Studies on Haemolytic Complement

by

Ralph Ernest Bernstein

Department of Physiology, University of the Witwatersrand

Thesis submitted for the Degree of Master of Science, in the University of the Witwatersrand, Johannesburg.

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INTRODUCTION.

The study of the processes involved in general immunity has provided one of the most interesting fields of research in preventive medicine. The popularity of this problem among research workers was due to the fact that the defence mechanisms of the blood were regarded as closely allied to general bodily resistance and it was hoped that, from a knowledge of the substances involved in these reactions, a basis for the treatment and prevention of infectious disease might be evolved. Immunity reactions have, however, proved to be complicated, involving the consideration of many factors about which there is very little exact knowledge. Tangible results will be few, until we are able to handle the "labile" protein molecules of the blood and appreciate the "chain" reaction by which immunity reactions appear to produce their effect.

Of the immuno-chemical substances present in serum complement is probably the most important and most elusive. "Extremely labile and incapable of increase by any process of immunisation, it is invariably inactivated when once combined, so that there appears to be little prospect of isolating and concentrating it by any means" (Browning,1925). In a review of the present state of knowledge of complement by Osborn (1936c), we find that there has been little advance

in our knowledge of the constitution of complement. In a massive literature of conflicting results and interpretations one finds an almost unexplored field with reference to the essential biochemistry and physical properties of complement and related substances. A quantitative analysis of the factors concerned in general immunity would be a very valuable asset, but at present, owing to inadequate methods of estimation, one is forced to base conclusions on a statistical analysis of results.

Scope of this Thesis.

It is evident that our knowledge regarding complement is in a very incomplete state. The ultimate solution of the problem would appear to rest with the biochemist rather than the bacteriologist. Before further advances can be made some of the fundamental unknowns regarding complement must be solved - its chemical nature, the mechanism of its action, its origin within the body, and finally, its accurate estimation. The main purpose of the present work was the study of the properties of amboceptor-complement haemolytic systems and the variations in complement activity produced by various chemical and physical agencies. At the same time the relevant literature has been studied, and the results and conclusions of other investigators, which have a bearing

on the subject, are reported. The significance of the data obtained is discussed, and an analysis made of the theories regarding the mechanism of complement action.

SECTION I.

THE CONCEPT OF HAEMOLYTIC COMPLEMENT.

Historical.

During the latter part of the 19th century Metchnikoff showed that unicellular amoeboid organisms were capable of ingesting foreign particles, and all theories of the natural resistance of the body to bacterial invasion were based on Metchnikoff's demonstration that the destruction of foreign cells in the blood was due solely to its leucocytic elements (1883). The phagocytic theory was firmly established by 1888, when Nuttall showed that cell-free serum was capable of destroying bacteria in vitro. This effect was confirmed by Buchner (1889), who found that the bactericidal effect disappeared on standing the serum at room temperature or heating it to 56°C. Such serum could be reactivated by the addition of a small amount of fresh serum. Buchner named this thermolabile bactericidal substance present in normal serum "alexin".

It was not until the work of Bordet (1895-9,1909) and Ehrlich (1906) that the mechanism of the humoral

bactericidal and haemolytic action of serum was understood. Their investigations, together with those of Muir, Mackie, Sachs, and others, showed that the bactericidal and lytic properties of serum were brought about by two agencies, a thermostable sensitising agent (variously called the antibody, amboceptor, sensitiser, substance sensibilisatrice) which was incapable of killing foreign cells or their products, e.g., bacteria, toxins, red blood cells from another animal species, but merely prepared the organisms for the action of the second factor, which was named "complement" by Erlich in 1905 and is to be identified with the "alexin" of Buchner.

The phagocytic theory of Metchnikoff was therefore no longer tenable in its original form, and although Metchnikoff (1899) and Gengou (1901) attempted to show that the bactericidal substances were within the leucocytes during life, this was disproved by Fränkel (1912) and Lippmann and Plesch (1913), who artificially depressed the leucocyte count and found no change in the complementary power of the serum while Schattenberg and Harris (1934) found that leucopenic animals had a slightly higher complement than normal animals and that the serum complement of animals with leucocytosis was similar to that of the controls. In our present state of knowledge it appears that the processes

involved in phagocytosis are indeed dependent on humoral activity, and Wright and Douglas (1903) found that, before any extensive phagocytosis occurred, the serum probably exerted some specific sensitising action on the foreign organisms through the agency of a thermolabile substance which they called "opsonin".

Factors concerned in Humoral Immunity.

Since the establishment of the humoral bactericidal power of blood as a prominent factor in immunity processes much work has been done in elucidating the nature of the substances involved and their mode of action. The subsequent description represents our present state of knowledge with regard to the various substances which play a part in bactericidal and haemolytic action.

ANTIGEN.

Antigens are foreign substances of protein constitution. Attempts to demonstrate antigenic properties in amino-acids have been unsuccessful; at the stage of proteoses, however, there may be some antigenic function. It is therefore essential to introduce the foreign protein parenterally (i.e. directly into the tissues or circulation). When introduced parenterally it gives rise to a specific

reaction in the blood, namely, the development of a specific antibody. A foreign protein is not homogeneous as far as its antigenic function is concerned, but exhibits slight variations in that part of its structure which stimulates antibody formation; so also the related antibody may not be homogeneous, but may show slight variations in its structure corresponding with those of the antigen. In this way we may account for the phenomena of tissue specificity and species specificity.

The antigenic power of various foreign proteins varies considerably. The serum proteins and the stromata of the red corpuscles of certain species, e.g., ox, sheep, act as potent antigens when injected into rabbits; on the other hand haemoglobin and tissue proteins are only feebly antigenic, while nucleoproteins have yielded variable results (Beebe,1906; Pearce and Jackson,1906).

The Antigen in Complement Haemolysis.

In the case of haemolysis by complement the question arises as to what constitutes the antigen. Muir and Ferguson (1906) expressed the view that the antigens of the red blood cells which, after sensitisation with the homologous antibody,lead to complement absorption, reside in the stromata of the corpuscles. The antibody developed in the blood of an animal on injecting red cell suspensions from another species is therefore an anti-corpuscle immune

body. The protein constituent of haemoglobin, viz. globin, can act as an antigen, but apparently it only does so when freed from its prosthetic group (Browning and Wilson, 1920; Browning, 1925). For example, an antiserum which reacted intensely with globin failed to give any complement fixation with the same specimen of haemoglobin as that from which the globin was derived and in corresponding concentration. Although most attempts to produce antibodies against haemoglobin appear to have been unsuccessful (Schmidt and Bennett, 1919; Browning and Wilson, 1920), more recent work has shown that haemoglobin possesses some antigenic properties (Hektoen and Schulhof, 1922; Heidelberger and Landsteiner, 1923; Hektoen, 1927).

