complement components, C'1 and C'2 are so called heat labile, while C'3 and C'4 can withstand a higher temperature and are called heat stable.

Whereas Kiamil and Rassim (1928) have attempted to explain

this heat inactivation by postulating an altered albumin/globulin ratio, the generally accepted theory is that it depends on the denaturation of the proteins; a theory which is backed up by the parallel action of such sugars as glucose, maltose, and sucrose in guarding against both the heat denaturation of the protein and the heat inactivation of complement (Silber,1930; Silber and Schafran, 1932; Kiamil and Rassim, 1928).

That complementary activity disappears from serum when it stands at room temperature was one of the earliest known characteristics of the substance. Sera of different species vary greatly in stability, and therefore the time needed for inactivation at room temperature also varies. Gramenitski (1912) observed a slow spontaneous return of some activity on standing at room temperature and a rather faster regeneration of activity at 37°C. Hecht (1923), and Gordon and Hoyle (1933), showed that it was not the thermolabile components that disappeared on standing. In both guinea pig and human sera the first component to disappear is C'3.

Complementary activity disappears in from 4 to 10 days when serum stands at 0°C. (Mise, 1929). Lower temperatures produce a better preservation. Frozen serum may show an unchanged activity after 14 days (Ruffner, 1929), and a temperature of -12°C. will maintain complement for a month (Browning and Mackie, 1913-1914). Temperatures of-25°C. preserve complement for long periods of time with only small losses in complementary titers.

As the temperature increases from zero to 37°C. the degree of hemolysis is also increased. At 0°C. the combining components

of complement are fixed by the sensitized red blood cells, but the cells are not lysed. Between 32 and 37°C. the hemolytic power of the complement is at a maximum, but at higher temperatures hemolysis declines due to an inactivation of the serum proteins (Mayer, Osler, Bier and Heidelberger, 1946).

b. Aging.

Pillemer, Seifter and Ecker (1942a) carried out experiments on complement fixation using anti-type III pneumococcus rabbit serum compounds. A pool of fresh guinea pig serum was divided into two portions, one of which was allowed to incubate for one hour with the specific antipneumococcus complexes, at the end of which time the fixability of the complement components was determined. The second portion of serum was allowed to stand for 19 hours in the ice box at 5°C., and was tested in a like manner. The serum lost 50 percent of its complementary activity during the 19 hours in the cold. Complete hemolysis was brought about by 0.07 ml. of 10 percent dilution of fresh serum, while 0.14 ml. of a 10 percent dilution of aged serum was required.

An inspection of the results of this experiment shows that when serum stood for 19 hours at 5°C. and lost 50 percent of its complementary activity, the following changes in the fixability of the complement components occurred: (a) C'4 became more resistant to fixation, (b) C'3 underwent no change in fixability, (c) there was less reactivation upon the addition of the CO2-insoluble fraction, and increased reactivation upon addition of the CO2-soluble fraction in the case of the aged serum.

In the past many investigators have allowed complement to

age or "cure" in an icebox overnight before using it in fixation experiments. However, it is apparent from the foregoing experiment that this procedure may lead to serious errors in the determination of the fixability of the various components of complement.

c. Shaking.

Jacoby and Schutze (1909-1910) reported that shaking destroys complement. Ritz (1912b) was able to produce complete inactivation in as short a time as 20 minutes, by the use of a specifically designed shaker. Courmont and Dufour (1912) tried to explain this inactivation as an oxidative process, but Schmidt (1913), in a careful piece of research, showed that the rate of inactivation was independent of the presence of oxygen. He found that the details of technique affected the results greatly; both increase in temperature and increase in dilution speed up the inactivation, and the size and shape of the vessel are unexpectedly important.

In the same paper Schmidt reports that shaken serum is reactivated by the endpiece (HCl separation). Kashiwabara (1913) finds shaken serum to be reactivated by midpiece alone, endpiece alone, and by some samples of heated serum. As a result he is inclined to the opinion that C'3 is destroyed by shaking. Schmidt (1919), however, is convinced that shaking produces a physical and not a chemical change; that it produces a disturbance of the colloidal equilibrium in the solution.

d. Adsorption.

As early as 1906 it was shown by Landsteiner and Stankovic (1906) that coagulated serum protein removed the complementary

activity from serum. Since that time many attempts have been made to adsorb complement on various adsorbing agents: Berkefeld filter, Kaolin, alumina, carbon, calcium triphosphate, barium sulfate, starch, inulin, calcium oxalate, calcium fluoride, magnesium hydroxide, agar, gelatin, and even bacteria have been used for this purpose.

Fuchs (1929) believed that the adsorbents removed the midpiece or euglobulin fraction, while Strong and Culbertson (1924) stated that there was no selective adsorption of any single component of the complement.

Ecker, Jones and Kuehn (1941) carried out experiments using lead phosphate, titanium dioxide, kaolin, magnesium hydroxide, aluminum hydroxide and zymin. They found that lead phosphate and titanium dioxide removed the midpiece of complement. Kaolin and magnesium hydroxide were less specific in their action and adsorbed both midpiece and endpiece. Small amounts of aluminum hydroxide gel removed the total complement complex. The action of zymin on serum complement was of a different nature, in as much as zymin in optimal amounts specifically removed the heat stable fraction of the midpiece. Zymin had no adsorptive effect in the presence of 10 percent sodium chloride.

Determinations of total nitrogen and calcium were made on the fresh complement and on the complement after adsorption. Determinations of total phosphorus were made on the midpiece by the Fiske and Subarrow method (Fiske and Subarrow, 1925). From their results it was evident that no relationship existed between the amounts of calcium adsorbed and the ability of the agent to

inactivate complement. However a definite correlation existed between the ability of these inorganic agents to remove a large percentage of the midpiece phosphorus, and their ability to inactivate specifically the midpiece and its associated third component. This would seem to indicate that the removal of phosphorus, which is supposed to be present in a combined form with euglobulin as cephalin, interferes with the activity of the midpiece. It was suggested that the midpiece of complement is closely associated with a phosphatide in the euglobulin fraction.

2. CHEMICAL FACTORS.

a. Oxidation and Reduction.

Valley in 1928 reported that spontaneous inactivation of complement is, at least partially, a reversible reaction, and succeeded in reactivating complement with sodium hydrosulphite.

Ecker, Pillemer, Wertheimer and Gradis (1938), showed that complement, denatured by yeast, improved with the addition of ascorbic acid, while ammonia treated serum did not show reactivation. Aeration of complement reduced its activity, which may be restored by ascorbic acid.

These observations suggest the possibility that the effect may be largely attributable to reversible chemical actions upon certain definite groups.

In the same year, Ecker, Pillemer, Martiensen and Wertheimer (1938a), investigated the reactivation of complement of vitamin C deficient guinea pigs, aged guinea pig complement, aerated complement and complement treated with various oxidants,

reductants and components of complement. They made the following observations: Complement obtained from scorbutic guinea pigs can be reactivated by various reductants, like hydrogen sulfide, ascorbic acid and sodium hydrosulfite. Aged complement, not more than 22 days old, can be reactivated by hydrogen sulfide or ascorbic acid. Normal complement can be inactivated by a variety of oxidizing agents, and provided that the oxidation is controlled it can be reactivated by the reductants mentioned above. Normal complement inactivated by controlled oxidation can also be regenerated by heat inactivated complement, and by complement lacking third or fourth components.

These observations all seem to point to the fact that complement is controlled by oxidation and reduction; in the reduced form it is active, while in the oxidized form activity is lost.

b. Hydrogen Ion Concentration.

For many years little attention was paid to the pH at which complement fixation was carried out. This led to numerous false conclusions in the experimental field.

The first investigation along this line was carried out by Pillemer and Ecker (1941b). After a series of experiments they reported the following facts:

1. Complement functions best at a neutral pH. Deviations on either side gradually inactivate complement; and at a pH above 9.5 or below 4.2 complement is inactive.

2. A pH above 9.5 and below 4.2 results in irreversible inactivation of complement so that the complement cannot be

selectively reactivated.

3. The midpiece of complement is relatively stable in acids and unstable in alkalies. This is readily seen from the selective reactivations brought about only by ammonia-treated serum and intact midpiece; and it indicated that both the third component and its carrier are attacked by alkalies.

4. The endpiece of complement is relatively unstable in acids and stable in alkalies. This is seen from the selective reactivation brought about by zymin-treated serum and intact endpiece. Therefore it appears that acids attack the fourth component carrier.

At a later date Pillemer, Seifter and Ecker (1942a) conducted experiments to determine the effect of hydrogen ion concentration on the fixation of the individual complement components. They reported that at a pH below 7.0 less fixation of C'4 and C'1 occurred than at a neutral pH, but there was an enhancement of the fixation of C'3. At a pH above 7.0 and up to 8.8 there occurred complete fixation of C'4 together with an increased fixation of C'1. Optimum fixation occurred at a neutral pH.

One point of interest evident from this paper was that complement activity was removed by specific aggregates over an extremely wide range of pH (5.3 - 8.8), although there was marked variation in the fixation of the individual components at different hydrogen ion concentrations.

c. Salt Concentration.

As early as 1890 Buchner and Orthenberger realized that

salts were indispensable in the lysis of bacteria by serum. Apart from the different effects of individual ions and apart from the osmotic pressure effects, inorganic salts, per se, are essential for hemolysis by complement. Guggenheimer (1910) reported that the failure of hemolysis was due to the endpiece, that sensitized corpuscles could combine with the midpiece in a salt free medium, but that the endpiece was prevented from playing its part.

Markl (1902) found that hypertonic solutions of sodium chloride and other salts inhibited hemolysis completely when a sufficiently high concentration was reached. The failure of hemolysis was due to an absence of complement binding; the combination between antigen and antibody was in no way affected. Topley (1915) confirmed this and added "if the concentration of antibody be markedly increased it is possible, up to a certain point, to counteract the effect of increased salt concentrations" Topley also demonstrated the reverse, that "if the salt concentration be decreased, a decreasing concentration of antibody serves to produce the union of cells and complement" .

Although physiological saline is generally used in the dilution of complement, Neter (1931) reported that an antigen-antibody complex bound complement more readily in 0.6 percent sodium chloride than in 0.9 percent. He suggested this as a means of increasing the sensitivity of complement fixation reactions.

As in the case of increased CO_2 tension, increased osmotic pressure preserves the latent complement for a

considerable time. The midpiece is unstable in 0.9 percent saline; in fact it loses its activity in a few hours. However, it remains active and stable if suspended in distilled water or in hypertonic salt solutions (Brand, 1907; Ecker and Pillemer, 1942). Hypertonic salt solutions also have a protective action on the removal of C'3 from whole serum by yeast, zymin, or the insoluble carbohydrate extracted from yeast (Ecker, Jones and Kuehn, 1941).

3. THE SPECIFIC EFFECTS OF VARIOUS AGENTS.

a. Amino Compounds.

As has been previously noted Wormall et al (1926a) observed that the addition of appropriate concentrations of ammonia will remove the complementary activity from whole serum and that it can be regenerated by the addition of either heated or zymin-treated serum. They also established that the ammonia effect is not due simply to alkalinity, but that it is a specific reaction involving a hitherto undescribed thermostable fraction of serum.

Pillemer, Seifter and Ecker, (1941a) adopted the hypothesis that ammonia-inactivation of C'4 is due to the conversion of the active carbonyl groups of the component to some less active structure. To test this hypothesis they used various amino compounds of two general classes, namely, those which are known to react readily with aldehydes and those which do not. Inspection of their results brings several interesting and important facts to light.

1. Only ammonia, methylamine, ethylamine, hydrazine,

phenylhydrazine and alpha-methyl hydroxylamine, specifically inactivated C'4. The other amino compounds, those that were not primary amines, had no effect on any portion of complement, except that due to the hydrogen ion concentration.

2. Simple alkalinity of amines was not the cause of the inactivating property. In water, the effective compounds dissociated to form bases of moderate strength, but it was readily seen that many of the inactive compounds dissociated in the same manner. Thus the dissociation constants for the latter (diethylamine, dimethylamine, trimethylamine, triethylamine and tetramethylammonium hydroxide) were greater than those of ammonia and phenyl-hydrazine, and greater than, or of the same order as methylamine, ethylamine and hydrazine. Furthermore, a pH of over 9.0 was destructive to midpiece, while the endpiece was relatively alkali stable.

3. C'4 was inactivated only by amino compounds that are known to have decided reactions with aldehydes. However, hydroxylamine, semicarbezide, and thiosemicarbazide, which are considered to be potent aldehyde reagents, had no demonstrable effect on C'4. These special cases are considered below.

4. The amino group was necessary for inactivation of C'4. There was no significant quantitative difference in the minimal effective molar concentrations of ammonia and its simple alkyl derivatives, methylamine and ethylamine, whereas hydrazine, with two free amino groups, required only one half the concentration. On the other hand, dimethylamines, Trimethylamines, and tetramethyl ammonium hydroxide, which do not contain free amino groups,

had no effect on C'4.

5. The type of substituent replacing a hydrogen atom of ammonia to produce the amino compound had a pronounced influence on the capacity of the compound to inactivate C'4. If the substituent group was positive (simple alkyl) as in methylamine and ethylamine, the action continued to be like the ammonia action. A negative substituent group (e.g. phenyl group) diminished the amino effect on C'4. Thus phenylhydrazine was about one half as active as methylamine. Polar groups which potentially lend acidic or redox properties to amino compounds completely abolished the effect on C'4. This was seen in such compounds as the carbazides, urea, glycine and hydroxylamine. Blocking of the polar groups restored the activity, as in the case of methyl-hydroxylamine. Amines which have negative polar groups apparently needed extra physiological conditions of pH and temperature in order to function as such toward aldehydes.

6. Amino compounds which have been treated previously with aldehydes did not affect C'4. This was demonstrated by the failure of methenamine to inactivate C'4, and the similar failure to activate amino compounds which were previously treated with equi-molar propionaldehyde. However, a mixture of equi-molar parts of ammonia and propionaldehyde still inactivated complement. This is not surprising since the ammonium aldehydes readily release ammonia.

In no case was the complement reactivated by the addition of formaldehyde or propionaldehyde to amino and ammonia-treated sera. If the union of amino compounds with C'4 is of a loose

nature, reactivation should have taken place. Furthermore, the apparent union of the amino compound with C'4 was sufficiently stable to resist dialysis against 0.9 percent sodium chloride, as shown by the failure of complementary activity to return after such treatment.

An attempt has been made to relate these findings with what is known of the serum constituents. C'4 in guinea pig serum is associated with C'2, which, after dialysis, contains albumins (Hewitt, 1938; Ogston, 1938; Rimington, 1933), pseudo-globulins, combined calcium (Gordon, Whitehead and Wormall, 1926b), bound carbohydrate (Parsons, 1926) and lipid. Albumins do not appear to be significant in the lytic action of serum (Ecker, Jones and Kuehn, 1941), and these same authors have shown that complement is entirely resident in the globulin fraction. Nor are lipids involved in C'4 (Pillemer, Seifter and Ecker, 1940). Carbohydrate is bound to the serum pseudoglobulin (Hewitt, 1938). In addition to the factor which is attacked by amino compounds, complement, it has been established, has a non-diffusable calcium factor (Gordon, Whitehead and Wormall, 1926b). All of these facts and the experimental findings reported by Pillemer, Seifter and Ecker (1941a), would seem to indicate that the end-piece of guinea pig complement is the calcium-carbohydrate-pseudoglobulin molecule. In the light of this, C'4 would be the carbohydrate (the assumption being made that it contains the reactive carbonyl group), and the complex calcium-pseudoglobulin molecule would be the carrier. Additional support for this view is to be found in the immunological experiments which

indicate that ammonia-treated-serum does not differ from normal serum in its serological specificity (Gordon and Marshall, 1929), and that bound carbohydrate of serum proteins does not influence their specificity (Heidelberger, 1938).

b. Yeast.

As has been previously mentioned von Dugern (1900) was the first to describe the inactivation of complement by treatment with yeast cells. His observation was confirmed by Ehrlich and Sachs, (1902), and Coca (1914) further showed that the inactivation was due to the removal of a relatively heat stable component and not to the addition of an inhibiting factor.

Whitehead, Gordon and Wormall (1925) inactivated serum using Fleischmann's yeast, which proved to be more effective than ordinary baker's yeast. The serum was mixed with a thin paste of yeast cells, and incubated for 2 - 3 hours, following which the yeast cells were removed by centrifugation. Serum treated in this manner was usually quite devoid of hemolytic activity. The activity could be restored by adding small amounts of guinea pig serum, which had been previously inactivated by heating at 56°C. The method was, however, by no means certain, since much depended upon the amount of yeast used, and the time of incubation. These authors reported that this inactivation by yeast was not due to enzymes destroying the third component, nor due to inhibiting substances released by the yeast. They theorized that the yeast may raise the salt content of the serum so much that hemolysis is inhibited by osmotic action, or that the yeast may cause an acidity in the serum

sufficient to precipitate the midpiece. Neither of these theories proved to be correct. They concluded therefore that yeast inactivated guinea pig serum by combining physically or chemically with the third component. The same authors demonstrated that while live yeast cells proved to be a most uncertain method of removing C'3, heated zymin was almost invariably successful.

Zymin was prepared by mixing fifty grams of Fleishmann's yeast with 300 ml. of acetone. The suspension was stirred for ten minutes and passed through a Buchner funnel. The residual mass was again stirred in 100 ml. of acetone for two minutes and refiltered. The residue was then kneaded with 25 ml. of ether for three minutes, filtered and spread on filter paper to dry. After thorough drying the zymin was powdered.

Pillemer and Ecker (1941c) extracted from Fleishmann's yeast a fine, almost white, hygroscopic powder, which was insoluble in hot water, organic solvents and cold alkali. Chemical analysis revealed carbohydrate 94 percent, nitrogen 1.78 percent, magnesium 2.43 percent and phosphorus 0.4 percent. This fraction was labelled the "insoluble fraction" and it proved to be the agent responsible for the inactivation of C'3. This insoluble fraction inactivated the third component in amounts only one-twenty-fifth of the required amount of fresh yeast.

c. Lipid Solvents.

Several early investigators reported that lipid solvents were able to inactivate complement. Ottolenghi and Mori (1905) and Guggenheimer (1910) showed that complement treated with ether

became inactive after the solvent was allowed to evaporate. It was later reported by Toda and Mituse (1933) that the fraction removed by extraction with chloroform or ether was the same as C'4. They further claimed that a similarity existed between C'4 and the lipid fraction since calcium compounds, known to combine with lecithin, inactivated this component. Benzène inactivated their complement and the inactive serum was regenerated by the fraction soluble in benzène, but not by C'4. Since this heat-stable fraction did not reactivate ammonia-treated complement they postulated a hypothetical fifth component.

Tokano (1936), however, could not demonstrate the existence of such a fifth factor, but noted that ether or chloroform disturbed C'4.

In summing up this work, it may be stated that little attention has been paid to the question of protein denaturation during the process of lipid extraction.