ANTIBODY.

Antibodies are specific thermostable substances present in the blood of normal animals and in greatly increased concentration in the blood of animals immunised by the injection of foreign cells (antigens). Each specific antibody consists of overlapping moieties which are recognisable by various phenomena which result from their interaction with their corresponding antigens, viz. (a) antitoxic, (b) bactericidal, (c) haemolytic, (d) opsonic, etc. It is still undecided whether the biological properties of a

serum containing antibodies are due to alterations in the physical reactions of normally occurring constituents or to the presence of chemical substances produced by immunisation. However, it is found that antibodies are colloidal in nature or are bound to colloids, since they do not dialyse through filters which retain colloids (Brodie, 1897). The antibody developed by antigen injection is associated with the pseudo-globulins of the serum (Diacono, 1933); this receives confirmation from the observation that there is an increase in the globulin content of the serum of an immunised animal (Ledingham,1907; Kirkbride and Murdick, 1927).

Antibodies show specific properties dependent on the species of animal which has developed them - Species Specificity. This is demonstrated by the properties of haemolytic immune bodies obtained by injecting the red blood corpuscles from the same species into different animals (Muir, 1912). Although the resulting antibodies combine with the same "receptors" of the corpuscles, the effects vary considerably, as is shown by the susceptibility of the sensitised red cells to different complements.

The occurrence of naturally occurring antibodies - haemolytic antibodies - in most mammalian sera is a

phenomenon which has as yet received no explanation. Without any contact or sensitisation, the horse, cat, rat, rabbit, ox, and human being carry in their blood antibodies specific to sheep red cells. Despite some properties in common, it is not certain whether they are to be identified with the antibodies developed by repeated injection of washed sheep erythrocytes into the respective animals (Sachs, 1902; v. Euler and Gard, 1931).

Antibodies which require complement for their action are designated by the generic term "immune-bodies", but are usually referred to as "amboceptors" (the name originally applied by Ehrlich).

The Antigen - amboceptor Combination.

The nature of the combination between the antigen and its specific antibody was regarded by Bordet (1899) as an adsorption process, whereby the antibody ("sensitiser") united with the foreign body and modified it so as to allow direct absorption of complement. About the same time Ehrlich (1906) applied the classical side-chain theory, developed by him to explain many biological reactions, to demonstrate the sequence of events in the reaction of the tissues to antigens. He regarded the process as the interlocking of a reactor group in the antibody with a receptor group on the antigen, followed by a similar

interaction between the antibody and the component fractions of the complement complex. Because of the link which the antibody provides between the foreign cell and complement Ehrlich named it "amboceptor".

Fundamentally these two theories do not differ on any material point, and they supply a simple exposition of a complicated process. The quantitative relationships exhibited by the combination of antigen and amboceptor were found by Ehrlich (1897) to follow the Law of Multiple Proportions, viz: that a molecule of fresh toxin combines firmly with a definite and invariable amount of antibody. The rate of combination is accelerated by increasing the concentration of the reagents or by raising the temperature. Arrhenius and Madsen (1904) and Arrhenius (1907), however, consider that the process is a reversible chemical reaction governed by the laws of mass action, which is exhibited by substances of work combining affinity. More recently Eagle (1929,b) and Ponder (1932,1934) have re-investigated the problem, but their results have unfortunately only produced further confusion. Eagle considered that all in vitro immune reactions involve the aggregation of immune - serum globulins upon the surface of the antigen, and that the subsequent "fixation" of complement follows the adsorption isotherm of Freundlich

for dilute solutions. This result is therefore an amplification of Bordet's original theory. Ponder, on the other hand, has, in a series of careful experiments, shown that, although the sensitising agent may be concentrated at the surface, the quantity concentrated is a linear function of the quantity introduced into the system, showing a constant ratio within experimental limits of 0·75:1.

The quantitative relationships established by experiments in vitro, however, will not necessarily hold in vivo. This is illustrated by the experiments of Muir and McNee (1912) in which the amount of haemolysis resulting from the introduction of an anti-corpuscle serum for the animal's own serum into its circulation was much greater than could have been expected from estimations in the testtube.

The antigen-amboceptor combination is essential before complement can be absorbed. The early experiments of Ehrlich and Morgenroth (1899) demonstrate that:-

(i) when complement containing serum is treated with excess red blood corpuscles of a species for which the serum contains no amboceptor and is subsequently centrifuged, the supernatant fluid contains as much complement as the serum.

(ii) where red blood cells are treated with immune-body and complement at 0°C. haemolysis does not occur and the supernatant fluid obtained after centrifuging contains only the complement; the same mixture at 37°C. undergoes haemolysis.

Complement, therefore, has no affinity for red cells in the absence of amboceptor (Eagle and Brewer, 1929; Ponder, 1932), and even in its presence complement does not combine with red cells at two temperatures. Further, in the absence of antigen complement does not combine with the amboceptor (Muir and Browning, 1908).

The antigen-amboceptor combination can be dissociated. Muir (1903) showed that amboceptor could be partially dissociated from its combination with red cells at 37°C., that the process took place more slowly at room temperature, while at 0°C. no dissociation occurred. Kosakai (1918) showed that amboceptor could be removed from sensitised red cells by means of isotonic sugar solutions.

COMPLEMENT.

Properties.

Complement is a thermolabile and non-specific constituent of normal blood; it is easily destroyed

undergoing rapid deterioration on shaking, at temperatures above that of the body, and by alterations in the physical and chemical constitution of the medium. It is not increased during the process of immunisation nor by any procedure known to chemists. Muir (1909) defined complement as "that labile substance of normal serum which is taken up by the combination of an antigen and its anti-substance ('amboceptor')."

Formation and Occurrence.

The liver appears to be the principal organ concerned in the production of complement, and Olsen (1922) and da Costa Cruz (1929,1933) showed that the liver forms the thermolabile components of complement, viz: midstuk and endstuk. Neither the reticulo-endothelial system nor the leucocytes form the complement complex, though there is evidence that the white cells carry the fourth component (Maltaner, 1935).

Complement is present in all the body fluids, and Müller (1910) gives the following series of complement concentrations :-

plasma > lymph > exudates > transudates > secretions, while the C.S.F. contains all components except the midstuk fraction (Okada, 1929). The presence of free, active complement in the circulating blood is

still disputed, and it is supposed that it is present in an inactive form which is readily liberated when required (Osborn, 1936,c).

Constitution of Complement.

The constitution of complement is not as yet clear, but the evidence points to some physico-chemical manifestation associated with the behaviour of the protein constituents of the serum, or to a complex composed of various protein aggregates onto which an enzyme has been adsorbed. Several different entities of the complement complex have been demonstrated, and their interaction and concentration in the serum play important rôles in the functioning of complement (Osborn, 1936c).

The methods of fractionating complement by treating serum with distilled water, carbon dioxide, or dilute acid (Ferrata, 1907; Sachs, 1909; Liefmann, 1909; Browning and Mackie, 1914) showed that complement could be split into two parts associated with the albumin and globulin fractions of the serum. Brand (1907) found that the action of the globulin fraction had to precede that of the albumin component in any complement reaction, and hence named the fractions midstuk and endstuk respectively. Browning and Mackie (1914), by a method of salting out proteins with ammonium salts, concluded that the midstuk

represents the euglobulin fraction of the serum, while the endstuk represents the albumin and greater part of the pseudo-globulin fractions.

It would appear that the complementary activity of a serum depends on these two factors. Fränkel,(1911), Marks, (1911,b), and Mackie (1925) concluded from their experiments that a decrease in the one component could be compensated for by increase of the other, but that haemolysis was inhibited if the midstuk fraction was increased out of proportion to the endstuk and vice versa. The addition of endstuk increased the complementary power of a serum to some extent; while the midstuk could be reduced considerably in the mixture and still produce haemolysis, the endstuk must be present in a relatively high proportion to be effective (Ledingham and Dean, 1912; Parsons, 1926). Endstuk would therefore appear to be the limiting factor in the complement reaction. Despite the fact that these fractions are not constant entities, we can only conclude that variations in complement activity must be due to inactivation or enhancement of one or other of the complement fractions, produced by the various physical and chemical agencies which can be employed to split complement.

The fraction of complement, which is present in the globulin precipitate, becomes attached to sensitised red

corpuscles. Further, red corpuscles, together with large quantities of amboceptor, remove the same fraction from whole serum at 0°C. (Sachs and Bolkowska, 1910). Even in the absence of amboceptor this midstuk component may become fixed to red corpuscles in isotonic sugar solution (Guggenheimer, 1910). Brand termed those sensitised corpuscles which have bound midstuk "persensitised." This is strong evidence in favour of midstuk being an actual chemical entity. In the case of endstuk the evidence that it is bound is not so direct, and it has often been dismissed as some physical state.

The part played by the thermostable and slowly filtrable third and fourth components of complement (Dungern, 1900; Braun, 1911; Whitehead, Gordon, and Wormall, 1925; Gordon, Whitehead, and Wormall, 1926; Strong and Culbertson, 1934) is not clear, except that the third component is associated with the midstuk of complement and the fourth component with the endstuk. The various parts act in the following sequence in the haemolysis of sensitised cells:- fourth component, thermolabile midstuk and endstuk, and finally, the third component (Osborn, 1936 c). This has been aptly termed the "chain reaction" of complement. The third component cannot be shown to be used up in the complement reaction (Nathan, 1913; Deissler, 1932), and is held to be some peculiar physico-chemical state.

Unity or Multiplicity of Complement.

Throughout the study of complement it has been assumed that the bactericidal and haemolytic complements are identical in their nature, since haemolysis by complement provides the same fundamental features as are characteristic of the bactericidal action of serum. Both Muir and Browning, (1908) and Ehrlich, (1906) held that there was a multiplicity of complement, but that they had much in common. Bordet, on the other hand, stated that in any one serum, the bacteriolytic alexin was identical with the haemolytic in a given species. Braun (1911), Liefmann (1909, 1911), and Boehncke (1912), by fractionating complement into midstuk and endstuk, showed that the separate components were inactive in respect of haemolytic and bactericidal power, but that the two fractions, when combined, possessed bactericidal power and haemolytic activity. Gordon and Wormall (1928) and Gordon (1930 a) showed that the loss of bactericidal power, whether produced in the serum by ammonia or Congo red inactivation, ran parallel with the destruction of haemolytic complement, and expressed the opinion that bactericidal and haemolytic complements are identical. Subsequent experiments by Gordon and Carter (1932) showed that variations in bactericidal power against different organisms are due to the varying sensitiveness of these organisms to non-specific factors in the serum (complement plus a heat-stable factor).

The Nature of Haemolytic Complement Action.

In the haemolysis of red blood corpuscles by complement there is general agreement that the complement is adsorbed onto the cell surface or concentrated at the cell-liquid interface (Eagle and Brewer, 1929; Ponder, 1932). The subsequent events leading to the liberation of haemoglobin are obscure. Since no lysis occurs under the action of amboceptor alone, the physical changes in the corpuscles leading to the escape of haemoglobin are attributed to the action of complement. The haemolytic process has been regarded as due to the enzymatic powers of complement. It was held by Ehrlich that when complement was brought into combination with a susceptible cell through the medium of a specific amboceptor some sort of digestion followed. According to such a conception complement was analogous to the ferments, and subsequent work by Walker (1906-6), Fuchs (1931a, 1933), and Osborn (1936c) appears to confirm this. Fuchs (1931a) showed that the midstuk of complement was able to activate a thermostable substance in heat-inactivated amylase, while Osborn (1936c) reported that in the kinetics of its action, complement has characteristics common to many enzymes. Finally Willstatter has defined an enzyme as "composed of a colloidal and a specific, active group, enabling it to be bound to the substrate, a conception which would apply to

the amboceptor-complement combination.

The fact that complement is destroyed during haemolysis does not preclude its enzymic nature. The midstuk component is generally regarded as responsible for the enzyme action of complement (Fuchs, 1931a). The destruction of midstuk is due to the production of products during haemolysis which inactivate complement (Basil and Suzuki, 1910; Liefmann and Cohn, 1910).

Though there is evidence that complement has enzymatic properties, it does not appear that complement acts by dissolving the lipoid envelope of red cells by lipolytic action (Brinkman and Szent-Györgyi, 1924; Gordon and Wormall, 1929), while the evidence in favour of complement producing a digestion of the corpuscular protein is not conclusive (Dick, 1913; McNeil and Kahn, 1918; McVey, 1918; Douris, Mondain, and Beck, 1932).

The fact that the stromata of the red blood corpuscles can be recovered and observed by microscopical examination after complement haemolysis raises the important question of whether the effect is due to a digestive mechanism or to an osmotic effect. The haemolysed stromata have altered properties, since they do not contract in hypertonic solution nor do they swell in hypotonic solution. Apparently the action of complement has increased their permeability so that the

usual osmotic phenomena are no longer seen.

A line of research which has not received much attention is the close similarity between the effects of various physical and chemical agencies on osmotic and amboceptor-complement haemolysis. It raises the possibility that the action of complement, as a result of its concentration at the cell surface, is to produce osmotic pressure changes within the cells, leading to the liberation of haemoglobin, Thus, the haemolytic effect of fresh guinea-pig serum on unsensitised red blood cells in isotonic sugar solutions has been attributed to the direct action of complement, since heated serum has no effect (Wright and MacCallum, 1922).