Ecker, Pillemer and Grabill (1938), working with active dehydrated complement, were not able to show any reduction in the power of the complement after it had been extracted in the cold with absolute alcohol, ether and petroleum ether. Fresh complement was readily denatured by these solvents. Treatment of active, dehydrated complement with chloroform, benzine and pyridine led to no change in initial titers. They found no evidence to suggest a fifth component.

Pillemer, Seifter and Ecker (1940) repeated the experiments of Toda and Mituse but could find no evidence to suggest that C'4 is associated with a lipid complex.

d. Anticoagulants.

The question of whether two of the important normal properties of the blood, complement and coagulative activity, are basically related, has long been of interest. Of the elements making up the two systems, it has been claimed by Fuchs (1929) that prothrombin and complement midpiece are the same entity, or at least the same molecule is involved, with different groups being concerned in the two reactions (Quick, 1935).

Wadsworth, Maltaner and Maltaner (1937) reported on the effect of many anticoagulants on complement. Many of these substances did inhibit complement activity, but there was no correlation between their anticomplementary activity and their anticoagulative powers. The authors concluded that substances which are anticomplementary and anticoagulative react with either cephalin or calcium to prevent the interaction of cephalin with calcium and protein, the mechanism which they believe to be responsible for the coagulative and complementary activities of the blood. Many of their experiments are now interpreted in a different light. No one has yet proven satisfactorily that cephalin or calcium have anything to do with complement activity. It has been suggested that heparin inhibits coagulation by virtue of its high molecular size, and sulfuric acid groups. Furthermore, Bergstrom (1936) and Chargaff, Bancroft and Stanley-Brown (1936) in their studies of synthetic sulfonated carbohydrates, attribute the anticoagulative action of these compounds to a similar mechanism. Ecker and Pillemer (1941) reported that very small quantities of synthetic sulfonated carbohydrates

inactivated complement (0.5-0.8 mg. inactivated 1 ml. of serum). Since these compounds contain strong acidic groups, these authors suggested that the complement was inactivated by virtue of these groups. Large quantities of heparin were needed to inactivate complement. As heparin has a large molecular size, and possesses acidic groups, two explanations for its inhibitory action present themselves. It either inactivates complement through these acidic groups, or through a disruption of the natural serum equilibrium. Ecker and Pillemer concluded that there was no definite correlation between the inactivation of complement and the inhibition of coagulation.

Rice and coworkers (Boulanger and Rice, 1951; Rice, Boulanger and Plummer, 1951a; 1951b) conducted a parallel study of complement and coagulation. They reported that dicumerol produced a marked increase in the prothrombin time values of plasma of guinea pigs, without any accompanying change in the whole complement titer of the serum or in the relative concentration in the four major complement components. When guinea pigs were on low protein diets, the mean complement titers were found to be lower and the prothrombin times longer in the group fed the diet containing the least protein, 2.3 percent. Of the four components of complement, C'4 seemed to be the most affected. Carbon tetrachloride was fed to guinea pigs to determine whether liver injury would result in a parallel decline in the complement titer and coagulative properties of the blood. Marked fatty degeneration of the liver, a decline in total serum protein and albumin, a decrease in complement activity,

and a prolongation of coagulation time was observed in the treated animals. A general relationship was noted between the albumin-globulin ratio and the complement titer of the serum, and between the complement titer and the coagulation time of the plasma.

e. Ascorbic Acid.

In the 1920's and 30's there were many contradictory reports on the effect of scurvy on complement titers (Hamburger, R. and Goldschmidt, L., 1922; Koch, M.L. and Smith, A.H., 1924; Simola, P.E. and Brunius, E., 1933; Marsh, F.R., 1936; Hargan, E.S., 1936) The majority of these papers reported that there was no decrease in complement titers during scurvy, in fact two authors (Hamburger and Goldschmidt, 1922; and Koch and Smith, 1924) found a slight increase in the titer at this time.

In 1937 Ecker, Pillemer, Wertheimer and Gradis carefully studied the relationship between ascorbic acid and complement. Since feeding of vegetables introduced numerous other factors, animals were given ascorbic acid; one group intraperitoneally, and the other groups orally. They were able to demonstrate experimentally that a correlation existed between complement activity and the ascorbic acid level of the blood of guinea pigs. They reported that complement titers rose as the ascorbic acid content of the blood was increased to 1.0 milligram percent, but that above this level no further increase occurred.

Kodicek and Traub (1943) tested groups of guinea pigs on diets supplemented with 0.5, 1.0 and 10.0 milligrams of ascorbic acid. Using the point of 50 percent hemolysis as the unit of comparison in their titrations, they found that, although the

animals on the vitamin-C-deficient diet had lost weight, their complement titers were not altered significantly.

Rice and Boulanger (1950a) carrying out similar experiments, obtained essentially the same results. They reported that the majority of guinea pigs on a basic pellet diet, without roots or vitamin C supplement, would develop symptoms of scurvy and a considerable number die without showing any significant decrease in complement titer. A few animals however, which had become greatly emaciated, had ceased to eat and were on the point of death, showed complement titers significantly lower than those of guinea pigs on the same basic diet with root supplement. Aside from the effect of scurvy in reducing the food intake of the animals, the omission of vitamin C from the diet from 2-4 weeks had no consistent reducing effect on activity.

These reports may not be as contradictory as they appear. From the work of Ecker, Kodicek and Rice it may be reasoned that a deficiency of vitamin C does not reduce the complement activity, but that a slight excess of vitamin C causes a rise in the titer.

f. Magnesium.

The enhancing effect of Mg^{++} on the lytic function of complement has long been known (Cernovodeaun and Henri, 1906; Kellogg and Wells, 1926), but for a long time its utility and significance was overlooked.

In search for possible clues concerning the enzymatic nature of complement, Mayer, Osler, Bier and Heidelberger, (1946) studied the effect of Mg^{++}, and they were able to show that this element is essential for the hemolytic action of

complement. The hemolytic system usually does not contain sufficient magnesium for optimal hemolytic activity, so that a marked enhancement can be obtained by addition of extra magnesium ions.

Substances which bind Mg^{++} were anticomplementary when added to the usual hemolytic system, which contained only a small quantity of the ion. This type of anticomplementary effect can be overcome by addition of extra Mg^{++}.

g. Calcium.

The role that calcium plays in complement function has long been a matter of discussion. In 1921 Liebermann expressed the view that the active complement responsible for immune hemolysis was none other than the soaps normally occurring in the serum. This author claimed to be able to prepare an artificial complement by adding to heat inactivated serum a methyl alcoholic solution of calcium oleate, the mixture obtained being hemolytic towards sensitized cells but having no effect on unsensitized cells. Several other authors have repeated these experiments, but state that the solutions obtained have no specific action on sensitized cells.

Gordon, Whitehead and Wormall (1926b), observed that the removal of the diffusable calcium did not influence complementary activity, but that a correlation existed between the bound calcium of serum fractions and their ability to reactivate ammonia-treated serum. However, attempts to reactivate ammonia-treated serum by the addition of calcium in various forms were unsuccessful. They also showed that when complement was split by carbon dioxide

all the calcium passed into the albumin fraction.

Wadsworth, Maltaner and Maltaner (1936) believed that ionized calcium was necessary for complement function, since the removal of bound calcium from the endpiece by acid dialysis resulted in a loss of complement activity. They claimed that the loss of endpiece activity could be restored by the addition of calcium salts. It is difficult to judge whether the loss of complement activity was due to the removal of calcium, or to the low pH at which dialysis was carried out.

In 1941 Pillemer and Ecker (1941b) studied the role of calcium in complement function. They reported that whenever calcium was removed on the acid side, there was always some degree of C'2 inactivation along with an effect on C'4; while its removal (up to 98 percent) in neutral or slightly alkaline media had no effect on either C'2 or C'4. Their results indicated that no definite relationship existed between bound calcium and C'4.

It is the opinion of Pillemer and Ecker (1941b) that calcium may be a structural part of the pseudoglobulin-fourth component-complex, but not vital to its function, and that removal of the calcium may be effected without disturbing C'4 activity.

h. Congo Red.

The action of congo red, and a number of other dyes, on complement was first reported by Klopstock in 1924. He suggested that his results supported the view that complement inactivation is a physico-chemical phenomenon in which the colloidal state

of the reacting substance is an important factor. The list of dye stuffs which he employed included members of various chemical groups.

Gordon (1930) also showed that congo red and related dyes inactivated complement as shown by tests on the bactericidal and hemolytic activity of normal guinea pig serum. This inactivation was unaccompanied by destruction of complement, since charcoal removed congo red from an inactive mixture of complement containing serum and dye, thereby restoring its hemolytic activity. This reversibility, which was readily demonstrated, indicated an adsorption phenomenon by the dye of the complement.

In 1945 Gordon and Walker concluded that the anticomplementary activity of the dyes of the congo red series appeared to be dependent more on the molecular size of the reacting substance than on any specific grouping within the molecule. They showed that replacement of the amino groups by hydroxyl groups in no way reduced the anticomplementary activity of congo red dyes. These authors were able to reverse anticomplementary effect by adsorbing the dyes on silk.

i. Gamma Globulins.

It has long been known that increased serum globulins are frequently anticomplementary. If suitable proportions of complement and globulin are incubated the hemolytic power of the complement is inhibited (Zinsser and Johnston, 1911).

Davis, Kabat, Harris and Moore (1944) reported on the anticomplementary effect of globulin fractions separated by electrophoresis. From their results it was apparent that the ability to interfere with the activity of complement was a

property of normal gamma globulin or some constantly present portion thereof. Electrophoretically separated gamma globulin from a number of normal human sera was highly anticomplementary, as little as 0.04 milligrams of protein giving complete inhibition of hemolysis. Purified Wassermann antibody, prepared by dissociation of the specific precipitate, was anticomplementary in similar amounts. The anticomplementary action was decreased by heating at 56°C. for one half hour, and was abolished by the addition of approximately equal amounts of albumin or beta globulin.

The mechanism of the complement-fixation reaction proposed by Heidelberger, Weil and Treffers (1941) involved the assumption that complement could enter into easily dissociable compounds with antibody molecules in the absence of antigen; when antigen was introduced the molecules of complement became firmly bound in the antigen-antibody aggregates. The observation that normal gamma globulin is highly anticomplementary in the absence of the inhibiting effect of other serum proteins, together with the current belief (Kabat, 1943) that antibodies are modified gamma globulins, suggests that these modifications in the cellular synthesis of gamma globulins which produce antibodies may not be necessary for the development of affinity for complement.

While participating in the study of the therapy of scarlet fever with human gamma globulin, Waring and Weinstein (1947) carried out experiments to see what effect this gamma globulin was having on the complement of their patients. They found this material to be anticomplementary in the test tube even after the

addition of considerable amounts of human albumin. Ten patients received intramuscular injection of the globulin, but no decrease in complement activity was noted. This was not surprising in view of the albumin content of human serum, the dilution by the circulating blood, and possibly the relatively slow rate of adsorption following intramuscular injection.

j. Mustard Gas and Related Compounds.

Complement is readily inactivated by processes which affect the state of the serum proteins, and since mustard gas, BB'-dichlorodiethyl sulphone (H-sulphone), and divinyl sulphone react with a variety of proteins (Banks, Boursnell, Francis, Hopwood and Wormall, 1946a; 1946b) it is not surprising that they inactivate complement.

Boursnell, Francis and Wormall (1946) reported that small amounts of mustard gas inactivated complement, as did divinyl sulphone. H-sulphone inactivated complement, but relatively slowly when compared with these other two substances. The inactivation appeared to affect all the components of complement. There was some indication, however, that the relatively heat labile components, C'1 and C'2 were more rapidly inactivated than the other two components. The authors suggested that mustard gas and divinyl sulphone differed considerably in the way in which they reacted with the protein molecule, and it was probable that they differed in their action on complement.

The combination of H-sulphone and divinyl sulphone with proteins was mainly concerned with a reaction of the sulphone with the free amino groups of the protein, and it was possible

that inactivation of complement by these two reagents was effected by this means. In the case of mustard gas, however, the reaction with proteins was apparently concerned with the carboxyl and iminazole groups (Banks, Boursnell, Francis, Hopwood and Wormall, 1946b).

Another possible mechanism for the inactivation of complement by these compounds is the oxidation of the -SH groups of the serum proteins. Mustard gas, H-sulphone and divinyl sulphone all readily react with -SH groups, and a reaction of this type might account for part at least of their inactivating action on complement. Ecker, Pillemer, Martiensen and Wertheimer (1938a) have suggested that complement may possess -SH groups, and that inactivation by oxidation might be due to their conversion to disulphide (-S-S-) groups.

4. SEASONAL FLUCTUATIONS.

It is a well recognized fact that the complement titer of guinea pigs tends to fall in the late winter and early spring. When the feeding of green grass is begun the general health of the animal improves rapidly and there is often an abrupt rise in complement titer. This would suggest that some dietary requirement is concerned.

This suggestion was investigated by Rice and Boulanger (1950b), who noted that in late March a certain proportion of their individual guinea pig sera had complement titers below the level accepted as satisfactory for routine complement-fixation tests. They planned therefore, to continue to feed the guinea

pigs the stock diet of pellets until May, when green grass would be available. It was then proposed to compare the relative effects of known vitamin supplements with that of green grass upon complement recovery. This carefully planned experiment was completely upset, however, by the unexpected behaviour of the test animals: Their mean complement titer began to return to normal levels about the middle of April; that is well before green grass could be secured. This was observed both in the group of animals receiving pellets supplemented by roots and in those given pellets alone. It would appear therefore that since the diet had not been altered, some other factor must have been involved in the complement recovery. What this seasonal factor is has not been discovered.

A similar fluctuation in horse serum was reported in the same paper. In May, although on the same diet as previously, the complement titer of this animal was three times what it had been in March. This higher titer was maintained during the summer months, then began to decline in November, and in February and March had reached the approximate level of the year before.

5. COMPLEMENT IN DISEASE.

Complement has been deemed in the past to be a normal and invariable constituent of blood serum. It is held to be a protective agency because of its destructive activities in respect to invading organisms, after the latter have been sensitized by the antibody which is produced in response to the antigenic action of the invader. The theory is that complement

is one of the factors which guards the body against pathogenic organisms, that it aids in the combat against established and well-seated infection, and that it tends in a way to supplement the combative activities of antitoxins, serums and vaccines. In short, complement is held to be a necessary factor in bacteriolytic immunity in respect to infection.

This latter point was reported upon by Rich (1923) who had a strain of guinea pigs in which he could detect no complement activity. These pigs were weaklings, and very hard to raise, due to the fact that minor infections proved fatal to them. Systematic immunization, however, would produce immunity to any specific organism, and lack of complement did not interfere with the production of agglutinating antibodies. It seems probable that the strain was not totally deficient in complement, but was lacking in one component only.

Ecker, Seifter, Dozois and Barr (1946) in a survey of complement in infectious diseases in humans, arrived at the conclusion that many of the apparent contradictions and inconsistencies encountered in the literature could be attributed to the lack of a uniform technique of complement assay and to an inadequate definition of the limits of "normal" and "abnormal" complement levels. In general, while the observed complement level often changes in the course of infection, the extent and direction of the changes were by no means always similar in different diseases, or even in individual instances of the same disease. Furthermore, little information existed concerning the variability of the complement components in the course of

infections.

Ecker and coworkers (1946) reported that in those cases showing a decrease of complementary activity, a marked decrease or even disappearance of C'4 occurred. In other cases, in addition to a diminution of C'4, a decrease of C'2 and C'1 was observed. C'3, however, except in one instance, did not show a loss in activity. In all cases showing no hemolytic complement, C'4 was strikingly reduced. It is interesting to note that in normal complement the latter component is one of maximum titer. In the cases studied, C'1 varied only slightly. Frequently, low complement activity was observed in epidemic meningococcal meningitis; ninteen of thirty-eight patients with this disease exhibited diminished complement. In cases of erysipelas, high complement levels were noted.

In a subsequent study, Seifter and Ecker (1946) identified complement components in the precipitated urinary proteins of patients with nephrotic syndrome, and in cases of acute and chronic glomerulitis, C'2 was consistently found in the urine, C'3 and C'4 were also detected, but C'1 was seldom present. As a possible explanation it was pointed out that C'2 is a highly soluble protein, whereas C'1 is a euglobulin. The excretion or retention of the components might therefore be determined by their solubilities.

Although it has long been known that a reduction in complement activity occurs during malarial paroxysms (Cathoire, 1910; Vincent, 1910; Wendelberger and Volavsek, 1934; Zermati and Vargues, 1947), further work employing more accurate methods,

was necessary to establish the loss in comparison with the complement activities of healthy individuals. Dulaney (1948) stated that the 50 percent unit of hemolytic complement for thirty normal individuals ranged from 0.0032 ml. to 0.006 ml. with a median of 0.0045 ml., and that day to day variations in these individuals appeared to be slight. No marked variation from the normal was noted in the sera of twenty-five patients with liver disease, or in thirty-two patients with non-infectious diseases of various types. However, in 178 titrations of the sera of 24 neurosyphilitics treated with malaria, complement was usually diminished. This decrease in some instances was slight, but in severe cases was great. Two of the patients showed reductions from 0.0048 ml. before inoculation to 0.08 ml. at the tenth day after inoculation, and 0.28 ml. on the twentieth day after inoculation. One of these cases had a preinfection titer of 0.0039 ml. and on the thirteenth day following inoculation a titer of 0.0134 ml. On the twentyfirst day the titer stood at 0.225 ml.

It is an interesting observation that the complement depression coincided with, or followed, the peak of parasitemia for that individual. Dulaney was, however, unable to correlate the complement values with the number of parasites.

McGhee (1952), working with malaria in ducks, reported a significant reduction in the titer of whole complement. This reduction was evident in all but one duck in which the infection never rose above 150 parasites per 10,000 erythrocytes. The percentage loss of complement in all infections, independent of

the number of organisms, was approximately the same. The reduction in titer was not always associated with the presence of in vivo hemolysis, caused by the parasites, but in ducks in which hemolysis did occur, there was a coincident drop of titer. When hemolysis occurred on successive days, the titer remained low. In ducks which recovered, it gradually increased to normal levels.

As with whole complement, hemolysis was indicative of changes in the components. Moreover, the component titers were affected on the terminal day of infection whether hemolysis was present or not. When hemolysis occurred on the terminal day, all components showed a mean loss of 71 to 86 percent in titer. In subterminal hemolysis there was never less than a 50 percent reduction of C'4, and C'2 was reduced in all ducks, from 20 to 85 percent. The loss of C'1 and C'3 varied from no loss at all to 60 percent in the different ducks.

It is the belief of the author that the in vivo hemolysis, the reduction in number of parasites, and the total or differential loss in component titers reflects an antigen-antibody combination, and that the percentage loss of the various components is an indication of the intensity of this reaction.