Function of Complement.

There is no doubt that complement represents a biochemical property of the highest importance. The presence of complement has been demonstrated generally in warm-blooded animals and it has also been shown to exist in the blood of certain lower vertebrates (Mazetti, 1913), and even in protozoa, e.g., Entamoeba histolytica (Craig, 1927). Ehrlich held that the complementary power of sera observed in vitro had its parallel in the animal body, and now in the light of many experiments it may be taken as proven that complement

action takes place in the blood (Muir and McNee, 1912), and there is every reason to believe that complement is indispensable to the animal organism in protecting it against the occurrence of infection. This was first brought into prominence by Lister (1880), who observed that blood removed aseptically from animals had no tendency to undergo "putrefaction", and that there was no detectable growth in the medium when bacteria were added. Lister was also aware of the importance not only of the purely bactericidal agents in serum, but also of phagocytosis by living cells.

The rôle of phagocytosis (cellular immunity) in the processes of infection is outstanding. The phagocytic power of leucocytes is dependent on a sensitisation of the antigen by a humoral substance in normal serum termed opsonin. The opsonic power of normal serum is due to a non-specific thermolabile substance which is not increased by immunisation, and which has been regarded as due to the presence of complement (Muir and Martin, 1906). Gordon, Whitehead, and Wormall, 1926, 1929; Gordon, 1930b; and Gordon and Thompson, 1935, have, however, shown important differences between the opsonic and complementary powers of sera, the difference being due to the inactivation of the fourth component of complement by techniques which leave the opsonic power unaltered, and the demonstration that this component is unnecessary

for opsonin action. The work of Maltaner (1935) has provided a simple explanation of these findings; he has shown that opsonin is complement minus the fourth component, and that the leucocytes contain this component. The phagocytes, therefore, ingest cells "buttered" with opsonin and thus the "chain reaction" of complement proceeds intracellularly.

In immune sera, besides the normal non-specific thermolabile complement opsonin, there are specific thermostable opsonins. One type acts without complement - bacteriotropin -, while in the second case the phagocytic action is brought about by a combination of an opsonic immune body plus complement. We are thus forced to conclude that phagocytosis is but a manifestation of the humoral agents present in the blood.

The part played by complement in bringing about bacteriolytic and bactericidal effects is exemplified by Pfeiffer's reaction, and there is little doubt that the amboceptor - complement reaction is one of the chief mechanisms which contribute to natural and acquired anti-bacterial immunity. Organisms such as B.typhosus, B.dysenteriae, V.cholerae, and various members of these groups, are rapidly killed if normal serum is added at body temperature. The lysis of sensitised red cells and other animal cells by normal sera and antisera has been

generally accepted as a similar phenomenon. The destruction of bacteria and foreign red cells without the addition of a specific amboceptor when normal serum is added, is due to a naturally occurring amboceptor, which is not necessarily identical with the amboceptor produced by immunisation processes.

There is evidence that complement is bound by toxin-antitoxin and viral-antiviral combinations.

The presence of complement in conjunction with specific or naturally occurring amboceptors in the blood will therefore, if the results of in vitro experiments hold in vivo, protect the individual against specific bacterial organisms and foreign substances. An anti-bacterial serum will produce a high degree of sensitisation of the bacterial organism, so that a smaller quantity of complement will be required in the humoral process of destruction. A deficiency in the blood complement concentration, on the other hand, may predispose the individual to serious infections even if anti-bacterial sera are injected, since complement is, as has already been described, the final link in the process of foreign cell destruction. In the presence of a low concentration of complement the foreign organisms may therefore be insensitive to its action. Such deficiency may well be responsible for a lowered

resistance to infection.

While there is little evidence that nutritional defects disturb the amboceptor-forming mechanism, there is ample proof that resistance both to spontaneous and induced infection is profoundly affected, and that the blood complement tends to be lowered. The precise parts played by particular vitamin deficiencies in producing this state of lowered resistance are not yet clear. With regard to vitamin A this is regarded by Mellanby as an anti-infectivity vitamin; Osborn (1931) found that Vitamin A deficient rats had a low complement and that feeding cod-liver oil to humans raised the complement above its normal level (Osborn, 1933).

SECTION II.

METHODS AND MATERIALS IN AMBOCEPTOR-COMPLEMENT HAEMOLYSIS SYSTEMS.

The methods of complement estimation which have been employed in this investigation were carried out on amboceptor-complement haemolytic systems containing sheep red blood corpuscles (antigen), anti-sheep corpuscle serum (amboceptor), and fresh guinea-pig or human serum or plasma (complement). The universal use of this system and the fact that the materials were readily available from the South African Institute for Medical Research led to their sole use throughout these investigations.

ESTIMATION OF HAEMOLYTIC COMPLEMENT.

The methods of estimation of the complementary activity of serum fall into three groups. The method most commonly used is the "serial tube" determination, in which graded volumes of serum are added to a series of tubes containing standard volumes of a suspension of sheep cells sensitised with the required number of minimal haemolytic doses of the appropriate decomplementised immune serum. The total volume in each tube is brought to the same volume by the addition of normal saline. The smallest amount of serum which is required to haemolyse this standard volume of

sensitised cells after an hour's incubation at 37°C. is read, and this represents the complement titre of the serum under the conditions prevailing. The complementary activity of the serum is assumed to be inversely proportional to the minimal haemolytic volume. This method presents certain definite disadvantages:-

(a) it yields stepped results, which cannot be expressed on a quantitative basis.

(b) large amounts of serum are necessary, unless the micro-method of Huntermüller (1929) is used.

(c) the serum is used in a dilution of 1:20 to 1:40. The rapid deterioration of complement in diluted sera is well-known; further it will be shown that, on dilution of the serum, the complementary activity takes a certain time to reach a stable equilibrium (table 5).

(d) the estimation by this method takes a long time.

The methods employed in this investigation were:-

(a) Timing Method.

This method consists in the estimation of the time for complete haemolysis at 37°C. in a haemolytic system containing standard volumes of sensitised sheep corpuscles and complement serum. This method was originally introduced by Eagle (1929a) and later modified by Osborn (1931,1933) and Ponder (1934). The modified technique of Osborn has been substantially followed in this investigation. The relation between this

and the "serial tube" method appears from the curves published by Eagle (1929a) and Osborn (1931), which show that the speed of haemolysis is, as is to be expected, a function of serum used and therefore to the amount of complement introduced into the system.