Huddleson (1948) reported that, in brucellosis, a rapid and complete termination of the disease can be accomplished either in the experimentally infected guinea pig or in the human being by making use of the potentiating property of certain of the sulfonamides on the serum antibody-complement system. If fresh serum from the infected animal or man, tested during the oral administration of the drug (sulfadiazine or sulfamerazine)

showed a bactericidal effect against the organisms in the test tube, and if this action persisted in vivo for at least six days, then all the organisms in the body fluid were destroyed and recovery ensued. On the other hand, if the disease had progressed to the point where a large portion of Brucella serum antibodies had apparently lost their power to bind complement (Neisser-Wechsberg phenomenon), the presence of the sulfadiazine in the blood in high concentration (30 milligram percent) had no appreciable effect on the antibodies. The solution of this problem was accomplished by the combined use (in transfusion) of normal whole blood, plasma, or serum, and in the oral administration of small quantities of the drugs.

Jordan (1942) in his studies on complement in disease, went so far as to state that "if, in patient with icterus, the complementary power of the serum is found to be high, this is an argument ..... against the existence of an atrophic cirrhosis". In some cases of Weil's disease, and in other patients with "catarrhal jaundice", normal or even high complement levels were observed. Strikingly high values were found in patients with mechanical obstruction of the bile ducts. Also, low values were obtained in patients with diseases of the bones (Kahler's, Schuller-Christian's, Albers-Schonberg's diseases and carcinomatosis). Local affections of the skeleton (tuberculosis, tumour), however, resulted in no changes. In lipoid nephrosis low values were observed.

Bieler, Spies and Ecker (1947) noted no significant deviations from the normal in the sera of eight persons with

hypoproteinemia without infections.

Although it has been known among serologists that the serum of a pregnant guinea pig was likely to be unsatisfactory for use in complement fixation, it was not until 1947 that Ecker, Hiatt and Barr attempted to delineate the range of variation of complement during pregnancy. From their data it is seen that pregnant guinea pigs have a complementary titer of about one half that of normal healthy animals. After parturition complement activity gradually increases towards the level normal for nonpregnant female guinea pigs.

D. SULFONAMIDES

Until Domagk in 1935 first reported the usefulness of prontosil in hemolytic streptococcal infections, and Trefouel et al (1935) established that the active part of the compound was the sulfanilamide molecule, specific antipneumococcal serum therapy had been the only means of combating pneumococcal infections. The first American report on the use of a sulfonamide in veterinary therapeutics appeared in 1937 (Allott, 1937). During the years which have elapsed since the appearance of that report, the sulfonamides have received clinical trial in a wide variety of infectious diseases in animals. From data compiled by Northey (1948) showing the relative order of effectiveness of the various sulfonamides against a number of infections, sulfamethazine, sulfamerazine, sulfathiazole and sulfadiazine are the most effective against the pneumococcus in animals. Sulfapyridine is also effective in pneumonia, but requires higher

dosages to maintain therapeutic blood levels (Thorp, 1944).

1. ABSORPTION, EXCRETION AND DISTRIBUTION.

The majority of the sulfonamides are given orally, and are absorbed through the gastro-intestinal tract. A notable exception is sulfaguanidine, which is only slightly absorbed, and is therefore used for the treatment of various intestinal infections. The rate and degree of absorption varies with the different sulfonamides; this means of course that the amount of drug required to give a certain concentration in the blood depends largely on which sulfonamide derivative is being used. In initiating the treatment of an acute infection, it may be desirable to establish therapeutic blood concentrations with greater rapidity than is possible with oral administration. In this case the initial dose may be given by subcutaneous, intramuscular, or intraperitoneal injection (Schneller and Foss, 1948).

The renal clearance of the sulfonamides varies with the different drugs. In man, and most mammals, the sulfonamide is acetylated in the liver and passed in the urine. Varying quantities of the non-acetylated drug may be reabsorbed by the tubules of the kidney. The resultant of these biochemical factors of absorption, acetylation and excretion, is the concentration of the drug which is achieved and maintained in the blood stream. A minimum effective blood level is considered to be five milligram percent, although somewhat higher levels are desirable, and in certain cases essential (Welsh and Schroeder, 1946).

A factor which conditions the distribution ratio of a sulfonamide in the various tissues with respect to the blood concentration, and which is probably concerned in the duration of blood levels, is the degree of plasma binding. The sulfonamides differ quite widely in this respect. It has been suggested that the drug bound to plasma protein is probably unavailable for antibacterial action (Davis, 1944). This hypothesis remains to be adequately tested, as also the corollary premise that the effective concentration of drug is that in or on the bacterial cell, rather than the concentration of filterable drug in plasma (Northey, 1948).

2. SULFONAMIDES AND COMPLEMENT ACTIVITY.

Until this time there has been no investigation into the effect of sulfonamides on complement activity. However, during investigations of complement activity during disease, mention has been made of these drugs in reference to complement titers.

In 1946 Ecker, Seifter, Dozois and Barr reported on the complement titers of patients with various communicable diseases. Several different infections were treated with sulfadiazine, and, whereas in a patient with bronchopneumonia and a sulfadiazine blood level of 3.6 milligrams percent, the complement titer was zero, a patient with scarlet fever, receiving 17 - 18 grams of sulfadiazine a day, had a very high complement titer. Healthy persons were dosed with sulfadiazine, as a control, and from their results the authors concluded that the highest blood levels of the sulfonamide drugs were without significant effect on the

complement titer.

3. SULFAMETHAZINE AND ITS SODIUM DERIVATIVE.

Sulfamethazine was one of the later members of the sulfonamide family to come into general use. On the basis of its wide spectrum of antibacterial effectiveness, its low toxicity, and the prolonged duration of therapeutic blood levels obtained with oral or parenteral dosage, sulfamethazine stands head and shoulders over the early sulfonamides used in veterinary medicine.

In vivo experiments on mice have shown that following a single intravenous dose, the concentration in the blood rises more rapidly and falls more slowly with this drug than with either sulfanilamide or sulfapyridine (Welsh et al, 1946). The latter is a result of its low excretion rate and the high degree to which the drug is bound in the plasma protein, and results in lower dosages maintaining effective blood concentrations.

In vivo studies in many experimental animals indicate that levels of 5 milligrams percent and over are therapeutically adequate. Furthermore, the time involved in administration is small because only once-a-day administration is required as compared to the three or four times a day administration necessary in the case of more rapidly eliminated sulfonamides (Schneller, Foss and Sullivan, 1948).

Sulfamethazine is less toxic than sulfapyridine and sulfathiazole, and no more toxic than the other pyrimidines. No signs

of histopathological changes in the kidneys of animals resulted from large repeated doses of sulfamethazine, despite the fact that this drug produced higher blood concentrations than other sulfonamides (Schneller et al, 1948).

Sulfamethazine is, therefore, of great use in veterinary medicine, and is used frequently to cure infections in small and large animals. It is the drug of choice against D. pneumoniae in guinea pigs, where a solution of the drug is usually administered orally.

Sodium sulfamethazine has all the properties of sulfamethazine and in addition is much more soluble in aqueous solutions. For this reason it is very often used in place of sulfamethazine.

EXPERIMENTAL

A. MATERIALS AND METHODS.

1. MATERIALS.

a. Reagents.

Veronal Sodium Chloride Buffer.

85.0 gm. NaCl.

5.75 gm. 5,5-diethyl barbituric acid.

3.75 gm. sodium 5,5-diethyl barbiturate.

The acid was dissolved in 500 ml. of hot distilled water, and added to the solution of the other two components. The solution was cooled and made up to 2,000 ml. with distilled water. The buffer was decanted into 250 ml. erlenmeyer flasks, autoclaved at 120°C. for 20 minutes, and stored at 5°C. Before using, one part of buffer was diluted up to five with distilled water. The pH of the diluted solution was 7.3 to 7.4

This buffered salt solution was used throughout all experiments for diluting complement and hemolysin and for washing cells.

Alsever's Modified Solution. (Bukantz, Rein and Kent, 1946)

Each 100 ml. of solution contained:

2.05 gm. dextrose.

0.80 gm. sodium citrate.

0.42 gm. sodium chloride.

0.055 gm. citric acid.

The solution was autoclaved at 120°C. for 10 minutes, and stored at 5°C., the final pH being 6.1.

b. Test Materials for Complement Titrations.

Complement.

Guinea pig serum was used throughout as the source of complement. All guinea pigs weighed over 700 gm., and were normal healthy animals. They were arranged in groups, and the sera of the guinea pigs in a group was pooled. All complement used contained the sera of at least three guinea pigs.

Blood was obtained by cardiac puncture of unanaesthetized animals. A 10 ml. glass syringe with a 20 guage needle was used, and amounts ranging from 4 to 8 ml. were withdrawn from each animal. Care was taken that the guinea pigs were not fed before being bled. To insure this the animals were always bled in the morning, and fed in the early afternoon. Water was available in the cages at all times.

The blood was placed in clean 15 ml. centrifuge tubes. After it had clotted it was rimmed, and placed at room temperature for one hour. The serum was separated from the clot by centrifugation, and was withdrawn with a pasteur pipette. Complement which was not tested immediately, was placed in 10 ml. ampoules, which were sealed, labelled, and placed at -25°C. At this temperature the complement could be kept indefinitely with very little reduction in hemolytic titer.

Hemolysin.

The hemolysin was prepared in normal, healthy rabbits. Two ml. of citrated sheep red cells were washed three times in cold saline, and resuspended in one ml. of saline. This dose was injected into the marginal ear vein of a rabbit, three times

a week, for three weeks. After the sixth dose a small amount of blood was taken from the ear vein and the antibody titer determined. If the titer was 1:6000 or greater, the final injections were not given.

Thirty ml. of blood were taken from each rabbit, by cardiac puncture, placed in sterile test tubes, and allowed to clot. The serum was separated from the clot by centrifugation, and the clear serum heated at 56°C. for thirty minutes. This temperature destroys all of C'1 and C'2, but does not greatly affect C'3 or C'4 (Dozois et al, 1949). The hemolysin was placed in vials, which were sealed with paraffin, and stored at 5°C.

Sheep Red Blood Cells.

50 ml. of sheep's blood was withdrawn from the external jugular vein and added to 60 ml. of modified alsever's solution; sterile technique was closely observed. The cells stored in this medium became constant in their susceptability to lysis after 48 hours at 5°C., and remained at the same level of susceptability for at least 10 weeks (Bukantz, Rein and Kent, 1946).

The amount of cells required for a days work was removed aseptically, and washed with buffered salt solution. Care was taken that there were always ten volumes of buffer to every one of cells. The cells were washed three times, and centrifuged at 2570 r.p.m. for 10 minutes between each washing. For the final washing the cells were suspended in graduated centrifuge tubes and centrifuged as above, and the volume of packed cells was noted. To the packed cells, 19 volumes of veronal NaCl buffer were added, resulting in a 5 percent suspension of red cells.

c. Sulfonamides.

The following sulfonamides were used in experimental work:

Sulfamethazine.

Sodium Sulfamethazine. (Trade name Coxine)

Sulfadiazine.

Sulfapyridine.

Sulfasoxizole. (Trade name Soxisol)

d. Reagents for Sulfonamide Determinations.

15% trichloracetic acid solution.

0.1% Sodium nitrate solution (freshly prepared each week).

0.5% Ammonium sulfamate solution.

0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride solution.
(Stored in a dark bottle.)

Stock solution of sulfamethazine, containing 200 milligrams/liter.

Dilute standards of sulfamethazine, prepared as follows;

5 ml. stock solution diluted to 100 ml. = 1 milligram %.

10 ml. stock solution diluted to 100 ml. = 2 milligrams %.

20 ml. stock solution diluted to 100 ml. = 4 milligrams %.

40 ml. stock solution diluted to 100 ml. = 8 milligrams %.

50 ml. stock solution diluted to 100 ml. = 10 milligrams %.

60 ml. stock solution diluted to 100 ml. = 12 milligrams %.

e. Media.

Yeast media.

2.0% bactopeptone.

2.0% cerelose.

0.1% inositol.

0.01% thiamin.

Modified Saberaud media.

1.0% neopeptone.

2.0% cerelose.

Blood agar plates.

Pneumo broth.

"L.S. broth.

2. METHODS.

a. Complement Titrations.

i. Titration of Hemolysin.

Hemolysin was titrated against a constant amount of complement and sheep red cells. The latter were used in a 3 percent suspension, and 0.4 ml. of a 1 : 10 dilution of complement provided a constant excess of this material.

0.1 ml. of hemolysin was added to 9.9 ml. of saline, and mixed by blowing in and out with a pipette 30 times. 0.5 ml. of the 1 : 100 dilution was added to 4.5 ml. of saline, to give a 1 : 1000 dilution, and mixed as above. A 10 tube titration was set up, as in Table I.

The tubes were shaken and incubated in a 37°C. water bath for 30 minutes. The highest dilution of hemolysin giving complete hemolysis at the end of this time was taken as the titer. A titer of 4,000 units per ml., or higher, was required for complement titrations. Throughout all experiments the hemolysin was diluted with veronal NaCl buffer to contain 5 units per ml.

Table I.

Titration of Hemolysin.

Tube.	Dilution.	Complement dil. 1/10.	Hemolysin dil. 1/1000.	Buffered saline.	Sheep cell suspension 3%.
		ml.	ml.	ml.	ml.
1.	$\frac{1}{1000}$	0.4	.4	0	0.4
2.	$\frac{1}{1500}$	0.4	.27	.13	0.4
3.	$\frac{1}{2000}$	0.4	.2	.2	0.4
4.	$\frac{1}{3000}$	0.4	.13	.27	0.4
5.	$\frac{1}{4000}$	0.4	.1	.3	0.4
6.	$\frac{1}{6000}$	0.4	.067	.333	0.4
7.	$\frac{1}{8000}$	0.4	.05	.35	0.4
8.	$\frac{1}{12000}$	0.4	.04	.36	0.4
9.	$\frac{1}{16000}$	0.4	.025	.375	0.4
10.	$\frac{1}{32000}$	0.4	.0125	.3875	0.4

ii. Sensitization of Sheep Cells.

The required amount of a 5% suspension of sheep red blood cells was placed in a flask, and to it was added an equal volume of hemolysin, containing five units per ml. The mixture was well shaken, and stood at room temperature for 15 minutes prior to use. The concentration of the sensitized red blood cells was approximately 250,000,000 erythrocytes per ml.

iii. 20 Tube Complement Titration.

To a series of 20 serology tubes, complement, diluted 1 : 30, was added in amounts ranging from 0.01 to 0.20 ml. A 0.2 ml. pipette graduated in 0.001 ml. was used, and the diluted complement was delivered to the bottom of each tube. Veronal NaCl buffer was added to give a volume of 0.2 ml. in each tube. The tubes were rotated and 1 ml. of the sensitized cells was added. The racks were thoroughly shaken and incubated in a 37°C. waterbath for 30 minutes. After incubation the tubes were either placed at 5°C. overnight and read in the morning, or centrifuged at 2,570 r.p.m. for 5 minutes, and read immediately against a standard.

iv. Hemolytic Standard.

Each day a fresh hemolytic standard was prepared. A washed, packed sediment of sheep red blood cells was diluted in distilled water to give a 5% suspension. To this suspension an equal volume of 1.7% NaCl was added, resulting in a 2.5% suspension of hemolysed red blood cells in physiological saline. To 21 serological test tubes the 2.5% suspension was added in amounts which increased from zero to one ml. by an arithmetic

progression of 0.05 ml. A 2.5% suspension of normal, unhemolysed, red blood cells in saline was added to each tube to make a total volume of one ml. 0.2 ml. of saline was added to each tube to make a final volume of 1.2 ml., the same volume as in the test titration. The result was a hemolytic standard which ranged from no hemolysis in the first tube, to complete hemolysis in the last tube, with each 5 percent change in hemolysis recorded between the two extremes. The standard tubes were centrifuged, and the test titrations compared with them.

v. Initial, 50 and 100 percent hemolysis.

The method most commonly used to determine the efficiency of complement, has been to measure the amount of complement necessary to produce complete hemolysis of a given volume and concentration of sensitized sheep red cells. Such a unit is a most variable quantity. According to Brooks (1920) "it is impossible to make a dependable interpolation between the lowest concentration producing complete hemolysis and that just failing to do so, for when hemolysis is nearly complete large changes in the amount of complement produce little change in the amount of hemolysis". Brooks therefore suggested determinations of the proportion of cells hemolysed in each mixture rather than the number of cells; i.e., the depth of the colour of the supernatant fluid. Wadsworth, Maltaner and Maltaner (1931) have also called attention to the inaccuracy of titration carried out to the point of complete hemolysis.

Brooks observed that a complement titration plotted on graph paper gave an "S" shaped curve (Graph I). The steepest

part of the curve lay between 30 and 70 percent hemolysis, indicating that between these two points small volumes of complement caused large changes in hemolysis. He therefore suggested that the 50 percent hemolytic unit was a more accurate measure of the complement titer.

Koopman and Falkner (1935-1936) insisted that titrations should be read where hemolysis began, so that they would be independent of the inevitable variations in cell concentrations and also to minimize the resistence to further complement action engendered by hemolysis. In complete agreement with Koopman et al, Ecker, Pillemer, Wertheimer and Gradis (1938) also found that the amount of hemolysis obtained was not directly proportional to the amount of complement present. They carefully observed the first tube to show faint hemolysis and took this as their titer. These authors stated that this initial unit of hemolysis was the most accurate measurement of the activity of the complement.

In the following experiments both initial and 50 percent hemolysis was noted, and both figures will be given in the results. In certain cases complete hemolysis was used as the point of comparison.

b. Fractionation of Complement.

Methods have been evolved to inactivate one or more of the components of complement and to leave the remaining components active. The active components are called "fractions" and are named after the method used in their preparation.

i. Preparation of the Midpiece (M.) and Endpiece (E.) Fractions by the Carbon Dioxide Method.

Distilled water was saturated at room temperature with carbon dioxide gas, which was allowed to bubble through the water for 30 minutes under slight pressure. To 9 ml. of the carbon dioxide saturated water 1 ml. of serum was added, and carbon dioxide gas was again passed through the mixture for 20 minutes. Foaming, produced by the bubbling of the gas through the serum, was kept at a minimum by gently waving a wooden applicator stick, dipped in capryl alcohol, over the liquid. Because of the hemolytic quality of the alcohol, care was taken to avoid allowing the saturated applicator stick to touch the liquid. The globulin precipitated after 20 minutes saturation of the serum with carbon dioxide was rapidly centrifuged in an international centrifuge, Type S.B.1, at a speed of 3,000 r.p.m. at room temperature, and resuspended three times in distilled water, after each time again being centrifuged as above. The precipitate was then redissolved in 10 ml. of veronal NaCl buffer.

The endpiece which was present in the supernatant, after the precipitation of the globulin, was made isotonic with 17 percent NaCl solution.

The two fractions were then placed in flasks in a dessicating jar, which was sealed and connected to a vacuum pump. The jar was evacuated under slight pressure for one half hour to remove the residual carbon dioxide from the fractions. Both fractions were then tested for neutrality with Accutint indicator paper, and the pH brought up to 7.0 with 0.1 N NaOH.

If the fractions were not to be used immediately, they were placed in glass ampoules, which were sealed and labelled, and stored at -25°C.

ii. Preparation of Fraction 56, Containing C'3 and C'4.