The haemolytic system is prepared so as to have a standard volume by means of a graduated micropipette. This consists of a fine capillary bore of 50 or 100 cu.mms. capacity, and this volume is divided into ten graduations. The capillary tube is connected through a glass bulb of about 1 c.c. capacity to a 5 c.c. syringe, which is fitted with a plunger whose threaded screw makes 15 turns per c.c. capacity. The syringe is filled with a coloured fluid to reduce the air volume of the pipette. It was found that such a pipette could measure small volumes of fluid with a high degree of accuracy. By rotating the screw of the syringe plunger the desired volumes of the various constituents of the haemolytic system can be drawn up the pipette with a bubble of air between each volume, and the mixture can then be expelled, by reversing the direction of the screw, into agglutination tubes of the Dreyer pattern. The mixture is thoroughly mixed and this is done by blowing small bubbles into the liquid from the pipette and shaking the tube so that the bubbles act as mixers. The tube is placed in a waterbath regulated by a gas-thermostat at 37°C. with 0.5°C. variation: and the time taken for the

complete haemolysis of the red blood corpuscles in the tube. This is evidenced by the change from an opaque suspension to a clear fluid.

The endpoint occurs in several stages of clearing, and it is customary to use as a standard the attainment of complete definition of a ruled black and white background placed behind the tubes. It has been found that haemolysis taking more than 360 seconds has a vague end-point, while haemolysis taking less than a minute appears very suddenly. The difference between two estimations may be quite appreciable on this account in the former instance, while in the latter case it is difficult to differentiate small differences in the complementary activity. Conditions are usually arranged so that the end-point lies between these two extremes, and duplicate estimations do not vary more than 0.5 - 5.0 seconds under such conditions.

The haemolytic system found suitable and so employed as standard in the timing method is:-

3.0 vols.	0.9% NaCl.
0.5 vols.	3% sheep cell suspension, sensitised with 5 units amboceptor.
0.5 vols.	fresh guinea-pig serum.
4.0 vols.	standard haemolytic system.

The quantities of the reagents employed may be expressed as absolute amounts or as a percentage volume of the system.

(b) Colorimetric Method.

This method consists in the estimation of the degree of haemolysis in a standard system at specified intervals of time. The methods devised to enable us to follow the progress of haemolysis from moment to moment are of two types:-

(i) The principle underlying the first type is that a suspension of red cells is opaque, while a haemolysed suspension is transparent. The intensity of light transmitted through the suspension is thus a function of the amount of haemolysis, and is an indirect measure of the number of cells haemolysed. The "indirect" methods differ in the way in which the intensity of the transmitted light is measured, e.g., opacimeter (Jacobs, 1930), radiometer (Ponder, 1923,1927), selenium photo-electric cell (Mellanby, 1925; Ponder, 1927), potassium photo-electric cell (Ponder and Yeager, 1930), and the Stuphenphotometer (Ponder, 1934). Ponder claims that the photometer exceeds all others in its accuracy for this type of work.

(ii) The principle underlying these methods is the direct estimation of the percentage of red cells destroyed either by counting the number which still remain intact or by estimating the quantity of haemoglobin liberated by the haemolysed cells. Vles, Prager and Bernstein (1931) employed a spectrophotometric determination of the absorption spectrum of haemoglobin at 542$\mu\mu$ to estimate the quantity of

haemoglobin liberated. But the simplest method, and the one employed, was a modification of a colorimetric method developed by Müller (1910). After incubating serum with a suspension of sensitised red cells he centrifuged down the non-haemolysed cells and compared the tint of the supernatant fluid with that of a series of standards containing known concentrations of haemoglobin. Brooks (1919), using a colorimeter, determined the amount of haemoglobin liberated in an amboceptor-complement haemolytic system by comparing the colour of the supernatant fluid with a standard preparation.

The author found that the colorimetric method was capable of considerable accuracy, and was especially useful in differentiating small variations in the haemolytic activity of complement. It was observed that the results were more reliable if the liberated haemoglobin was converted to the stable acid haematin by means of N/10 HCl.

The requisite volumes of the various constituent elements of the haemolytic system are measured out in the micropipette and the mixture is incubated at 37°C. in a Dreyer agglutination tube. After specified intervals of time the tubes are removed from the waterbath and placed in an ice-salt mixture to prevent further "binding" of complement. The tube is then centrifuged for one minute at 3,000 r.p.m. to throw down the non-haemolysed erythrocytes. A certain volume of the supernatant fluid is then treated

with dilute hydrochloric acid and matched in a Klett colorimeter against acid haematin standards prepared by laking the same suspension of sheep cells with dilute HCl, or against the Newcomer acid haematin standard.

In order to obtain a sufficient depth of tint to cover haemolysis values ranging from 0% - 100%, the following haemolytic system was employed:-

4.0 vols. 3% sheep cell suspension, sensitised 5 units amboceptor.
1.0 vol. fresh guinea-pig serum, diluted 1:10 to 1:30.
5.0 vols. haemolytic system.

After incubation four volumes of the supernatant fluid is treated with 1 c.c. N/10 HCl. and the mixture stood for 15 minutes until the tint of the acid haematin becomes of uniform intensity. The following acid haematin standards are prepared:-

(i) 12.5% haemolysis - 0.4 vols. sensitised corpuscles plus 3.6 vols. distilled water plus 1 c.c. N/10 HCl.

(ii) 25% haemolysis - 0.8 vols. sensitised corpuscles plus 3.2 vols. distilled water plus 1 c.c. N/10 HCl.

(iii) 50% haemolysis - 1.6 vols. sensitised corpuscles plus 2.4 vols. distilled water plus 1 c.c. N/10 HCl.

(iv) 75% haemolysis - 2.4 vols. sensitised corpuscles
plus 1.6 vols. distilled water
plus 1 c.c. N/10 HCl.

(v) 100% haemolysis - 3.2 vols. sensitised corpuscles
plus 0.8 vols. distilled water
plus 1 c.c. N/10 HCl.

Koopman and Falker (1935) have criticised the use of the time for complete haemolysis as a function of complementary activity of the serum. They consider that the time for the complement to produce the first trace of haemolysis should be used as an index of its concentration, since only a small fraction of the complement introduced into the system is adsorbed at the cell surface, and once haemolysis has commenced, various side-reactions inhibitory to complement action occur. They have, however, not taken into account the fact that the absorption of complement does not occur instantaneously, that the fraction adsorbed is dependent on the degree of sensitisation, and that the "latent period" during which no haemolysis occurs covers a relatively short period of time, which may prove difficult of estimation.

THE CONSTITUENTS OF AMBOCEPTOR-COMPLEMENT HAEMOLYTIC SYSTEMS.

(a) Antigen - amboceptor suspensions.