To inactivate the first and second components, the required amount of complement was placed in a 56°C. waterbath for 20 minutes. Following incubation it was diluted 1 : 5 with veronal NaCl buffer, and if not used immediately it was placed in glass ampoules, which were sealed and stored at -25°C.

iii. Preparation of Fraction H, Containing C'1, C'2 and C'3.

Although dilute ammonia is commonly used for the specific inactivation of C'4, it has been the practice here to use hydrazine, as suggested by Pillemer, Seifter and Ecker (1941 a). Ammonia, although a specific inactivator of C'4, has often damaged the midpiece of complement, by virtue of its high pH. Hydrazine has a pH of between 7.2 and 7.4 and is therefore not destructive to the midpiece.

A 0.16 M solution of hydrazine was used. Amounts ranging between 0.05 and 0.15 ml. of the hydrazine solution were added to 1 ml. of serum, thoroughly mixed, and incubated in a waterbath at 37°C. for one hour. The smallest quantity giving complete inactivation of the complement was used for further inactivation. To each ml. of hydrazine inactivated serum, 4 ml. of veronal NaCl buffer were added, and if the fraction was not to be used immediately it was stored in sealed glass ampoules at -25°C.

iv. Preparation of Fraction Z, Containing C'1, C'2 and C'4.

Several methods for the inactivation of C'3 were tried,

before a successful one was found.

(1) Inactivation with yeast cells.

Following the method of Robinson, Whitehead and Gordon (1925), a cake of L'Allemand's yeast was mixed with saline to form a thin paste. 5 ml. of the paste was placed in each of 4 test tubes, 3 of which were placed in a boiling water bath for 45 minutes, while the fourth tube was left at room temperature. At the end of this time the tubes were centrifuged at 2,560 r.p.m. in an angle centrifuge, and the supernatant decanted. 0.5 ml. of serum was added to each tube, the contents stirred with a glass rod, and the tubes incubated at 37°C. for varying lengths of time. After incubation 4.5 ml. of veronal NaCl buffer was added to each tube and the tubes were centrifuged for 20 minutes at 2,560 r.p.m. The supernate, complement diluted 1 : 10, was decanted, and tested for inactivation and reactivation (Table II).

It can be seen from Table II, that the complement, incubated with yeast cells which had not been boiled in a waterbath, was anticomplementary. The complement, treated with yeast cells which had been in a boiling waterbath, was not anticomplementary, but it was not completely inactivated.

It had been noted by Robinson et al (1925) that Fleischmann's yeast was the most successful in removing C'3.

Fleischmann's dried yeast was added to 0.9 percent saline until a thin paste was formed. Three tubes, each containing 5 ml. of the paste were placed in a boiling waterbath for 45 minutes. The tubes were centrifuged at 2,560 r.p.m. for 20 minutes, and the supernatant removed. 0.5 ml. of guinea pig

Table II.

Complement Treated with L'Allemand's Yeast.

Tube.	Treatment of yeast cells.	Incubation time of complement & yeast in hrs.	% Hemolysis	
			Treated complement	Reactivated complement
			0.2 ml. of a 1:30 dilution.	0.2 ml. of a 1:30 dil. of treated complement 0.2 ml. of a 1:30 dil. of fraction 56.
1.	22°C.	1	0	0
2.	100°C.	1	60	100
3.	100°C.	2	60	100
4.	100°C.	3	50	100

Table III.

Complement Treated with Dried Fleischmann's Yeast.

Tube.	Time of incubation at 37°C. in hours.	% Hemolysis	
		Treated complement	Reactivated complement
		0.2 ml. of a 1:30 dilution	0.2 ml. of a 1:30 dil. of treated complement 0.2 ml. of a 1:30 dil. of fraction 56.
1.	1	60	100
2.	2	30	100
3.	3	40	100

serum was added to each tube; the tubes were incubated at 37°C., the first for one hour, the second for two hours and the third for three hours. 4.5 ml. of veronal NaCl buffer was added to each tube, the tubes were centrifuged and the complement, diluted 1 : 10, was decanted. The serum was tested for inactivation and reactivation.

The results, shown in Table III, indicated that serum treated in the above manner was not anticomplementary, nor was it completely inactivated.

Coca (1914) reported that he was able to inactivate C'3 with a 20 percent suspension of yeast cells. A 20 percent suspension was prepared as follows: Saline was added to dried Fleischmann's yeast, and a drop of the suspension was transferred to a blood-agar plate. The yeast was spread evenly over the plate and was left at room temperature for 24 hours. Single yeast colonies were picked off and transferred to Saberaud slopes; smears were made to see that pure cultures were obtained. These stock cultures were transferred to fresh Saberaud slopes once a week, and were used throughout the year when Fleischmann's yeast was needed. A pure 48 hour culture of Fleischmann's yeast was washed twice with 20 volumes of 0.45 percent NaCl, rapidly centrifuged, and the supernatant withdrawn. After the final centrifugation, the content of yeast cells was noted, and made up in a 20 percent suspension with 0.9 percent NaCl.

Amounts varying from 0.05 ml. to 2.0 ml. of the 20 percent yeast suspension were placed in serological test tubes, centrifuged, and the supernatant decanted. The guinea pig serum was

added to the yeast cell sediment, mixed thoroughly, and stood at room temperature, or incubated at 37°C. for varying lengths of time. To each tube enough veronal NaCl buffer was added to make a 1 : 10 dilution of the complement, the tubes were centrifuged at 2,560 r.p.m. for 15 minutes, and the diluted complement decanted. The complement was tested for inactivation and reactivation (Table IV).

From the results it is seen that complement treated in this manner became anticomplementary, and reactivation was relatively unsuccessful. As the amount of yeast cells decreased, so did the anticomplementary effect, but small quantities of yeast cells did not completely inactivate C'3. This method did not appear to be satisfactory, so another method was tried.

Robinson et al (1925) noted, that while a yeast cell suspension proved a most uncertain agent, and while yeast heated at 100°C. in saline was a better agent, zymin powder was almost invariably successful in removing C'3 from serum. As yeast, heated in saline, and a suspension of yeast cells had been tried without success, zymin was prepared.

Fleischmann's yeast was only available in a dried form, so enough had to be grown to provide a suitable amount of zymin. 12 liters of yeast media were placed in each of two, 20 liter carboys, which were then autoclaved at 120°C. for one hour. 8 tubes of modified Saberaud's media were inoculated from the pure yeast cultures and kept at room temperature for 48 hours. The contents of 4 tubes were used to inoculate each of the two carboys. The cultures were kept at room temperature for 48 hours, while air,

Table IV. Complement Treated with a 20 percent Suspension of Fleischmann's Yeast.

Tube.	Complement in ml.	20% yeast suspension in ml.	Time of incubation in min.	Temperature of incubation °C.	Treated Complement, 1:30 dil. in ml.		% Hemolysis. Reactivation of treated complement. in ml.					
							Fraction 56 1:30 dil.	0.3	0.6	0.3	0.6	0.6	1.2
							Treated serums 1:30 dil.	0.3	0.6	0.6	0.3	1.2	1.2
					0.6	1.2							
1.	1.0	2.0	60	37	0	10		10	30	20	10	30	40
2.	1.0	2.0	60	22	0	15		20	30	40	10	40	45
3.	1.0	1.0	60	37	0	-		25	30	-	-	20	-
4.	1.0	1.0	30	37	0	-		20	30	-	-	30	-
5.	0.5	1.0	60	37	0	-		10	20	-	-	10	-
6.	0.5	0.5	30	37	0	-		15	25	-	-	20	-
7.	1.0	0.5	60	37	0	-		20	30	-	-	25	-
8.	1.0	0.5	30	37	20	20		25	50	-	-	-	70
9.	1.0	0.25	30	37	40	40		30	60	-	-	-	90

filtered through a Berkfeldt filter, no. 13, was run through glass tubing to the bottom of the carboys. A piece of glass tubing, sealed at the end, with many small holes in it, was attached to the bottom of the air tube, thus allowing better dispersion of the air bubbles and increased aeration. The cultures were shaken by hand every 2 - 3 hours during the day time, to help keep the yeast in suspension. After 48 hours, the carboys were given a final shake, and the contents were siphoned into the de Laval centrifuge . The rate of flow was regulated by means of a saline drip and a screw clamp. The yeast cells lined the bowl of the centrifuge, where they were collected and twice suspended in 2 liters of 0.45 percent NaCl and centrifuged as before. After the final centrifugation the yeast sediment was suspended in one liter of 0.9 percent NaCl and filtered through a Buchner funnel. The cells packed down in a hard cake, and the pressure was held until no more liquid passed through the filter. The yeast was weighed immediately, and zymin prepared. 210 grams of yeast cells were harvested from the two carboys.

Zymin was prepared, following the method described by Ecker, Jones and Kuehn (1941). 50 grams of the packed yeast cells were suspended in 300 ml. of acetone. The suspension was stirred for 10 minutes, and passed through a Buchner funnel. The residual mass was again stirred in 100 ml. of acetone for 2 minutes and refiltered. The cells were then kneaded with 25 ml. of ether for 3 minutes, filtered as above, and spread on filter paper to dry. After drying thoroughly the zymin was ground to a powder with a pestle and mortar.

Amounts, varying between 1 - 3 grams of the zymin powder were placed in test tubes and 10 ml. of saline added to each. The contents were mixed into a paste with a glass rod, and the tubes placed in a boiling water bath for 45 minutes. At the end of this time the tubes were centrifuged at 2,560 r.p.m. for 20 minutes, and the supernatant decanted. To each tube 1 ml. of guinea pig serum was added with stirring, and the tubes were incubated in a 37°C. water bath for 2 hours. Following incubation 4 ml. of veronal NaCl buffer were added to each tube, and the tubes were again centrifuged at 2,560 r.p.m. for 20 minutes. The complement, diluted 1 : 5, was decanted and tested for inactivation (Table V).

It was interesting to note that whereas 1 gram of zymin inactivated 1 ml. of serum, as the amount of zymin increased above 1 gram the inactivation became less. The complement inactivated by 1 gram of zymin was then tested for anticomplementary properties (Table VI).

It is seen that complement so treated was not anticomplementary, and could therefore be used for reactivation purposes.

In all further preparations of fraction Z, one gram of zymin was used to inactivate one ml. of serum.

c. Titration of Complement Components.

Hegedus and Greiner (1938) defined the titer of complement as the titer of the component present in least amount. Any component can be present in least amount if an excess of the other three components is added to the serum.

To titrate the individual components of a serum serial

Table V.

Test for Inactivation of complement treated with zymin.

Tube.	Weight of Zymin in grams.	% Hemolysis.			
		Treated Complement. 1:30 dilution, in ml.			
		0.05	0.1	0.2	0.4
1	3.0	80	85	95	100
2	2.5	50	70	90	95
3	2.0	10	30	50	60
4	1.5	5	10	15	20
5	1.0	0	0	0	0

Table VI.

Test for Anticomplementary Properties of Fraction Z.

	Zymin treated Complement in ml.											
	0.4	0.4	0.4	0.4	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1
Guinea pig serum diluted 1:30 in ml.	0.5	0.75	1.0	1.5	0.5	0.75	1.0	1.5	0.5	0.75	1.0	1.5
% Hemolysis	100	100	100	100	100	100	100	100	100	100	100	100

dilutions are made as in an ordinary complement titration except that a constant amount of the other three components are added to each tube. This provides an excess of all components except the one to be tested. The added components are referred to as "reagents", and are made up as follows:

R1 -- midpiece plus complement heated at 56°C. Contains C'1, C'3 and C'4.

R2 -- endpiece plus complement heated at 56°C. Contains C'2, C'3 and C'4.

R3 -- zymin treated complement. Contains C'1, C'2 and C'4.

R4 -- hydrazine treated complement. Contains C'1, C'2 and C'3.

The amount of reagent to be employed must be determined in a preliminary experiment. The quantity used must be inactive when tested with sensitized sheep cells, and it must not be anticomplementary.

Upon the addition of R2 and R4 an enhancing effect is usually observed, since these reagents supply components which are present in low titer in human and guinea pig serum. R1 is the reagent which most commonly exhibits anticomplementary effects, especially when the midpiece, which is part of this reagent, is old and has been allowed to become too alkaline.

Each reagent should supply an excess of these components it is designed to furnish. A sequence of tests is carried out to determine how much of each fraction to employ in making up the reagents. The fractions are labelled as follows:

M -- midpiece.

E -- endpiece.

Z -- complement lacking C'3.

H -- complement lacking C'4.

56 -- complement lacking C'1 and C'2.

The tests employed and a typical set of results are seen below. All tests were incubated in a 37°C. water bath for 30 minutes, following which they were centrifuged for 5 minutes and read against a hemolytic standard.

Test No. 1. - Test for complete inactivation.

Each fraction, with the exception of fraction E, was diluted 1 : 5, and 0.05, 0.10, 0.20 and 0.40 ml. were added to 1 ml. of sensitized sheep red blood cells. Fraction E was diluted 1 : 10, and 0.1, 0.2, 0.4 and 0.8 ml. were added to sensitized cells.

The results (Table VII) indicated that each fraction was inactive by itself.

Test No. 2. - Test for anticomplementary activity.

The possibility of fractions M, E, Z, H and 56 possessing anticomplementary properties was checked by placing the largest quantity of each fraction which proved to be inactive in test 1, with previously tested guinea pig serum. The guinea pig serum was used in a dilution of 1 : 30, and 0.5, 0.75, 1.0 and 1.5 100 percent units were placed with each fraction (Table VIII). Not more than one half the least anticomplementary dose of a fraction was used in the following tests.

It can be seen from Table VIII that fractions E. Z, H and 56 were not anticomplementary in the amounts used here,

Table VII. Test for Inactivation of fractions.

	Fraction M. diluted 1:5				Fraction E. diluted 1:10				Fraction Z. diluted 1:5				Fraction H. diluted 1:5				Fraction 56. diluted 1:5			
	ml.				ml.				ml.				ml.				ml.			
	0.05	0.1	0.2	0.4	0.1	0.2	0.4	0.8	0.05	0.1	0.2	0.4	0.05	0.1	0.2	0.4	0.05	0.1	0.2	0.4
2.5% sensitized sheep red cells in ml.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
% hemolysis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table VIII. Test for Anticomplementary Properties.

	Fraction M. diluted 1:5				Fraction E. diluted 1:10				Fraction Z. diluted 1:5				Fraction H. diluted 1:5				Fraction 56. diluted 1:5			
	ml.				ml.				ml.				ml.				ml.			
	0.4	0.4	0.4	0.4	0.8	0.8	0.8	0.8	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Complement: dil. 1:30 in 100% units.	0.5	0.75	1.0	1.5	0.5	0.75	1.0	1.5	0.5	0.75	1.0	1.5	0.5	0.75	1.0	1.5	0.5	0.75	1.0	1.5
2.5% sensitized sheep red cells in ml.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
% hemolysis	0	0	0	0	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

and could therefore be tested further. Fraction M, however, was anticomplementary, and it remained to be seen whether this anti-complementary effect could be removed by using smaller quantities of this fraction (Table IX).

The anticomplementary properties of fraction M were greatly reduced when 0.1 ml. of a 1 : 5 dilution was used, so 0.05 ml. of this dilution was the amount used in further tests.

Table IX. Test for anticomplementary properties of fraction M.

	Fraction M. 1:5 dilution in ml.								
	0.4	0.4	0.4	0.2	0.2	0.2	0.1	0.1	0.1
Complement diluted 1 : 30, in 100% units.	1	2	3	1	2	3	1	2	3
% Hemolysis	0	0	0	30	80	90	60	90	100

Test No. 3. - To determine the lower limits of activity of fraction 56.

Fraction 56 was titrated against fraction Z and H. All fractions were used in a 1 : 5 dilution. 1 ml. of sensitized sheep red cells was then added to each combination and the tubes incubated (Table X).

It was seen that 0.1 ml. of 56 contained sufficient C'4 to cause complete hemolysis. 0.2 ml. of fraction 56 could be reactivated to cause 70 percent hemolysis, but greater reactivation could not be accomplished. From these titrations 0.2 ml. was

taken as the lower limit for this fraction when used in R1 and R2. It has been found best to keep M and 56 and E and 56 apart, and to add them separately to the titrations requiring these reagents.

Table X. The lower limits of activity of fraction 56.

Fraction	ml.	% hemolysis obtained from reactivation of fraction 56 with fraction							
		Z. diluted 1:5				H. diluted 1:5			
		ml.							
		0.1	0.2	0.3	0.4	0.1	0.2	0.3	0.4
56 diluted 1:5	0.1	30	40	60	60	100	100	100	100
	0.2	30	50	70	70	100	100	100	100
	0.3	30	50	70	70	100	100	100	100
	0.4	30	50	70	70	100	100	100	100

Test No. 4. To determine the lower limits for fractions Z and H as R3 and R4 respectively.

Fractions Z and H, used in a 1 : 5 dilution, were titrated against each other; 1 ml. of sensitized sheep red cells being added to each tube (Table XI).

0.2 ml. of 1 : 5 dilutions of fraction Z and H contained sufficient C'4 and C'3 respectively to cause complete hemolysis, and were therefore considered to be the lower limit for these two fractions.

Table XI. The lower limits of activity of fractions Z and H.

Fraction	ml.	% hemolysis obtained from reactivation of fraction			
		Z. diluted 1:5			
		ml.			
		0.1	0.2	0.3	0.4
H diluted 1:5	0.1	80	80	85	85
	0.2	90	100	100	100
	0.3	100	100	100	100
	0.4	100	100	100	100

Test No. 5. To determine the lower limits for fractions M and E.

Fractions M and E were titrated against one another in the presence of an optimal amount of fraction 56, as determined in test No. 3. Fraction M could not be used in quantities greater than 0.5 ml. of a 1 : 5 dilution (test No. 2). Fraction E was used in a 1 : 10 dilution. 1 ml. of sensitized sheep red cells was added to each tube (Table XII).

Throughout all fractionation and reactivation experiments, fraction M was anticomplementary in amounts exceeding 0.05 ml. of a 1 : 5 dilution. It can be seen from Table XII that when used in this amount fraction M did not contain sufficient C'1. For this reason R1 was never successful.

Table XII. The lower limits of fractions M and E.

Fraction	ml.	% hemolysis obtained from reactivation of fraction			
		E. diluted 1:10			
		ml.			
		0.2	0.4	0.6	0.8
M diluted 1:5	0.1	0	0	0	0
	0.2	0	0	0	0
	0.3	5	20	20	20
	0.4	10	40	40	40

Table XIII. The reactivation of R1 and R2 with fractions Z & H.

Fraction	ml.	% hemolysis obtained from reactivation of fractions							
		Z diluted 1:5				H diluted 1:5			
		ml.							
		0.1	0.2	0.3	0.4	0.1	0.2	0.3	0.4
M* diluted 1:5	0.05	0	0	10	20	60	60	60	60
E* diluted 1:5	0.2	10	15	20	25	100	100	100	100
	0.4	15	20	30	40	100	100	100	100
	0.6	30	40	45	50	100	100	100	100
	0.8	30	40	50	60	100	100	100	100

* 0.2 ml. of a 1:5 dilution of fraction 56 was added to each tube.

Test No. 6. To determine whether fractions Z and H contain sufficient C'1 and C'2.

Fractions Z and H were titrated against R'1 and R'2. 1 ml. of sensitized sheep red cells was added to each combination, and the tubes incubated (Table XIII).

0.1 ml. of fraction H contained sufficient C'1, but 0.4 ml. of this fraction caused only 60 percent reactivation of R'1. As fraction H contained sufficient C'1, the small quantity of this component present in 0.05 ml. of a 1 : 5 dilution of fraction M did not account for the poor reactivation. It was felt that fraction M was slightly anticomplementary in this test.

Fraction Z was only able to reactivate R'1 to 20 percent of its former activity. This may have been due partially to anti-complementary properties of fraction M, but it also shows that fraction Z, in the amounts used here, does not contain sufficient C'2. 0.4 ml. of a 1 : 5 dilution of fraction Z reactivated R'2 to 60 percent of its former activity. This reactivation should have been higher, and it was concluded that while fraction Z contained some C'2, 0.4 ml. of a 1 : 5 dilution of this fraction did not contain sufficient to warrant its use as a reagent.

The foregoing tests have shown that whereas fractions E, H and 56 could be used to make up reagents, fractions M and Z were not satisfactory. As has been mentioned, all preparations of fraction M were anticomplementary, and minor changes in the technique of preparing this fraction did not overcome this property. Fraction Z did not appear to be anticomplementary, but treatment with zymin not only removed C'3 but also damaged

C'1 and C'2.

d. Sulfonamide Determinations.

i. Dosage.

During sulfonamide therapy small animals usually receive 1½ grains per pound body weight the first day, and 1 grain per pound body weight for the following two days (Swales, 1950); or 97 milligrams per pound body weight the first day, and 65 milligrams per pound body weight for the following two days.

A suspension of sulfamethazine in water was made up so that 1 ml. contained 65 milligrams of the drug. As has been noted, sulfamethazine is not very soluble in water, and the administration of a suspension was not considered to be accurate enough. It was replaced with sodium sulfamethazine, which is readily soluble in water, and a solution of this drug containing 65 milligrams per ml. was used in all the in vitro experiments. The dosage varied in different experiments and will be discussed with these experiments.

ii. Administration.

All drugs were administered orally. The guinea pigs were held in an upright position with their heads back, and an 8 F rubber catheter was inserted down the oesophagus into the stomach. The animals objected so strenuously to this operation that they were in danger of asphyxiation, and after the tube was removed they were weak for several hours. As all guinea pigs were being dosed frequently there was some danger that several of them might die, and in addition it was thought that the weakened condition of the animals might give an additional unknown factor to the experimental

results. For these reasons the stomach tube was abandoned after the first week.

The use of an ordinary syringe proved to be a more successful method. The needle was removed from a 5 ml. glass syringe and the required dose drawn up. The tip of the syringe was placed at the back of the guinea pig's tongue, and the contents slowly ejected. The throat of the animal was gently stroked to aid swallowing, and as long as the dose was given slowly enough, so the guinea pig did not choke, this method was very successful.

iii. Sulfonamide levels in the blood.

The standard laboratory method for the determination of sulfonamides is that of Bratton and Marshall (1939). It involves the diazotization of the para-amino group of the sulfonamide followed by coupling with N-(1-naphthyl)-ehtylenediamine. A purplish colour is produced, the intensity of which is proportional to the sulfonamide concentration.

As the guinea pigs were divided into groups for the experiment, one half ml. of blood was taken from four animals in a group, and combined to give the required 2 ml. necessary for sulfonamide determinations. The sulfonamide level obtained was, therefore, representative of the group. The sulfonamide level was determined at the same time as the complement titer, so it was not necessary to bleed the animals more than once for the two determinations.

To a flask containing 30 ml. of distilled water, one half ml. of blood from each of the four guinea pigs was added. The flask was shaken gently to insure hemolysis.

8 ml. of 15 percent trichloracetic acid were added with shaking, and the flask stood at room temperature for 10 - 15 minutes. The blood was then in a 1 : 20 dilution. The contents were filtered through a No. 576 analytical filter paper.

10 ml. of clear filtrate was placed in an Evelyn colorimeter absorption tube.

1 ml. of sodium nitrate solution was added, mixed, and the tube stood for 3 minutes.

1 ml. of ammonium sulfamate solution was added, mixed, and the tube stood for 2 minutes.

1 ml. of N-(1-napthyl)-ethylenediamine dihydrochloride solution was added, and the tube was allowed to stand for 10 minutes to allow optimum colour development.

With each unknown a blank and a standard were prepared. The blank was used to set the galvanometer of the colorimeter at zero, and the standard to check that the reagents had not deteriorated.

Blank tube:

8 ml. of distilled water were placed in an Evelyn colorimeter absorption tube. 2 ml. of 15 percent trichloracetic acid were added. The reagents were added as above, with 1 ml. of distilled water substituted for 1 ml. of sodium nitrate solution.

Standard tube:

2 ml. of the standard 10 milligram percent sodium sulfamethazine were added to 30 ml. of distilled water. 8 ml. of trichloracetic acid solution were added and mixed well.

10 ml. of this mixture was placed in an Evelyn colorimeter absorption tube, and the reagents were added as for the test.

iv. Calibration of the Evelyn Colorimeter.

A stock solution of sodium sulfamethazine was prepared and from it dilute standards were made. A blank tube was placed in the colorimeter, and the galvanometer reading was adjusted to zero. Readings of the dilute standards were then taken and the results plotted on a piece of graph paper. The points were joined, and from the straight line curve which resulted, all unknowns could be determined. A wave length of 545 was used in the colorimeter for all sulfonamide readings.

B. EXPERIMENTS.

1. TO SEE WHAT EFFECT THE COMPONENTS PRESENT IN RABBIT HEMOLYSIN HAVE UPON COMPLEMENT TITRATIONS.

Apart from its lower hemolytic titer, rabbit complement behaves essentially like guinea pig complement. Heating at 56°C. for 30 minutes destroys all of C'1 and C'2, but does not greatly affect C'3 or C'4 (Dozois et al, 1949).

Before starting the experimental work with the sulfonamides it was decided to check on the possibility of C'3 and C'4 in rabbit hemolysin supplementing these components in guinea pig serum, and masking slight changes that might occur.

a. Removal of C'3 and C'4 from hemolysin.

It is known that in hemolysis C'3 is not fixed to the sensitized red blood cell, but acts like a catalyst, and as such is not used up in the reaction (Nathan, 1913; Pillemer, et al, 1942a). It was suggested that this component could be eliminated by washing the red blood cells, after they had been sensitized with

the appropriate dilution of hemolysin. Treatment of the hemolysin with hydrazine was the method used to remove any C'4 that was present.

Method:

Antisheep hemolysin was prepared in a rabbit, and heated at 56°C. for 30 minutes. 1 ml. of the hemolysin was placed in a test tube and to it was added 0.5 ml. of an 0.16 M solution of hydrazine. The contents of the tube were mixed and placed in a 37°C. water bath for 1 hour. A titration of the treated hemolysin showed that the hydrazine had in no way affected its hemolytic titer.

The treated hemolysin was diluted so that 1 ml. contained 5 hemolytic units. 16 ml. of the diluted amboceptor were added to an equal volume of 5 percent sheep red cells, and the mixture was stood at room temperature for 15 minutes.

A similar aliquot of cells was sensitized with untreated hemolysin, diluted so that 1 ml. of the hemolysin contained 5 units. Both lots of sensitized cells were then centrifuged, the supernatant decanted, washed with veronal NaCl buffer, centrifuged, the supernatant decanted and the cells resuspended in the buffer solution to give a 2.5 percent suspension.

As a control, another 16 ml. of a 5 percent suspension of sheep red blood cells were sensitized with untreated hemolysin, but were not washed after sensitization.

A 20 tube titration of guinea pig complement was set up in triplicate. To the first titration the control cells were added; to the second the cells sensitized with untreated hemolysin and then washed; and to the third tube were added the cells

which had been sensitized with hydrazine treated hemolysin (Table XIV).

Discussion of Results:

In guinea pig complement C'3 is the component present in least amount, the titer of the complement is therefore a direct measurement of the titer of C'3. If there was enough C'3 present in the hemolysin to supplement this component in the guinea pig serum, the titer of the complement should have increased in the titration where the sensitized cells were not washed. This was not the case; the complement titer was essentially the same in all titrations where the three different cell preparations were used.

C'4 is present in excess in guinea pig serum. The titration of guinea pig complement does not indicate the amount of this component present, so that if there was enough C'4 in the hemolysin to supplement this component it would not be shown in this experiment.

b. The addition of complement, equal to the amount of hemolysin used in each tube.

When hemolysin with a titer greater than 1:4000 was used, the amount added to each tube of a complement titration was so minute that it did not seem possible for any components present in the hemolysin to supplement those in the complement. To test this point an additional amount of guinea pig serum was added to each tube in a complement titration - equal to the amount of hemolysin found in a tube in an ordinary complement titration. Guinea pig complement has a higher titer than rabbit complement,

Table XIV. Percent hemolysis obtained in complement titrations when hydrazine treated hemolysin was used and the sensitized red blood cells were washed.

Tube	Complement dil. 1:30 (ml.)	Buffer (ml.)	Untreated hemolysin. Sensitized cells not washed.	Untreated hemolysin. Sensitized cells washed.	Hydrazine treated hemolysin. Sensitized cells washed.
1.	0.01	0.19	0	0	0
2.	0.02	0.18	0	0	0
3.	0.03	0.17	0	0	0
4.	0.04	0.16	20	20	10
5.	0.05	0.15	25	20	20
6.	0.06	0.14	30	30	30
7.	0.07	0.13	35	45	35
8.	0.08	0.12	50	50	50
9.	0.09	0.11	55	60	55
10.	0.10	0.10	70	65	65
11.	0.11	0.09	70	70	70
12.	0.12	0.08	75	75	70
13.	0.13	0.07	85	90	80
14.	0.14	0.06	90	90	95
15.	0.15	0.05	95	95	95
16.	0.16	0.04	95	100	95
17.	0.17	0.03	100	100	100
18.	0.18	0.02	100	100	100
19.	0.19	0.01	100	100	100
20.	0.20	0	100	100	100
21.	0	0.20	0	0	0

so that if the addition of guinea pig serum did not cause an increase in hemolysis, the components present in rabbit hemolysin could not be expected to do so.

Method:

The hemolysin used had a titer of 1:8000. In order to obtain the hemolysin in a dilution containing 5 hemolytic units per ml., 0.01 ml. of the hemolysin was diluted to 16 ml. with veronal NaCl buffer. The guinea pig complement was diluted likewise: 0.01 ml. made up to 16 ml. with veronal NaCl buffer.

In all complement titrations each tube receives 0.5 ml. of diluted hemolysin; an equal quantity of red blood cells and hemolysin are mixed, and each tube receives 1 ml. of the mixture.

A 20 tube complement titration was set up in triplicate; the complement was used in a dilution of 1:30.

To the first titration 1 ml. of 2.5 percent sensitized red blood cells were added, and this titration was used as a control.

To the second titration an additional 0.5 ml. of the diluted complement was added to each tube, the tubes rotated and 1 ml. of 2.5 percent sensitized red blood cells added.

To the third titration additional complement was also added. To the hemolysin, diluted so that 1 ml. contained 5 units, 0.01 ml. of guinea pig complement was added. The hemolysin-complement mixture was used to sensitize an equal amount of red blood cells. 1 ml. of the resulting 2.5 percent suspension of sensitized cells was added to each tube.

With each titration a control tube was run, to see that

the sensitized red blood cells did not hemolyse on their own.

Table XV.

The Effect of Additional Complement, Equal to the Amount of Hemolysin Used, on Complement Titers.

	Total volume per tube in ml.	Initial hemolytic units	50% hemolytic units	100% hemolytic units	Cell control tube % hemolysis.
Titration 1. control	1.2	1500	375	166	0
Titration 2. 0.5 ml. dil. complement added to each tube.	1.7	750	333	150	0
Titration 3. Additional complement present in sensitized cell mixture.	1.2	1000	428	176	0

Discussion of results:

From Table XV it can be seen that the addition of 0.5 ml. of the diluted guinea pig complement to each tube caused a reduction in the complement titer. This reduction was probably due to the larger total volume per tube in this titration, causing a greater dilution of the complement. Where the additional complement was added with the sensitized cells (titration 3), there was a slight decrease seen in the initial hemolytic units, but a slight increase in the 50 and 100 percent units.

From these results it cannot be said that the addition of complement, in amounts equal to that of hemolysin, increased the guinea pig complement titer. Therefore one could not expect any components present in rabbit hemolysin to mask changes that might occur in these components in guinea pig serum. The minute quantities of hemolysin that were used in complement titrations did not contain sufficient C'3 or C'4 to supplement these components in the complement titrated.

2. IN VITRO STUDIES

Before starting in vivo experiments it was thought that an indication of what may happen in the intact animal could be arrived at by an investigation into the effect of the sulfonamide drugs on complement in the test tube. The complement used for these experiments was the pooled sera from normal healthy guinea pigs.

a. The Effect of Sodium Sulfamethazine.

As sodium sulfamethazine was the drug to be used in in vivo experiments, the effect of this drug on one and two units of complement was first investigated. A unit of complement was taken as the smallest amount necessary to cause complete hemolysis of 1 ml. of 2.5 percent sensitized sheep red blood cells.

Method:

A portion of the pooled complement was titrated, by the method previously described, to determine the amount necessary to cause complete hemolysis.

A series of 10 tubes was set up in duplicate, containing

sulfamethazine in amounts varying from 0.25 to 20.0 milligrams. To each tube in the first series one 100 percent unit was added, while to the tubes in the second series two 100 percent units were added. The complement and sulfamethazine were mixed with a glass stirring rod, and 1 ml. of 2.5 percent sensitized red blood cells were added to each tube. The tubes were well shaken, and placed in a 37°C. water bath for 30 minutes.

With each series two control tubes were set up; the first containing complement and sensitized red blood cells, and the second containing sulfamethazine and sensitized red blood cells.

Table XVI. Percent hemolysis obtained from one and two 100 percent units of complement following treatment with sulfamethazine.

	% hemolysis after treatment with the following amounts of sulfamethazine.											
	milligrams											
	¼	½	1	2	4	6	8	10	15	20	C 0	C 20
Two 100 percent units of complement.	100	100	100	100	100	100	100	100	100	100	100	0
One 100 percent unit of complement.	100	100	90	90	90	75	70	50	20	0	100	0

Discussion of Results:

The results can be seen from Table XVI. When two 100 percent units of complement were added to the sulfamethazine complete hemolysis was seen in all tubes. However when only one

100 percent unit was used there was a decrease in hemolysis as the quantity of the drug increased.

This experiment does not show whether the sulfonamide inactivated the complement in any way, or whether the decrease in hemolysis was due to a mechanical blocking by the drug, so that the complement was not able to come in contact with the sensitized red cells enough to cause complete hemolysis.

b. Treatment of complement with various sulfonamides.

In the last experiment the sulfamethazine was added to the complement and sensitized sheep red blood cells. In order to see if these drugs had an inhibiting effect on the complement itself, the complement must be treated with the sulfonamide prior to the addition of the sensitized cells.

Method:

Varying amounts of sulfamethazine, sodium sulfamethazine, sulfadiazine, sulfapyridine and sulfasoxizole were weighed out and placed in test tubes.

A portion of the pooled complement to be used in this experiment was titrated, by the method previously described. The amounts necessary to cause 50 and 100 percent hemolysis were noted.

The pooled sera was diluted 1:10, and 3.0 ml. of this diluted complement added to each tube of sulfonamide. The tubes were well shaken, the contents stirred with a glass rod where necessary, and were then placed in a 37°C. water bath for one hour, with shaking every 10 minutes. Following incubation the tubes were centrifuged at 2,560 r.p.m. for 20 minutes, and the

supernate filtered through hardened filter paper. The pH of the filtrate was adjusted to 7.0 with 0.1 N hydrochloric acid. The filtrate, containing a 1:10 dilution of complement, was further diluted with veronal NaCl buffer to give a final dilution of 1:30.

As a control, 3 ml. of a 1:10 dilution of complement from the same pool was incubated, and filtered through hardened filter paper in the same manner as the test complement, except that in the control no sulfonamide was added. The control was to show if any deterioration of complement activity was caused by the incubation and filtering procedures.

In the untreated sera 0.08 ml. of a 1:30 dilution caused 50 percent hemolysis, and 0.30 ml. caused 100 percent hemolysis. The power of 0.08, 0.30 and 0.60 ml. of a 1:30 dilution of the treated complements to lyse 1 ml. of sensitized red blood cells was tested, and compared with the untreated complement. In the same manner 0.08, 0.30 and 0.60 ml. of the diluted control complement was added to 1 ml. of a 2.5 percent suspension of sensitized sheep red blood cells.

Table XVII shows the results of this experiment.

Discussion of results:

Complement treated with large quantities of sulfonamides lost some of its hemolytic power in vitro. The larger the amount of the drug, the greater seemed to be the inactivation.

The hydrogen ion concentration caused by the drug must be considered. The sulfonamides in solution are very alkaline, and those drugs which are most soluble, sodium sulfamethazine

and sulfasoxizole, have a high enough pH to inactivate the mid-piece of complement. The low hemolytic power of complement treated with 500 milligrams of sulfasoxizole was probably due to the high pH.

Sodium sulfamethazine completely dissolved in the complement, and the pH of the solution was between 10.0 and 10.5. The quantity of 0.1 N of hydrochloric acid required to bring the pH to 7.0 was so great that it caused a precipitation of the proteins when the sensitized cells were added.

There is no reason to suppose that complement is affected in vivo because large amounts of sulfomamides reduce complement activity in vitro. Following a single dose of one half grain per pound, the blood level in rabbits varies between 3.4 and 0.8 milligrams percent, depending on the sulfonamide used (Welsh et al, 1947). In guinea pigs, following a single dose of one and a half grains per pound, levels as high as 12-14 milligrams percent have been reached in these experiments. A blood level of 14 milligrams percent, however, is very different from 3 ml. of a 1:10 dilution of complement being treated with 50 to 500 milligrams of the drug. The latter is far in excess of the amount of sulfonamide ever found in a guinea pig.

3. IN VIVO EXPERIMENTS.

The animals used for the in vivo experiments were healthy guinea pigs of both sexes, all of which weighed over 700 grams. The experiments were planned to investigate the stability of the complement within the animals, under different conditions of

Table XVII. The effect of varying amounts of sulfonamides on guinea pig complement in vitro.