The experiments described were all carried out on systems containing sheep cells sensitised with anti-sheep

corpuscle serum. The cell suspension was prepared as follows:- two parts of sheep's blood were drawn into one part of a 2% sodium citrate solution. The corpuscles were thrown down by centrifuging and washed three times with three volumes of normal saline, being spun for 10 minutes at 3,000 r.p.m. between each washing. A volume of corpuscles was then suspended in 0.9% saline to give a 3% suspension (more concentrated suspensions were relatively unstable and underwent auto-agglutination even when moderately sensitised). This sheep cell suspension constituted the antigen throughout this investigation.

The red blood cells were then sensitised by adding the anti-sheep corpuscle serum. This was prepared at the South African Institute for Medical Research by injecting increasing doses of washed sheep erythrocytes into rabbits over a period of three months. The rabbits' blood was withdrawn, and the concentration of amboceptor or titre estimated. This is the smallest amount of amboceptor that will cause complete haemolysis of an amount of red cells, arbitrarily selected as a standard, in the presence of an excess of complement, in one hour at 37°C. This is the unit of amboceptor or its minimal haemolytic dose. The antiserum was finally put up in á titre strength of 1:8,000, and the degree of sensitisation employed was 2,5,6, or 10 M.H.D. In certain cases the Burroughs Wellcome 1:1,000

haemolytic anti-sheep serum, a glycerinated and stable preparation of the serum of an immunised horse, was employed. Kilduffe (1924,1933) found that glycerinated specimens of amboceptor showed no change in titre strength (1:20,000) after a period of 16 years without any special precautions to ensure a consistent temperature. The mixture was allowed to stand for an hour at room temperature or for half-an-hour at 37°C. before use to ensure complete sensitisation, though there is evidence that the absorption of amboceptor by the corpuscles is rapid and complete within thirty seconds (Eagle and Brewer, 1929; Ponder, 1932). Examination of the suspension microscopically shows that the cells tend to aggregate in clumps; further, the suspension tends to sediment more rapidly than is found in the case of an unsensitised suspension.

The sensitised suspension was freshly prepared each day as the corpuscles deteriorated within a few days even in the refrigerator. This was especially so in the case of highly sensitised suspensions, due to sedimentation and auto-agglutination phenomena. When tested against a standard conserved complement (vide infra) these daily prepared suspensions showed some variation in their rate of haemolysis. The variability

of the cell suspensions had no bearing on the estimations of complement except when the experiment covered a period of time and necessitated the use of different batches of sensitised cells. It was then found necessary to apply a correction to the results, calculated from a graph for the haemolysis time of the various suspensions against a standard conserved complement (table I). The time for complete haemolysis in a standard system was plotted daily, and a line drawn through the average distribution of the points. This yielded the haemolysis time for each day corrected for the individual daily variation, and from this all complement estimations could be corrected.

Three possible causes for this variability in the sensitised suspensions suggested themselves:-

(i) The variation in the number of red corpuscles in the suspension. 3% sheep cell suspensions, whose red cell counts had been determined, were tested against a standard conserved complement. The results (table 2) showed that, although the red cell count varied considerably in the daily suspensions, no definite correlation could be related between the number of red cells and the resulting time for haemolysis.

(ii) The variation in the resistance of the red cell to haemolysis; Ponder (1934) has shown that the red cells possess varying degrees of resistance to haemolysis

Conservation of complement:-
100 ccs. guinea-pig serum
10 gms. Sodium chloride.
4 gms. Boric acid.
Diluted 1:10 with distilled water for use.

Conservation of sheep corpuscles:-
3 vols. blood corpuscles.
5 vols. 5.4% Glucose.
2 vols. 3.8% Sodium citrate.
A 3% suspension was prepared in normal saline.

The haemolytic system employed consisted of:-
2.0 vols. diluted serum.
1.5 vols. 0.9% saline.
0.5 vols. sheep cell suspension, sensitised 5 units amboceptor.

Date.	Haemolysis Time, in Seconds.	
	Prepared cell suspension.	Conserved corpuscles.
8/3/34.	230.	-
9/3/34.	240.	-
10/3/34.	121.	-
13/3/34.	168.	-
14/3/34.	123.	-
15/3/34.	186.	222.
16/3/34.	136.	277.
17/3/34.	152.	235.
18/3/34.	-	247.
19/3/34.	248.	265.
20/3/34.	179.	262.
21/3/34.	-	253.
22/3/34.	-	267.
24/3/34.	263	256.
26/3/34.	214.	239.
27/3/34.	-	255.
28/3/34.	235.	254.
29/3/34.	247.	-
30/3/34.	205.	264.
31/3/34.	115.	245.
1/4/34.	-	224.
3/4/34.	212.	238.
4/4/34.	163.	253.
5/4/34.	195.	256.
6/4/34.	180.	240.

Table I. The Daily Variation in the Haemolysis Time of Conserved and Prepared Corpuscles when tested against Conserved Complement.

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Haemolytic System:-

0.5 vols. conserved complement, 1:10 dilution.

0.5 vols. 3% sheep cell suspension, sensitised 5 units amboceptor.

3.0 vols. 0.9% NaCl.

4.0 vols. standard haemolytic system.

Date.	Red Cell Count.	Haemolytic time in seconds.
7/8/34	345,000.	220.
8/8/34	489,000.	235.
9/8/34	258,000.	203.
10/8/34	316,000.	172.
13/8/34	385,000.	155.
14/8/34	522,000.	165.
15/8/34	374,000.	207.
16/8/34	302,000.	195.
18/8/34	364,000.	174.
20/8/34	281,000.	223.
21/8/34	276,000.	219.
22/8/34	410,000.	204.
23/8/34	365,000.	152.
24/8/34	317,000.	136.
27/8/34	368,000.	186.
28/8/34	270,000.	111.
31/8/34	392,000.	235.

Table 2. The Relation Between the Red Cell Count of a 3% Sheep Cell Suspension and Its Time for Haemolysis by a Haemolytic System Containing Conserved Complement.

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during amboceptor-complement haemolysis. From fragility test reactions it is found that the sheep cell commences to haemolyse in 0.6 - 0.5% NaCl, and that there is considerable variation from sample to sample. It is suggested that the susceptibility to haemolysis may vary from one sheep to another and from day to day in the same animal.

(iii) The variation in the agglutinogens of the sheep cell suspensions. Since the sheep cell suspension is not prepared from the same sheep, the agglutinogens of the corpuscles may show great variation, and therefore varying degrees of incompatibility with the agglutinins of the sensitising serum.

In order to overcome the variability of the sensitised cell suspension to haemolysis the use of "conserved standard" corpuscles has been advocated. The method employed in their preservation was that of Fea (1933). Three volumes of blood corpuscles were added to five volumes of 5.4% glucose and two volumes of 3.8% sodium citrate. Observations were made daily of the time for haemolysis of these corpuscles against a standard conserved complement simultaneously with observations on the prepared cell suspensions (table 1). The conserved suspension yielded results more constant in character, and remained stable for a considerable period.