Sulfonamide.	Drug in milligrams.	pH after treatment.	% hemolysis caused by complement diluted 1:30		
			ml.		
			0.08	0.30	0.60
Original untreated complement.	0	-	50	100	100
Sulfamethazine.	500	8,2	15	90	95
	300	8.1	25	90	100
	200	8.0	35	95	100
	100	8.0	35	95	100
	50	8.0	50	95	100
Sodium sulfamethazine	500	10.5	Precipitation of red blood cell proteins.		
	300	10.5			
	200	10.2			
	100	10.0			
	50	10.0			
Sulfadiazine.	500	8.2	15	90	100
	300	8.2	25	95	100
	200	8.0	25	95	100
	100	8.0	30	95	100
	50	8.0	35	95	100
Sulfapyridine.	500	7.5	15	90	100
	300	7.5	20	90	100
	200	7.4	25	95	100
	100	7.4	35	95	100
	50	7.3	35	100	100
Sulfasoxizole.	500	9.5	5	30	100
	300	9.0	20	90	100
	200	8.5	25	95	100
	100	8.5	35	95	100
	50	8.5	35	95	100
Control complement.	0	7.1	50	100	100

sulfonamide therapy.

a. The effect of normal doses of sodium sulfamethazine on guinea pig complement.

The purpose of this experiment was to see whether normal doses of sodium sulfamethazine had any effect on complement in the intact guinea pig. As many guinea pigs as possible were used in order that a fair sample would be obtained, and so that the animals would not have to be bled too frequently.

Method:

48 guinea pigs were divided into 8 groups of 6, four of which were test groups, and four controls.

Three weeks before the first administration of the sulfonamide all test and control groups were bled. A portion of the serum from each group was titrated for complement activity, establishing a base line titration for each group, against which further titrations could be compared.

Sera from the test groups, I, II, III, IV, were placed in 0.5 ml. glass ampoules, which were sealed and stored at -25°C. These sera, collected prior to treatment, were used as controls for their respective groups.

Sodium sulfamethazine was administered orally to the test groups, while the control groups, V, VI, VII and VIII received no drugs. The doses used were those suggested by Swales (1951); 220 milligrams/kilogram body weight the first day, followed by 150 milligrams/kilogram body weight the second and third days. This schedule was repeated seven times during the experiment.

The guinea pigs were bled from the heart. 0.5 ml. of

blood from four guinea pigs in each test group was added to distilled water, for sulfonamide level determinations. The remainder of the blood was allowed to clot for one hour at room temperature, centrifuged, and the serum drawn off. The sera collected from the guinea pigs in a group were pooled, and what was not used immediately was sealed in glass ampoules and stored at -25°C.

The four groups receiving the drug were dosed throughout the experiment, but were rotated for bleeding. This prevented any group from being bled often enough to cause changes in the complement due to overbleeding.

BLEEDING AND DOSING SCHEDULE FOR TEST AND CONTROL GROUPS

Date.	Groups bled.	Sodium sulfamethazine fed to test groups in mg./kg. body weight.
Nov. 8	All groups	
Dec. 3		220
Dec. 4		150
Dec. 5		150
Dec. 6	Groups I, V.	150
Dec. 8		150
Dec. 10	Groups II, VI.	
Dec. 14	Groups III, VII.	
Dec. 17		220
Dec. 18		150
Dec. 19	Groups IV, VIII.	150

Date.	Groups bled.	Sodium sulfamethazine fed to test groups in mg./kg. body weight.
Dec. 27	Groups I, V.	
Jan. 7		220
Jan. 8		150
Jan. 9		150
Jan. 11	Groups II, VI.	
Jan. 14		220
Jan. 15		150
Jan. 16		150
Jan. 18	Groups IV, VIII.	
Jan. 21		220
Jan. 22		150
Jan. 23		150
Jan. 25	Groups I, V.	
Jan. 28		220
Jan. 29		150
Jan. 30		150
Feb. 1	Groups II, VI.	
Feb. 4		220
Feb. 5		150
Feb. 6		150
Feb. 8	Groups IV, VIII.	

Following each bleeding the sulfonamide level in the blood was determined by the method previously described.

The sera from the test groups were titrated by the method described under "Methods". The quantity of serum required for initial and 50 percent hemolysis of the standard quantity of maximally sensitized sheep red cells was noted. With each test titration two control titrations were run. The first was the titration of the complement from the control group; any variations in complement levels due to seasonal fluctuations, infections, etc., were controlled in this way. The second control was a titration of the complement from the test group, which had been collected prior to the administration of the drug and stored at -25°C.

Discussion of results:

It was found that there was very little change in the complement titers of the guinea pigs undergoing sulfonamide therapy. The results summarized in tables XVIII, and XIX, showed that the number of initial hemolytic units per ml. of serum remained practically unchanged over the two month period. There was a little more variation in the number of 50 percent hemolytic units per ml. of serum, but not enough to suspect any inhibition of complement activity.

Table XVIII shows that on Jan. 25 there appeared to be a slight diminution in the complement titer of the test group. In Table XIX, however, it is seen that the control group and the frozen serum control titers were also low on this date.

There was no evidence in these results to suggest any connection between the complement titers and the sulfonamide blood levels.

Table XVIII. The complement titers of guinea pigs receiving normal doses of sodium sulfamethazine.

Date of bleeding.	Number of guinea pigs bled.	Sulfur level in mg. %.	Hemolytic units/ml. serum.	
			Initial	50%
Dec. 6	6	1	1000	375
Dec. 10	5	10	1000	375
Dec. 14	5	0	1000	500
Dec. 19	5	14	750	375
Dec. 27	6	0	750	428
Jan. 11	5	0	1000	428
Jan. 18	4	-	1000	428
Jan. 25	6	0	750	333
Feb. 1	4	0	1000	428
Feb. 8	3	0	1000	428

Table XIX. The complement titers of the individual groups.

Groups.	Date.	Test serum		Control serum		Frozen serum control	
		Hemolytic units per ml. serum.					
		Init.	50%	Init.	50%	Init.	50%
Test group I.	Nov. 8 *	1000	428	1000	428	-	-
	Dec. 6	1000	375	1500	750	600	272
Control group V.	Dec.27	750	428	1000	428	1000	428
	Jan.25	750	333	750	428	750	200

Table XIX. continued.

Groups.	Date.	Test serum		Control serum		Frozen serum control	
		Hemolytic units per ml. serum.					
		Init.	50%	Init.	50%	Init.	50%
Test group II.	Nov. 8	750	428	1000	428	-	-
	Dec.10	1000	375	1000	375	1000	250
Control group VI.	Jan.11	1000	428	750	333	750	333
	Feb. 1	1000	428	750	375	750	250
Test group III.**	Nov. 8	1000	500	1000	428	-	-
	Dec.14	1000	500	1000	428	1000	500
Control group VII.							
Test group IV.***	Nov. 8	1500	428	1000	428	-	-
	Dec.19	750	375	1500	750	750	272
Control group VIII.	Jan.18	1000	428	1000	333	1000	375
	Feb. 8	1000	428	1500	600	750	250

* Nov. 8 base line titrations determined prior to sulfonamide therapy.

** Group III was discontinued after January 14.

*** It was very difficult to determine the number of 50 percent hemolytic units per ml. of serum in group IV.

When the serum from group IV was titrated on Nov. 8, prior to the administration of sodium sulfamethazine, the titration gave a normal "S" shaped curve when plotted on graph paper.

Since Nov. 8, Group IV was bled three times during the experiment, and each time gave a jagged inverted curve (graphII). The first time this curve was seen, on December 18, it was thought to be due to inaccurate pipetting. The complement was again titrated, and the curve obtained proved to be identical with the former inverted one. On Jan. 18 and Feb. 8 jagged inverted curves were again found.

The last dose of sodium sulfamethazine was given on Feb. 6, and on Feb. 28 group IV was bled and the complement titrated. The resulting curve resembled the inverted curves previously obtained.

It was suggested that the serum of one of the guinea pigs of the group might be responsible for the peculiar curve obtained from the pooled sera. The three remaining guinea pigs in group IV were separated, and bled. Two 20 tube titrations were set up for the serum of each guinea pig. It was thought that by running two titrations on each serum, any inaccuracy in pipetting would be shown. 1 ml. of serum from each guinea pig was placed in a glass ampoule, sealed, and stored at -25°C. A control titration was run on previously tested complement.

Two of the guinea pigs had low complement levels, with normal "S" shaped curves. Both titrations of the serum from the third guinea pig gave abnormal jagged curves (graph III).

The following day, the frozen serum from the guinea pig giving the abnormal curve was titrated in duplicate. Both titrations gave identical normal "S" shaped curves. The guinea pig was bled again, and the fresh serum was titrated in duplicate.

Not only were the resulting curves identical, they were also identical with the "S" shaped curve obtained from the frozen serum.

Why the sera from group IV gave abnormal curves was never discovered. It may have been inaccurate pipetting, but this seems doubtful as duplicate titrations always gave the same results. Dirty test tubes were another possibility. This does not seem probable, however, because only the first 8 tubes in the group IV titrations were ever affected. All other groups gave perfectly normal curves, as did larger quantities of group IV serum. If dirty tubes were causing the irregular curves, one would expect to find them scattered through the other group titrations also.

b. The effect of excess doses of sodium sulfamethazine and frequent bleeding on guinea pig complement.

As normal doses of sodium sulfamethazine appeared to have no effect on the complement titer the effect of large doses was investigated.

If the drug were in any way to inhibit the manufacture of one or more of the components of complement, it would not be until the available store of this component within the animal had been used up that one would expect to find a reduction in titer. The guinea pigs were bled frequently, therefore, to make visible any interference with the production of components that might occur.

Method:

16 guinea pigs were divided into 4 groups, with 4 animals in each. Groups I and II were test groups and the other two

were controls.

Sodium sulfamethazine was dissolved in distilled water so that 2.5 ml. of the solution contained 330 milligrams. Groups I and II received this dose 5 times a week for 4 weeks.

Group I and its control group were bled every 3 to 4 days, while group II and control were bled once a week. Complement titrations and sulfonamide blood level determinations were carried out after each bleeding by the methods previously described.

BLEEDING AND DOSING SCHEDULE.

Date.	Test group and control bled.	Sodium sulfamethazine administered to test groups in mg./kg. body weight.
Feb. 11		330
Feb. 12	II	330
Feb. 13		330
Feb. 14	I	330
Feb. 15		330
Feb. 18	I	330
Feb. 19	II	330
Feb. 20		330
Feb. 21	I	330
Feb. 22		330
Feb. 25	I	330
Feb. 26	II	330
Feb. 27		330
Feb. 28		330
Feb. 29	I	330
Mar. 3		330
Mar. 4	II	330
Mar. 5	I	330

Discussion of results:

The results are summarized in Tables XX and XXI.

There was very little change in the number of initial and 50 percent hemolytic units per ml. of complement in group I over a three and a half week period, during which time the guinea pigs were constantly receiving sodium sulfamethazine and were bled six times.

Group II, which were only bled once a week, showed a constant number of initial hemolytic units for the first three weeks, but in the fourth week a slight diminution was seen. The titer of this group, measured by 50 percent hemolytic units, decreased steadily from 500 to 300 units per ml.

The average titer, measured in 50 percent hemolytic units, of the guinea pigs used in this laboratory, was between 428 and 375 units per ml. In the first week group II was therefore well above average, while in the second week it possessed an average titer. In the last two weeks, however, the complement level fell below average. It can be seen in Table XXI that the control group was also slightly below average. Only in the fourth week was the titer of the test group lower than the titer of the control group.

Both tables show that no correlation existed between the actual sulfonamide blood levels and the complement titers.

c. The effect of sublethal infections of D. pneumoniae on guinea pig complement.

The few studies which have been published regarding complement activity in pneumonia present conflicting conclusions.

Table XX. The complement titers of guinea pigs receiving excess doses of sodium sulfamethazine. Group I.

Date.	Sulfonamide level in milligrams percent.	Test group.		Control group.	
		Units per ml. of serum.			
		Initial	50%	Initial	50%
Feb. 14	10	1000	375	1000	375
Feb. 18	9	1500	333	1500	428
Feb. 21	13	1500	333	1500	375
Feb. 25	13	1000	333	750	375
Feb. 29	-	1500	333	750	375
Mar. 5	11	1000	333	750	333

Table XXI. The complement titers of guinea pigs receiving excess doses of sodium sulfamethazine. Group II.

Date.	Sulfonamide level in milligrams percent.	Test group.		Control group.	
		Units per ml. of serum.			
		Initial	50%	Initial	50%
Feb. 12	14	1000	500	1000	375
Feb. 19	9	1000	375	600	250
Feb. 26	13	1000	333	1000	333
Mar. 4	14	750	300	1000	333

Dick (1912) reported that in cases of pneumonia, complement was low before crisis and high on the second or third day following crisis. Rutstein and Walker (1942) noted that 16.9 percent of their pneumonia cases had low complement levels on admission to hospital, and all of these titers were normal following recovery. On the other hand, Ecker, Seifter, Dozois and Barr (1946), reported that two cases of pneumococcus Type III meningitis exhibited extraordinarily high complement levels in the days preceding death.

In this experiment complement levels of guinea pigs infected with D. pneumoniae were compared with those of similarly infected animals which were receiving sulfonamide therapy.

Method:

A virulent strain of D. pneumoniae, Type I, was used. Strain No. 710 is a stock strain in this laboratory, and its virulence for guinea pigs was tested by Nunes in 1949. Hereafter it will be referred to as S-710.

S-710 was transferred from a lyophilizing tube to "L.S." broth, and from this suspension blood-agar plates were inoculated. After 18 hours incubation at 37°C. the plates were covered with smooth colonies which were surrounded by areas of alpha hemolysis. Gram stains showed typical gram positive cocci, in pairs, surrounded by a definite capsule. Bile solubility tests were positive, and capsular swelling and agglutination were seen when the organisms were typed with Type I antipneumococcus rabbit serum.

Before injection into the animals the organism was

transferred to "pneumo broth" and incubated for 18 hours. One ml. of this culture was diluted in 9 ml. of peptone broth, and was twirled between the hands to insure good mixing. 1 ml. was then removed and placed in another 9 ml. of peptone broth, with shaking, resulting in a 10^{-2} dilution. To infect the guinea pigs, 1 ml. of the 10^{-2} dilution of the pneumococcus was injected intraperitoneally.

It has been demonstrated by Besredka (1899) and Murray (1924) that in the presence of carmine, when given either at the time of injection, or previous to the injection of living meningococci intraperitoneally, the apparent virulence of the organism is markedly raised. Following the method of Nunes (1949), a stock solution of carmine in physiological saline was prepared, so that 1 ml. of the solution contained 100 milligrams. Immediately prior to the injection of the pneumococci 1 ml. of the carmine solution was injected intraperitoneally.

A solution of sodium sulfamethazine was prepared so that 1 ml. contained 150 milligrams.

14 healthy guinea pigs, all weighing between 800 and 1100 grams, were divided into 5 groups as follows:

Group I, 3 guinea pigs; experiment control.

Group II, 2 guinea pigs; carmine control.

Group III, 2 guinea pigs; sulfonamide control.

Group IV, 3 guinea pigs; infected with pneumococci and treated with sodium sulfamethazine.

Group V, 4 guinea pigs; infected with pneumococci.

Complement titrations and sulfonamide blood level deter-

minations were carried out as previously described. Following each bleeding, sera from each group were placed in glass ampoules which were labelled and stored at -25°C.

Complement reagents were prepared and tested as previously described, and the titers of the individual components were determined in groups IV and V.

Schedule of procedures.

Date.	Time.	Group.	Treatment.
Mar. 11	9:30 A.M.	I	None
		II	1 ml. carmine solution intraperitoneally.
		III	1½ ml. sodium sulfamethazine orally.
		IV	1½ ml. sodium sulfamethazine orally. 1 ml. carmine solution intraperitoneally. 1 ml. 10^{-2} dilution of pneumococci intraperitoneally.
	1:30 P.M.	I,II,III, IV,V.	Bled. Complement titers and sulfonamide levels determined.
Mar. 12	9:30 A.M.	III & IV.	1½ ml. sodium sulfamethazine orally.
		I,II,III, IV,V.	Bled. Complement titers and sulfonamide levels determined.
Mar. 13	9:30 A.M.	III & IV.	1½ ml. sodium sulfamethazine orally.
Mar. 14	9:30 A.M.	I,II,III, IV,V.	Bled. Complement titers and sulfonamide levels determined.
Mar. 25	9:30 A.M.	I,II,III, IV,V.	Bled. Complement titers and sulfonamide levels determined.

Discussion of results:

The results are summarized in Tables XXII and XXIII.

The guinea pigs experimentally infected with D. pneumoniae showed a definite reduction in their complement titers.

The infected guinea pigs which received sulfonamide therapy showed a reduced complement titer three days after the infection started. Two weeks after the date of infection the complement titer of this group was close to the original titer, when measured by 50 percent units, but the number of initial hemolytic units was still low. Unfortunately the sera showing the reduced titer had deteriorated during storage, due to a crack in the glass ampoule. The titers for the individual components on this date were not listed in Table XXIII because of the deterioration. The titers of the individual components on March 25 were similar to the base line titrations of this group.

Two weeks after the date of infection the guinea pigs which did not receive sodium sulfamethazine showed a complement level one half that of the baseline level. The number of initial hemolytic units was only one third of the number found in the original titer. The 50 percent hemolytic units were thought to give a better indication of the complement levels in this experiment, because all the titers in the control and test groups were low when measured by initial hemolytic units.

The titers of the individual components were measured in 50 percent hemolytic units per ml. of serum. When the complement level of group V was diminished by one half the titer of C'2 was also reduced by one half, and 40 percent of C'4 activity was lost. The titers of C'1 and C'3 remained unchanged.

Table XXII. The complement titers of guinea pigs experimentally infected with D. pneumoniae, Type I, Strain 710.

Date.	Group number and treatment.									
	I. Experiment control.		II. Carmine control. 1 ml. carmine intraperitoneally.		III. Sulfonamide control. 220 mg. sodium sulfamethazine orally.		IV. 220 mg. sulfamethazine orally. 1 ml. carmine intraperitoneally. 1 ml. 10^{-2} dil. pneumococcus intraperitoneally.		V. 1 ml. carmine intraperitoneally. 1 ml. 10^{-2} dil. pneumococcus intraperitoneally.	
	Hemolytic units per ml. of complement.									
	Initial.	50%.	Initial.	50%.	Initial.	50%.	Initial.	50%.	Initial.	50%.
Mar. 3*	750	250	1000	333	1000	230	1000	272	1000	333
Mar. 11	1000	230	600	230	600	230	1000	250	750	272
Mar. 12	600	200	1000	272	600	230	1000	272	1000	300
Mar. 14	1000	200	1000	230	1000	230	428	150	750	272
Mar. 25	750	272	500	272	500	272	500	266	333	170

* Base line titration, prior to infection.

Table XXIII. The titers** of the individual components of guinea pig complement before and after infection.