The concentration of amboceptor present, that is, the degree of sensitisation, played an important part in the

estimations. Within limits the more the amboceptor the greater the complementary power and the less its minimal haemolytic dose. Hence, in order that a series of complement estimations might be comparable it was necessary to use equally sensitised corpuscles. This is however complicated by the fact that most mammalian sera with the exception of the guinea-pig contain naturally occurring amboceptor to sheep red corpuscles. It is uncertain whether these amboceptors are identical with those formed by repeated injection of washed sheep erythrocytes into the bloodstream of another species (v. Euler and Gard, 1931), but they have been found to act in a similar manner and they yield a potential source of error in complement estimations. This is due to the fact that the degree of sensitisation is increased to a variable amount above that of the artificial sensitisation owing to an unknown amount of naturally occurring amboceptors.

In the case of guinea-pig sera "true" complement estimations may be carried out without any pre-treatment of the serum, and that is the reason why guinea-pig serum was employed throughout these investigations. With regard to human sera it is necessary to remove these naturally occurring amboceptors before carrying out estimations since they not only vary from individual to individual but probably in the same individual from time to time. The

removal of these amboceptors is a simple matter (Osborn, 1936a); the method depends on the fact that sheep red cells bind these amboceptors at 0°C.; complement is not bound at this temperature, though highly sensitised corpuscles bind midstuk at 0°C. (Sachs and Bolkowska, 1910). Conditions can therefore be arranged so that unsensitised corpuscles can remove naturally occurring amboceptors quantitatively without reducing the complement in a serum containing both. This is done by treating the serum for thirty minutes at 0°C. (in an ice-salt mixture) with an equal volume of a 3% suspension of unsensitised cells and estimating the complement activity of the supernatant fluid. An analysis of Osborn's results (1936a) shows that the degree of sensitisation produced by naturally occurring amboceptors in human and rat sera was equivalent to 5 units of artificial sensitisation.

(b) Diluting Fluid.

The diluting fluid was prepared by dissolving sufficient "pro analysi" sodium chloride in glass distilled water to give a 0.9% solution, whose osmotic pressure corresponded most closely to the osmotic pressure of mammalian blood (p.110). Its only disadvantage lay in the fact that its pH was on the acid side of neutrality (pH 6.8), and buffered solutions were therefore tried, viz., Ringer-Locke and Lamb's "equilibrated pH 7.4" solutions. As can

be seen from the results, (table 3), they possessed no advantage over 0.9% NaCl., which was simpler to prepare. Faber and Black (1936) have made an exhaustive study of the influence of various physiological salines in complement fixation reactions. Their results indicated the necessity for a uniform saline in any proposed standard procedure for complement estimations. They found that saline prepared from tap-water (pH 7.6 - 7.8) required less complement to produce haemolysis than that prepared from distilled water (pH 6.4 - 6.6), and they attributed it to the difference in pH, an assumption which has been proved correct. The addition of phosphates or magnesium chloride to the distilled water saline decreased the complement titre about one-half, and it would appear that is not expedient to steam, heat, or autoclave the salines, since increased evaporation and subsequent concentration of the salts affected the titre. The conclusions of Brooks (1920), Mason and Sanford (1924), Rockwood (1925), Kellogg and Wells (1926), Wadsworth (1927), Kolmer (1928) and Gradwohl (1935) should also be consulted in this connection.

(c) Complement.

A healthy guinea-pig is killed by a sharp blow on the head at the junction with the neck; the throat is cut and the blood is caught in a clean funnel leading

Date.	Sample.	Haemolysis Time in Seconds.		
		Ringer-Locke.	Lamb.	0.9% NaCl.
15/3/34.	Fresh Human Serum.	65.	64.	68.
	Conserved Guinea-pig Serum.	108.	107.	107.
16/3/34.	Fresh Human Serum.	70.	72.	75.
	Conserved Guinea-pig Serum.	-	129.	125.
19/3/34.	Fresh Human Serum.	66.	67.	61.
28/3/34.	Fresh Human Serum.	118.	115.	113.
	Fresh Human Serum.	160.	164.	166.
4/4/34.	Fresh Human Serum.	112.	110.	97.
	Fresh Human Serum.	107.	110.	103.

Table 3. The Effect of Ringer-Locke, Lamb and NaCl. Isotonic Solutions on the Complementary Activity of Sera.

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into a clean testtube. The serum is allowed to separate in a refrigerator, and is ready for use eighteen hours later. In other cases (human, rabbit, rat, cat) the blood is drawn by various techniques and allowed to clot at room temperature for one hour. It is then centrifuged, and the serum stored in a refrigerator. Before use, the serum is treated for thirty minutes at 0°C. with an equal volume of a 3% suspension of unsensitised sheep erythrocytes to remove naturally occurring amboceptors.

The unit or minimal haemolytic dose of complement is the smallest amount that will haemolyse one unit of sheep cells, arbitrarily selected as a standard, in the presence of an excess of amboceptor in an hour at 37°C. The titre usually found varies from 1:60 to 1:120, i.e., 60 - 120 M.H.D. per c.c., so that the 0.5 volumes of serum used in this investigation contains 3-5 M.H.D. of complement.

Owing to the extreme lability of complement success in quantitative work depends largely on the precautions taken to reduce its "rate of deterioration" to a minimum during the time of the experiment. It is essential to observe absolute cleanliness of the containing vessels, equable conditions of temperature, and to avoid shaking the sample. An effective means of preserving the complementary activity of a serum is to bubble carbon dioxide

through it; this produces a reversible inactivation of the complement, the mechanism of which is not clearly understood (p. 105.)

Standard conserved complement may be prepared by hypertonic inactivation of serum; this preserves its original activity in latent form, so that subsequent dilution to isotonicity restores its activity. The methods employed are:-

(i) Addition of sodium acetate and boric acid to serum to make their final concentration 10% and 4% respectively (Ruffner, 1929; Sonnenschein, 1930).

(ii) Addition of sodium chloride and boric acid to serum to make their final concentration 10% and 4% respectively (Ginsberg and Kalinin, 1929).

(iii) Addition of sodium sulphate and boric acid to serum to make their final concentration 5% and 4% respectively (Kalinin and Ginsberg, 1933).

The serum is diluted with nine volumes distilled water before use. It was found that on restoring the serum to isotonicity, the complementary activity took about ten minutes to attain an equilibrium (table 4). Little deterioration was noted in conserved samples kept for sixty days, and the method is of considerable value when a constant complement is required.