Date.	Group IV.					Group V.				
	C'.	C'1.	C'2.	C'3.	C'4.	C'.	C'1.	C'2.	C'3.	C'4.
Mar. 3***	272	1600	272	800	1333	333	2000	300	500	1333
Mar. 25	266	1600	266	800	1333	170	2000	145	500	800

** Measured in 50 percent hemolytic units.

*** Base line titration.

In both groups IV and V, C'2 was the component present in the least amount before and after infection. As has been mentioned, R'1, the complement reagent used to determine the titer of C'2, was never very satisfactory, and the low C'2 titers obtained may be due to the poor reagent. The drop in titer of C'2 in group V, however, could not have been caused by the reagent, as the same R'1 was used for all C'2 titrations.

The control groups showed large variations in their complement titers, some of which were markedly low. Only those titers, therefore, which were significantly lower than the controls were considered to be indicative of complement inhibition.

DISCUSSION.

Evidence has been presented showing that normal and excess doses of sodium sulfamethazine have no effect on guinea pig complement in vivo. Sodium sulfamethazine was the sulfonamide used in these experiments, because of the popularity of sulfamethazine in the treatment of infections in small animals.

In vitro experiments have shown that large quantities of the sulfonamide drugs partially inhibit complement activity. Several different sulfonamides were tested. The drugs which are most soluble in aqueous solutions showed the greatest inhibitory action, and the more soluble the drug, the higher was the pH of the complement sulfonamide mixture. Pillemer and Ecker (1941b) have shown that complement functions best at a neutral pH, and deviations on either side gradually inactivate it. A pH of above 9.5 completely and irreversibly inactivates complement, while a pH of 7.5 to 9.5 destroys the midpiece. It is difficult to tell in this case how much of the inactivation was due to the high pH, and how much to the sulfonamide itself.

A group of guinea pigs experimentally infected with Diplococcus pneumoniae, and receiving sulfonamide therapy, showed a 45 percent reduction of complement activity three days after the date of infection. Two weeks after the start of infection the complement level of this group was practically as high as the original titer. A control group of similarly infected guinea pigs, receiving no sulfonamides, showed a 50 percent reduction in complement activity two weeks after the

start of infection. At the time of the reduction in titer, C'2 possessed only 50 percent of its original activity, while 40 percent of C'4 activity was lost.

If the low hemolytic activity of the complement, encountered in this experiment, was due to the employment of the complement in bactericidal activity, it would explain the difference in time intervals between the date of infection and the reduction in complement titer seen in the two groups. The sodium sulfamethazine would help overcome the organisms, allowing the antibodies and complement to overpower the infection in a relatively short period. One would expect, therefore, a drop in complement titer soon after the infection started in the guinea pigs receiving sulfonamide therapy. The infected guinea pigs receiving no therapy had only the natural body defences, ie. antibodies and complement, and a longer period would ensue before the organisms were killed.

Among the earliest workers who studied the role of complement in bactericidal action of blood and serum were Liefmann and Stutzer (1910). They split guinea pig serum into midpiece and endpiece, and noted that in the presence of rabbit antiserum only the endpiece was required for bacteriolysis, whereas both fractions were needed for hemolysis. Dozois, Seifter and Ecker (1943) showed, however, that the bactericidal action of complement was destroyed by the inactivation of any one of the four components. They reported that the bactericidal action of human complement against V. comma was fortified by the addition of endpiece.

In the guinea pigs infected with D. pneumoniae the drop in the hemolytic activity of the complement appeared to be due to a reduction in the activity of C'2 and C'4, the components, which make up the endpiece of complement.

It is of interest to note that during the period of infection the only symptom that was noted among the infected guinea pigs was the reduction in complement activity. The animals remained healthy to all outward appearances, they ate and drank the normal amount and no weakness developed. None of the guinea pigs died during this period, nor in the two weeks following the experiment.

SUMMARY.

1. The presence of C'3 and C'4 in rabbit hemolysin in no way affected the titration of guinea pig complement.

2. Large quantities of sulfamethazine, sulfapyridine, sulfadiazine and sulfasoxizole partially inhibited the hemolytic activity of guinea pig complement in vitro.

3. The inhibition of complement activity was directly proportional to the increase in pH caused by the sulfonamides.

4. Normal and excess doses of sodium sulfamethazine had no effect on guinea pig complement in vivo.

5. Guinea pigs experimentally infected with D. pneumoniae, Type I, Strain 710, showed a 50 percent reduction in complement activity two weeks after the date of infection.

6. At this time C'2 possessed only 50 percent of its former activity, while 40 percent of C'4 activity was lost. C'1 and C'3 remained unchanged.

7. Guinea pigs, infected with D. pneumonia and receiving sulfonamide therapy, lost 45 percent of their complement activity.

8. The 45 percent loss in complement activity was seen three days after the start of the infection. Two weeks after the infection date the complement level had returned to normal.

BIBLIOGRAPHY

Allott, A.J., 1937; J. Am. Vet. Med. Assoc., 91, 588.

Banks, T.E., Boursnell, J.C., Francis, G.E., Hopwood, F.L., and Wormall, A., 1946a; Biochem. J., 40, 734; 1946b; Biochem. J., 40, 745.

Bergstrom, S., 1936; Ztschr. f. physiol. Chem., 238, 163.

Besredka, 1899; Ann. Inst. Pasteur, 13, 361.

Bieler, M.M., Spies, T.D., and Ecker, E.E., 1947; J. Lab. and Clin. Med., 32, 130.

Bordet, J., 1895; Ann. Inst. Pasteur, 9, 462.

Bordet, J., and Gay, F.P., 1906; Ann. Inst. Pasteur, 20, 467.

Boulanger, P., and Rice, C.E., 1951; Can. J. Med. Sci., 29, 5.

Boursnell, J.C., Francis, G.E., and Wormall, A., 1946; Biochem. J., 40, 774.

Brand, 1907; Klin. Wschr., 44, 1075.

Bratton, A.C., and Marshall, E.K., 1939; J. Biol. Chem., 128, 537.

Braun, H., 1911; Biochem. Z., 31, 65.

Brooks, S.C., 1919-20; J. Med. Research, 41, 399.

Browning, C.H., and Mackie, T.J., 1913-14; J. Path. and Bact. 18, 442.

Browning, C.H., and Mackie, T.J., 1914; Z. Immunitätsforsch, 21, 422.

v. Buchner, H., 1889; Zbl. Bakt., 6, 1.

v. Buchner, H., 1893; Arch. Hyg. Berl., 17, 179.

v. Buchner, H., and Orthenberger, M., 1890; Arch. Hyg. Berl., 10, 149.

Bukantz, S.G., Rein, C.R., and Kent, J.F., 1946; J. Lab. and Clin. Med., 31, 394.

Bulloch, W., 1901; Zbl. Bakt., 29, 724.

Cathoire, E., 1910; Compt. rend. Soc. Biol., 69, 562.

Cernovodeaun, P., and Henri, V., 1906; Compt. rend. Soc. Biol., 60, 571.

Chargaff, E., Bancroft, F.W., and Stanley-Brown, M.J., 1936; J. Biol. Chem., 115, 155.

Coca, A.F., 1914; Z. Immunitätsforsch, 21, 604.

Coca, F., 1920; Proc. Soc. Exper. Med., 18, 71.

Cohn, E.J., McMeekin, T.L., Oncely, J.L., Newell, J.M., and Hughes, W.L., 1940; J. Am. Chem. Soc., 62, 3386.

Courmont, P., and Dufour, A., 1912; Compt. rend. Soc. Biol., 72, 916, 1014, 1058.

Davis, B.D., Kabat, A.E., Harris, A., and Moore, D.H., 1944; J. Immun., 49, 223.

Deisler, K., 1932; Z. Immunitätsforsch, 73, 365.

Dick, G.F., 1912; J. Infect. Dis., 10, 383.

Domagk, G., 1935; Deutsche Med. Wchnschr., 61, 250.

Dozois, T.F., Seifter, S., and Ecker, E.E., 1943; J. Immun., 47, 215.

Dozois, T.F., Wagner, J.C., Chemerda, C.M., and Andrew, V.M., 1949; J. Immun., 62, 319.

v. Dugern, E., 1900; Münch. Med. Wschr., 47, 677.

Dulaney, A.D., 1948; Personal communication to Ecker, J. Clin. Invest., 27, 320.

Ecker, E.E., Hiatt, C.W., and Barr, L.M., 1947; J. Lab. and Clin. Med., 32, 287.

Ecker, E.E., Jones, C.B.,and Kuehn, A.O., 1941; J. Immun., 40, 81.

Ecker, E.E., and Pillemer, L., 1941; J. Immun., 40, 73.

Ecker, E.E., and Pillemer, L., 1942; Annals of the New York Acad. of Sci., 43.

Ecker, E.E., Pillemer, L., and Grabill, F.J., 1938; Soc. Exper. Biol. and Med., 38, 318.

Ecker, E.E., Pillemer, L., Martiensen, E.W., and Wertheimer, D., 1938a; J. Biol. Chem., 123, 351; 1938b; J. Biol. Chem., 123, 359.

Ecker, E.E., Pillemer, L., and Seifter, S., 1943; J. Immun., 47, 181.

Ecker, E.E., Pillemer, L., Wertheimer, D., and Gradis, H., 1938; J. Immun., 34, 19.

Ecker, E.E., Seifter, S., and Dozois, T.F., 1945; J. Lab. and Clin. Med., 30, 39.

Ecker, E.E., Seifter, S., Dozois, T.F., and Barr, L.M., 1946; J. Clin. Invest., 25, 800.

Ehrlich, P., and Sachs, H., 1902; Klin. Wschr., 39, 297, 335, 492.

Ferrata, 1907; Klin, Wschr., 44, 366.

Fiske, C.H., and Subarrow, Y., 1925; J. Biol. Chem., 66, 375.

Fuchs, H.J., 1929; Z. Immunitätsforsch, 61, 342.

Gordon, J., 1930; J. Path. and Bact., 33, 689.

Gordon, J., and Hoyle, L., 1933; J. Hyg., 33, 411.

Gordon, J., and Marshall, P.G., 1929; Brit. J. Exper. Path., 10, 249.

Gordon, J., and Walker, N., 1945; J. Path. and Bact., 57, 451.

Gordon, J., Whitehead, H.R., and Wormall, A., 1926a; Biochem. J., 20, 1028; 1926b; ibid, p. 1036.

Gramenitski, M., 1912; Biochem. Z., 38, 501.

Guggenheimer, H., 1910; Z. Immunitätsforsch, 8, 295.

Hamburger, R., and Goldschmidt, L., 1922; Jahrb. f. Kinderh., 100, 210.

Hargan, E.S., 1936; Nature, 137, 872.

Hecht, H., 1923; Z. Immunitätsforsch, 36, 321.

Hegedus, A., and Greiner, H., 1938; Z. Immunitätsforsch, 92, 1.

Heidelberger, M., 1938; Symp. Quant. Biol., 6.

Heidelberger, M., Weil, A.J., and Treffers, H.P., 1941; J. Exper. Med., 73, 695.

Hewitt, L.F., 1938; Biochem. J., 32, 26, 1540, 1554.

Huddleson, I.F., 1948; Am. J. Vet. Research, 9, 277.

Hyde, R.R., 1923; J. Immun., 8, 267.

Jacoby, M., and Schutze, A., 1909; Klin. Wschr., 46, 2139.

Jonas, W., 1913; Z. Immunitätsforsch, 17, 539.

Jonsen, J., Manski, W., and Heidelberger, M., 1951; J. Immun., 67, 385.

Jordan, F.L.J., 1942; Acta. Med. Scand., 111, 372.

Kabat, E.A., 1943; J. Immun., 47, 513.

Kellogg, W.H., and Wells, L.A., 1926; J. Lab. and Clin. Med., 12, 153.

Kiamil, S., and Rassim, A., 1928; Compt. rend. Soc. Biol., 98, 1419.

Klopstock, F., 1924; Biochem. Z., 149, 331.

Koch, M.L., and Smith, A.H., 1924; Proc. Soc. Exper. Biol. and Med., 21, 366.

Kodicep, E., and Traub, B., 1943; Biochem. J., 37, 456.

Koopman, J., and Falker, I.D., 1935-36; J. Lab. and Clin. Med., 21, 312.

Landsteiner, K., and Stanovic, R., 1906; Ztschr. f. Bakt., 41, 108.

Liebermann, 1921; Deutsch med. Woch., 43, 1283.

Liefmann, H., and Stutzer, M., 1910; Berl. Klin. Woch., 42, 1929.

Markl, 1902; Z. Hyg. Infektkr., 39, 86.

Marsh, F.R., 1936; Nature, 137, 618.

Mayer, M.M., Osler, A.G., Bier, O.G., and Heidelberger, M., 1946; J. Exper. Med., 84, 535.

Mise, S., 1929; Fukuoka Acta Med., 22, 46, 47, 65.

Moore, H.D., 1919; J. Immun., 4, 425.

Muir, R., and Browning, C.H., 1906; J. Hyg., 6, 20.

Murray, E.G.D., 1924; J. Hyg., 22, 175.

McGhee, R.B., 1952; J. Immun., 68, 421.

Nathan, P., 1913; Z. Immunitätsforsch, 19, 216.

Neter, E., 1931; Z. Immunitätsforsch, 72, 136.

Northey, E.H., 1948; "The Sulfonamides and Allied Compounds", New York, Reinhold (1948).

Nunes, D.S., 1949; Monograph. Department of Bacteriology and Immunology, McGill University.

Nuttall, G., 1888; Z. Hyg. Infekt. Kr., 4, 353.

Ogston, A.G., 1938; Nature, 141, 1057.

Ormakov, L., 1911; Z. Immunitätsforsch, 10, 285.

Osborn, T.W.B., 1937; "Complement or Alexin", Oxford Press, London.

Ottolenghi, D., and Mori, M., 1905; Centralbl. Bakt. I., 38, 338.

Parsons, E.I., 1926; J. Immun., 12, 47.

Pillemer, L., and Ecker, E.E., 1941a; Science, 94, 437; 1941b; J. Immun., 40, 101; 1941c; J. Biol. Chem., 137, 139.

Pillemer, L., Ecker, E.E., Oncely, J.L., and Cohn, E.J., 1941; J. Exper. Med., 74, 297.

Pillemer, L., Seifter, S., and Ecker, E.E., 1940; Soc. exper. Biol. and Med., 45, 130.

Pillemer, L., Seifter, S., and Ecker, E.E., 1941a; J. Immun., 40, 89; 1941b; ibid, p. 97.

Pillemer, L., Seifter, S., and Ecker, E.E., 1942a; J. Exper. Med., 75, 421; 1942b; J. Exper. Med., 76, 93.

Pillemer, L., Seifter, S., San Clemente, C.L., and Ecker, E.E., 1943; J. Immun., 47, 205.

Quick, A.J., 1935; J. Immun., 29, 87.

Rice, C.E., 1950a; Can. J. Comp. Med., Dec. 1950, 369; 1950b; J. Immun., 65, 499.

Rice, C.E., and Boulanger, P., 1950a; Can. J. Research, 28, 262; 1950b; Can. J. of Comparative Med., Sept. 1950, 296.

Rice, C.E., Boulanger, P., and Plummer, P.J.G., 1951a; Can. J. Med. Sci., 29, 13; 1951b; ibid, p. 48.

Rice, C.E., and Crowson, C.N., 1950; J. Immun., 65, 201.

Rich, F.A., 1923; Bulletin No. 230 University of Vermont and State Agricultural College, Vermont Agricultural Experimental Station, Burlington, Vermont.

Rimington, C., 1933; Ergebn. d. Physiol., 35, 712.

Ritz, H., 1912a; Z. Immunitätsforsch, 13, 62; 1912b; Z. Immunitätsforsch, 15, 145.

Ruffner, E., 1929; Z. Immunitätsforsch, 60, 166.

Rutstein, D.D., and Walker, W.H., 1942; J. Clin. Invest., 21, 347.

Sachs, H., and Teruchi, Y., 1907; Klin. Wschr., 44, 467, 520, 602.

Schmidt, H., 1913; J. Hyg., 13, 291, 314.

Schmidt, H., 1919; Z. Hyg. Infektkr., 88, 495.

Schneller, G.H., Foss, N.E., and Sullivan, C.F., 1948; Calco Technical Bulletin No. 723, Calco Chemical Division, American Cyanid Co., Boundbrook, N.J.

Seifter, S., and Ecker, E.E., 1946; J. Clin. Invest., 25, 1946.

Silber, L.A., 1930; Z. Immunitätsforsch, 65, 285.

Silber, L.A., and Schafran, A.S., 1932; Z. Immunitätsforsch, 77, 514.

Simola, P.E., and Brunius, E., 1933; Biochem. Ztschr., 258, 228.

Strong, P.S., and Culbertson, J.T., 1934; J. Hyg., 34, 522.

Swales, W.E., 1951; Personal communication.

Thorp, W.T.S., 1944; M.S.C. Vet., 4, 110.

Thorsch, M., 1914; Biochem. Z., 68, 67.

Toda, T., and Mituse, B., 1933; Z. Immunitätsforsch, 78, 62.

Tokano, Y., 1936; Z. Immunitätsforsch, 87, 29.

Topley, W.W.C., 1915; Proc. Roy. Soc. B., 88, 396.

Trefouel, J., Trefouel, Mme. J., Ni Hi, F., and Bouet, D., 1935; Compt. rend. Soc. Biol., 120, 756.

Valley, G., 1928; J. Immun., 15, 325.

Vincent, H., 1910; Compt. rend. Soc. Biol., 69, 563.

Wadsworth, A., Maltaner, E., and Maltaner, F., 1931; J. Immun., 21, 313.

Wadsworth, A., Maltaner, F., and Maltaner, E., 1936; J. Immun., 30, 417.

Wadsworth, A., Maltaner, F., and Maltaner, E., 1937; J. Immun., 33, 297.

Waring, G.W., and Weinstein, L., 1947; Science, 105, 479.

Weil, E., 1913; Biochem. Z., 48, 347.

Welsh, M., Schroeder, C.R., Vroman, D.F., Reddin, L., Burkhart, R., and Langer, P., 1946; Proceedings Fiftieth Annual Meeting of the United States Livestock Sanitary Association, p. 213.

Wendelberger, J., and Volavsek, W., 1934; Wien. Klin. Wochschr., 47, 967.

Whitehead, H.R., Gordon, J., and Wormall, A., 1925; Biochem. J., 19, 618.

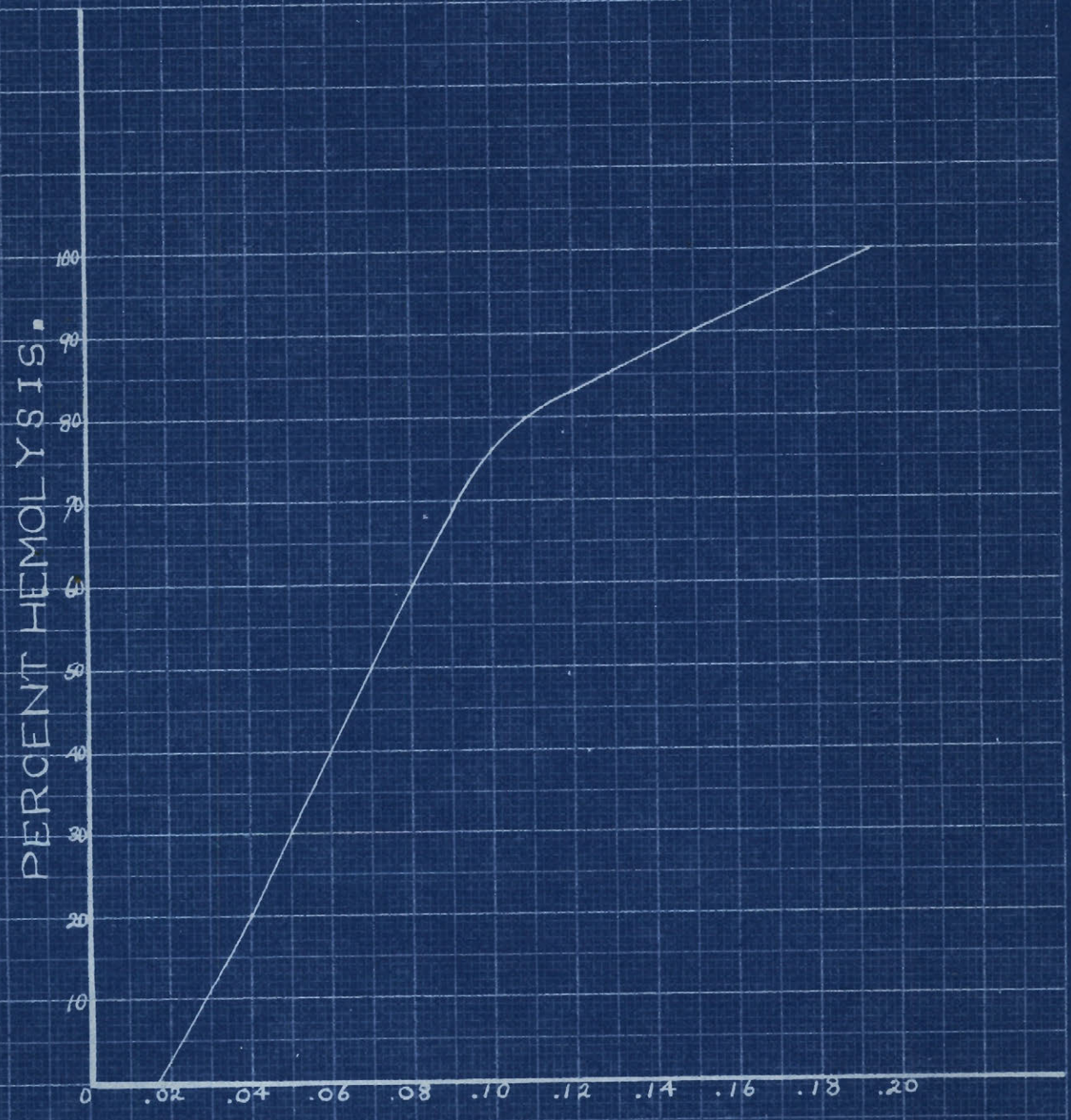
Wormall, A., Whitehead, H.R., and Gordon, J., 1926; Biochem. J., 20, 1028.

Zermati, M., and Vargues, R., 1947; Compt. rend. Soc. Biol., 141, 406.

Zinsser, H., and Johnston, W.C., 1911; J. Exper. Med., 13, 31.

GRAPH I.

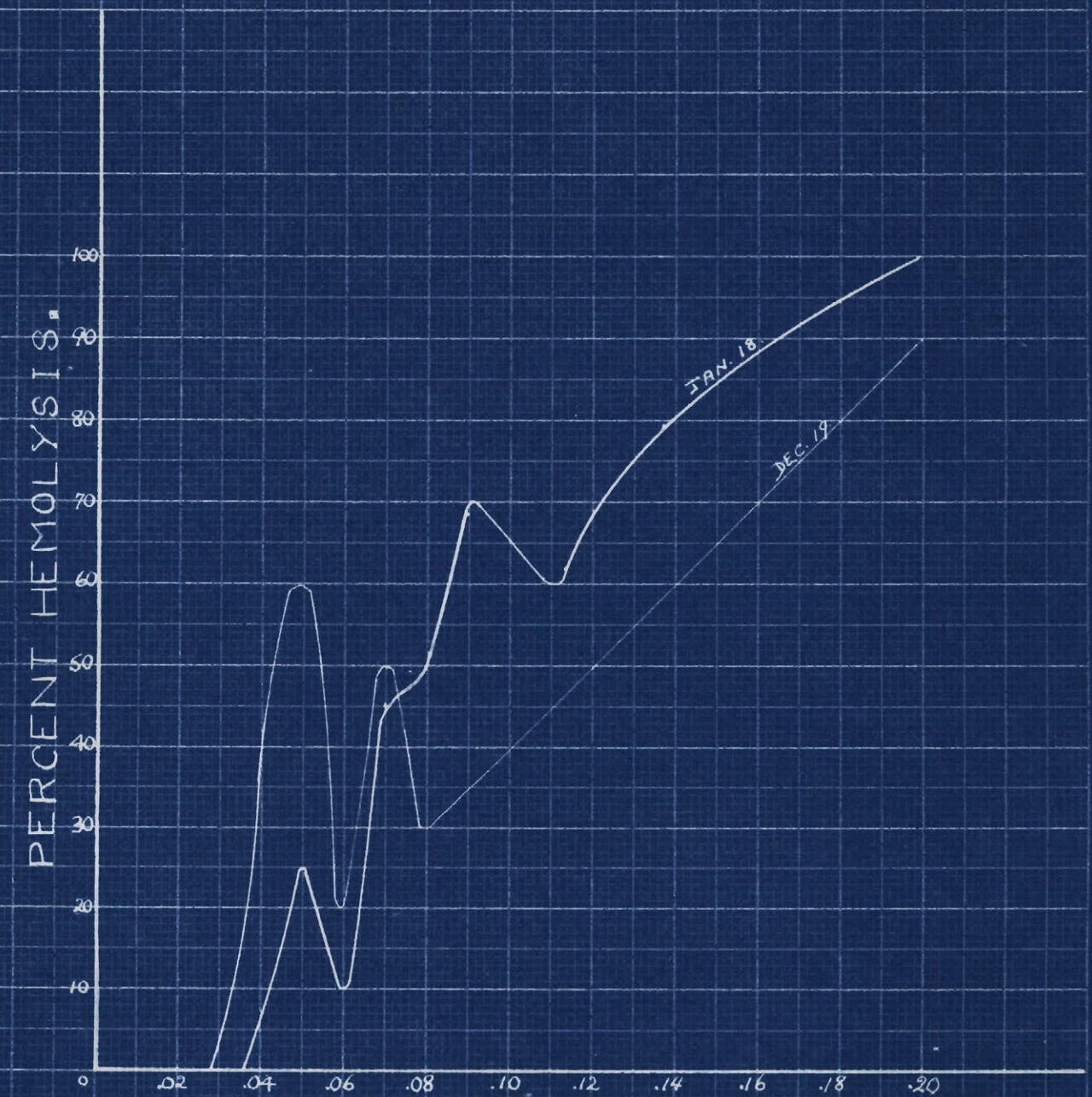
A NORMAL COMPLEMENT TITRATION CURVE.



GRAPH II.

ABNORMAL TITRATIONS.

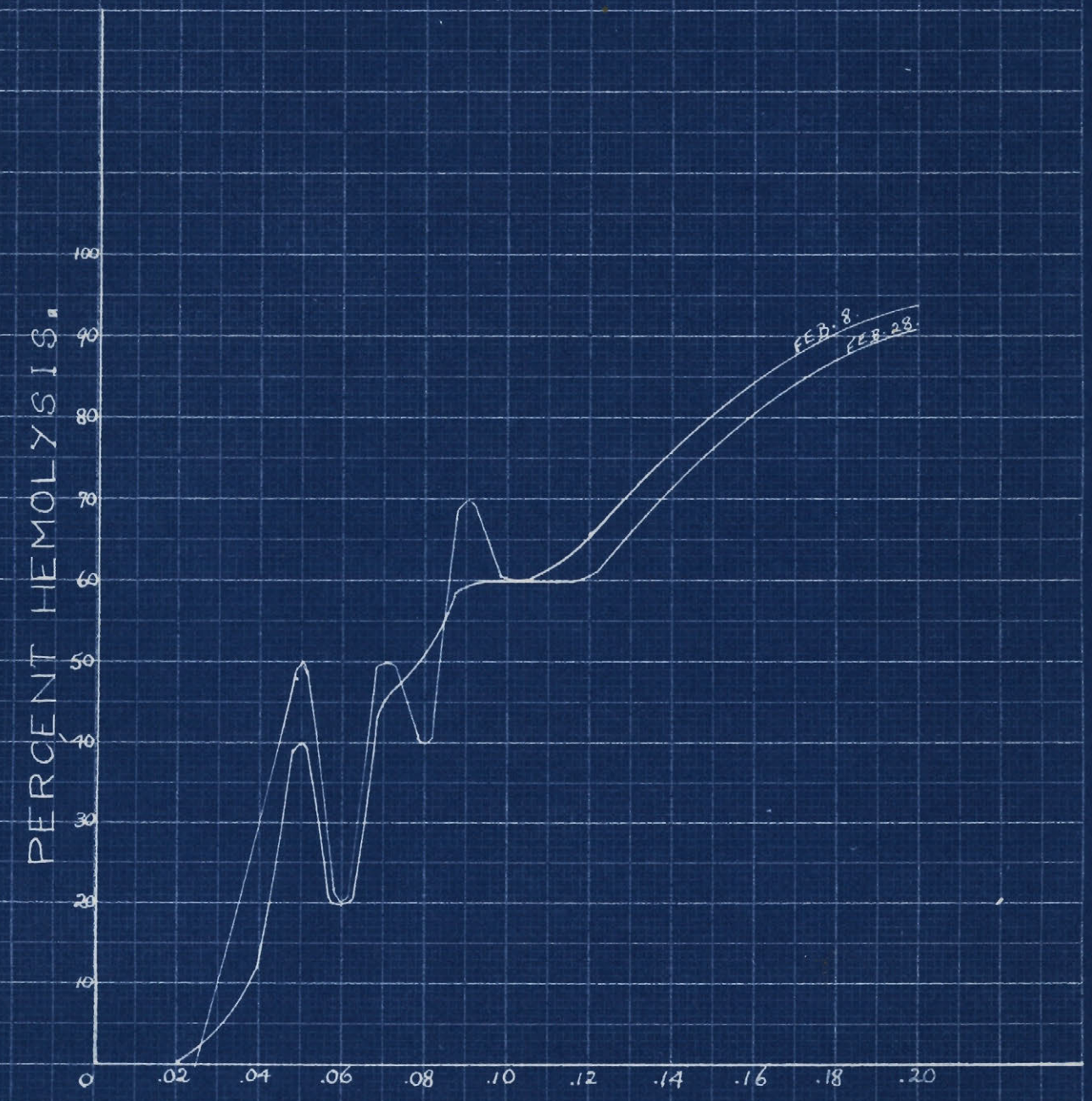
GROUP IV. EXPERIMENT 2 a.



GRAPH II CONTD.

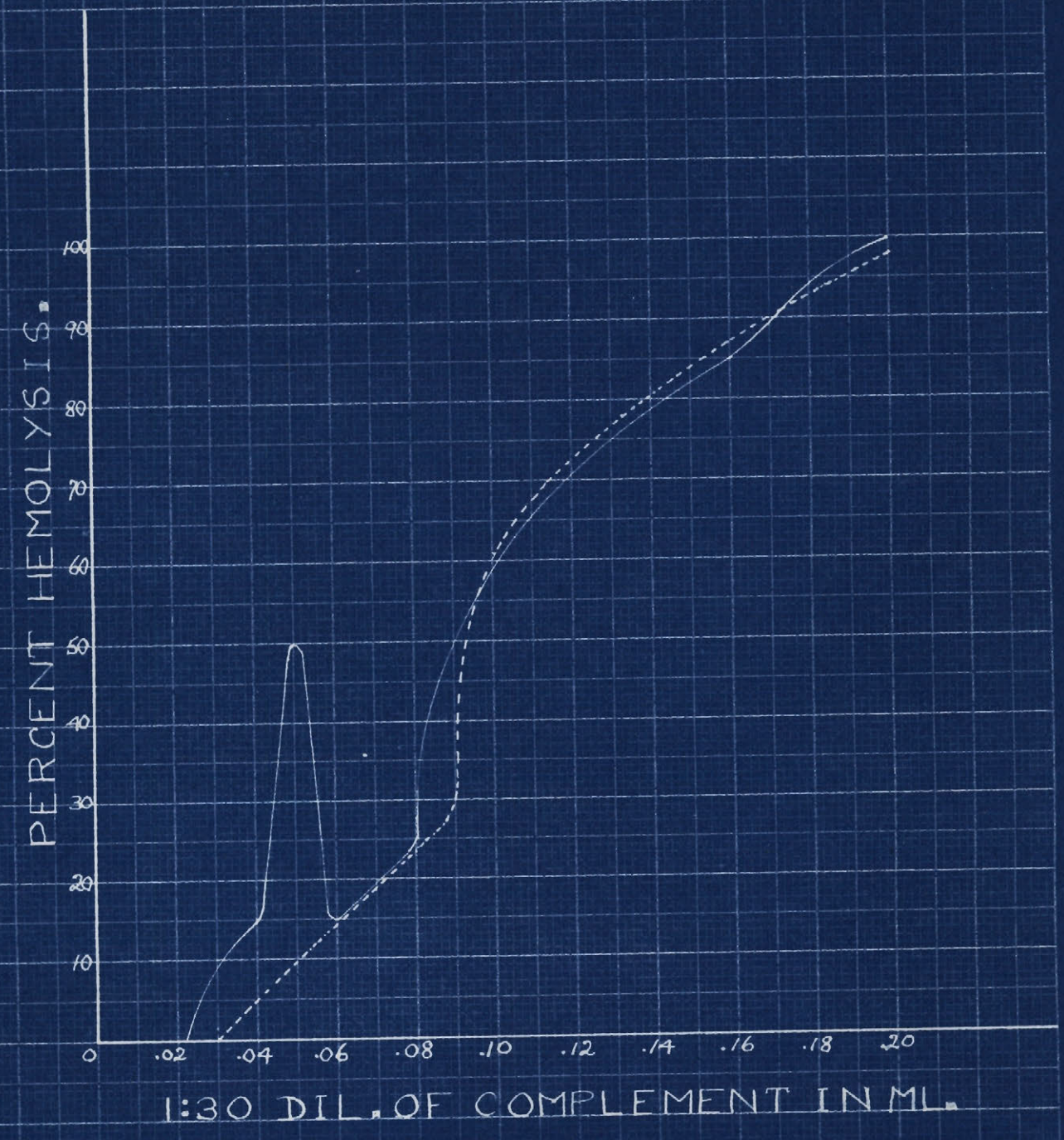
ABNORMAL TITRATIONS.

GROUP IV. EXPERIMENT 20.



GRAPH III.

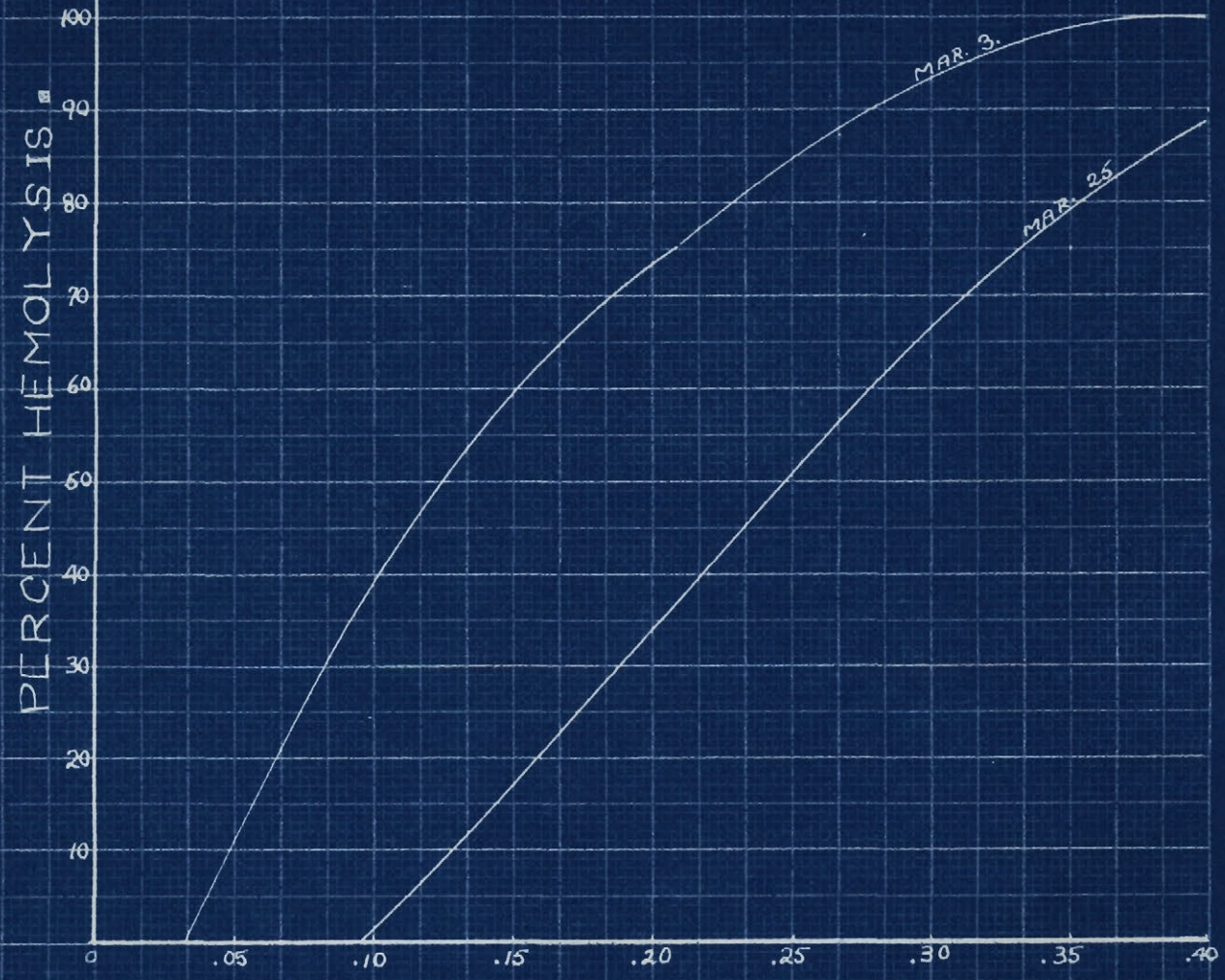
TITRATIONS OF COMPLEMENT TAKEN ON CONSECUTIVE DAYS FROM A SINGLE GUINEA PIG.



GRAPH IV.

COMPLEMENT TITRATIONS

BEFORE AND AFTER INFECTION.



GRAPH V.

C'2 AND C'4 TITRATIONS

BEFORE AND AFTER INFECTION.