The use of serum in undiluted form for complement

estimations is recommended since, immediately the serum is diluted, the complement loses its property of acting like a stable haemolytic agent. The greater the degree of dilution the faster is the rate of deterioration of the complementary activity of the serum. Further, it has been observed that in the dilution of a serum the complement activity takes a certain time to reach a stable equilibrium (table 5). It is essential that any dilutions of complement required should be made at $0^{\circ}C$. immediately prior to the experiment.

Conserved Complement is diluted 1:10 with distilled water.

	Haemolysis Time in Seconds.
Before dilution.	460.
After dilution.	
30 secs.	376.
1 min.	301.
2 mins.	193.
5	181.
10	176.
15	179.
20	172.
25	183.
30	174.

Table 4. The Haemolytic Activity of Conserved Complement on Dilution to Isotonicity for Use.

Diluted fresh guinea-pig serum is estimated for haemolytic activity at intervals:-

	Haemolysis Time.		
Before Dilution.	60 secs.	61 secs.	61 secs.
After dilution.	1:5	1:10	1:15
30 secs.	73 secs.	92 secs.	152 secs.
1 min.	78.	126.	169.
2 mins.	85.	127.	188.
5	89.	134.	191.
15	91.	132.	185.
20	90.	-	-
25	92.	-	-
30	95.	135.	194.

Table 5. The Haemolytic Activity of Fresh Guinea-pig on Dilution with Isotonic Saline.

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SECTION III.

THE PROPERTIES AND KINETICS OF AMBOCEPTOR - COMPLEMENT HAEMOLYSIS SYSTEMS.

The study of the kinetics of haemolysis in amboceptor - complement systems presents considerable interest, since the haemolytic agent is composed of a compound haemolysin, namely, amboceptor and complement. It is now generally accepted that the sequence of events in complement haemolysis of the red cells is the initial absorption or concentration of the amboceptor at the cell surface with a consequent alteration in the properties of the cell membrane. This enables the cell to absorb complement which liberates the haemoglobin from its structure by chemical and/or physical reactions which are at present not clearly understood. The points of interest are the mechanism of absorption of the amboceptor and complement and the method whereby complement causes haemolysis of the red blood cells. There has been much conflict of opinion in the literature on this particular subject, as is apparent from the account given in Section I. This has been due to the inadequate methods of estimation and experimentation. The recent work of Ponder (1932, 1934, 1936) has done much to show the line of research that is necessary in this problem.

EXPERIMENTAL.

The properties of each agent in the heamolytic

system, namely, antigen, amboceptor, and complement, can be studied by varying the concentrations of the haemolytic components of the system:-

(i) antigen varying, amboceptor and complement constant.

(ii) antigen constant, amboceptor varying, complement constant.

(iii) antigen constant, amboceptor constant. complement varying.

(iv) antigen constant, amboceptor and complement varying.

The analysis of the effects of complement haemolysis is not completed by the study of the results produced by an alteration in the concentration of one of the reacting substances; in addition it is necessary to study the phases of haemolysis during the haemolytic action by means of percentage haemolysis curves. The timing and colorimetric methods described in Section 2 are available for the study of time-dilution and percentage haemolysis curves respectively.

The selection of a standard haemolytic system for reference was considered essential. In the case of the timing method the most suitable system was found to be:-

3.0 vols. 0.9% NaCl.

0.5 vols. sensitised sheep all suspension amboceptor).

0.5 vols. guinea-pig serum.

The concentration of antigen and amboceptor is such that no auto-agglutination occurs for many hours, a common occurrence if the concentration of either substance is increased more than 200%. A more dilute sheep cell suspension gives a poor haemolysis tint and increases the time for haemolysis unduly, leading to some difficulty in ascertaining the endpoint. The endpoint is also delayed if the degree of sensitisation is lowered.

The amount of complement present in this system yields an endpoint between 60 - 100 seconds, a suitable time for estimation. Any increase in the amount of serum used does not accelerate the haemolysis time on account of the effect of serum protein inhibition, while a lower concentration prolongs the endpoint considerably. The advantages of this system for standard reference are therefore manifest.

For the colorimetric method the system employed for standard reference is:-

4.0 vols. sensitised corpuscles (5 units amboceptor).

1.0 vol. guinea-pig serum, 1:20 dilution.

Haemolysis occupies 12 - 16 minutes, so that an adequate percentage haemolysis curve can be obtained.

The reagents required in the investigation,

viz: sensitised sheep cell suspensions, guinea-pig serum, and normal saline, are kept in an ice plus salt mixture at -5^{o} to $-10^{o}C$. By this means the rate of decay of the complement is reduced to a minimum, and the complementary power of the serum is found to be unchanged after eight hours.

(i) Concentration of Antigen varied in standard Haemolytic System.

A series of sheep cell suspensions were made up varying in strength from 1% - 20%, and sensitised with 5 M.H.D. of amboceptor in the usual manner. The time taken to haemolyse a volume of these sensitised suspensions by an equal volume of guinea-pig serum was estimated in the following haemolytic system:-

3.0 vols. 0.9% NaCl.

0.5 vols. sensitised sheep cell suspension.

0.5 vols. conserved complement (p. 44).

The results are recorded in table 6, and show that the time for haemolysis increases as the strength of the sheep cell suspension is raised, until there is incomplete clearing in the 20% sensitised suspension due to the presence of an insufficient concentration of complement.

Strength of Suspension.	Haemolysis Time in Seconds.
1%.	123.
3%.	166.
5%.	225.
10%.	420.
20%.	incomplete haemolysis, 60 minutes.

Table 6. The Haemolysis Times of Sensitised Sheep Cell Suspensions by a Conserved Complement.

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(ii) Amboceptor varying, Complement constant.

A series of 3% sheep cell suspensions are sensitised with quantities of amboceptor varying from 2-100 units amboceptor. The time required to haemolyse these sensitised suspensions is tested in the usual haemolytic system consisting of:-

3.0 vols. 0.9% NaCl.

0.5 vols. sensitised sheep cell suspension.

0.5 vols. fresh undiluted guinea-pig serum.

The results are plotted in the form of a graph (fig.1). The time - concentration graph for amboceptor expresses the relation found, in any haemolytic system, between the initial dilution of the amboceptor and the time taken by it to produce complete haemolysis in the presence of a constant and adequate concentration of complement. It is found that time for complete haemolysis increases in a regular manner as the concentration of the amboceptor is decreased. This relation breaks down in the presence of low or very high amounts of amboceptor. As the quantity of amboceptor becomes less, so the quantity of complement required to produce complete haemolysis of the sheep erythrocytes becomes greater. A high concentration of amboceptor will lead to a retardation of haemolysis, which Ponder (1932) ascribes to the inhibition produced by the increased amount of serum proteins introduced in

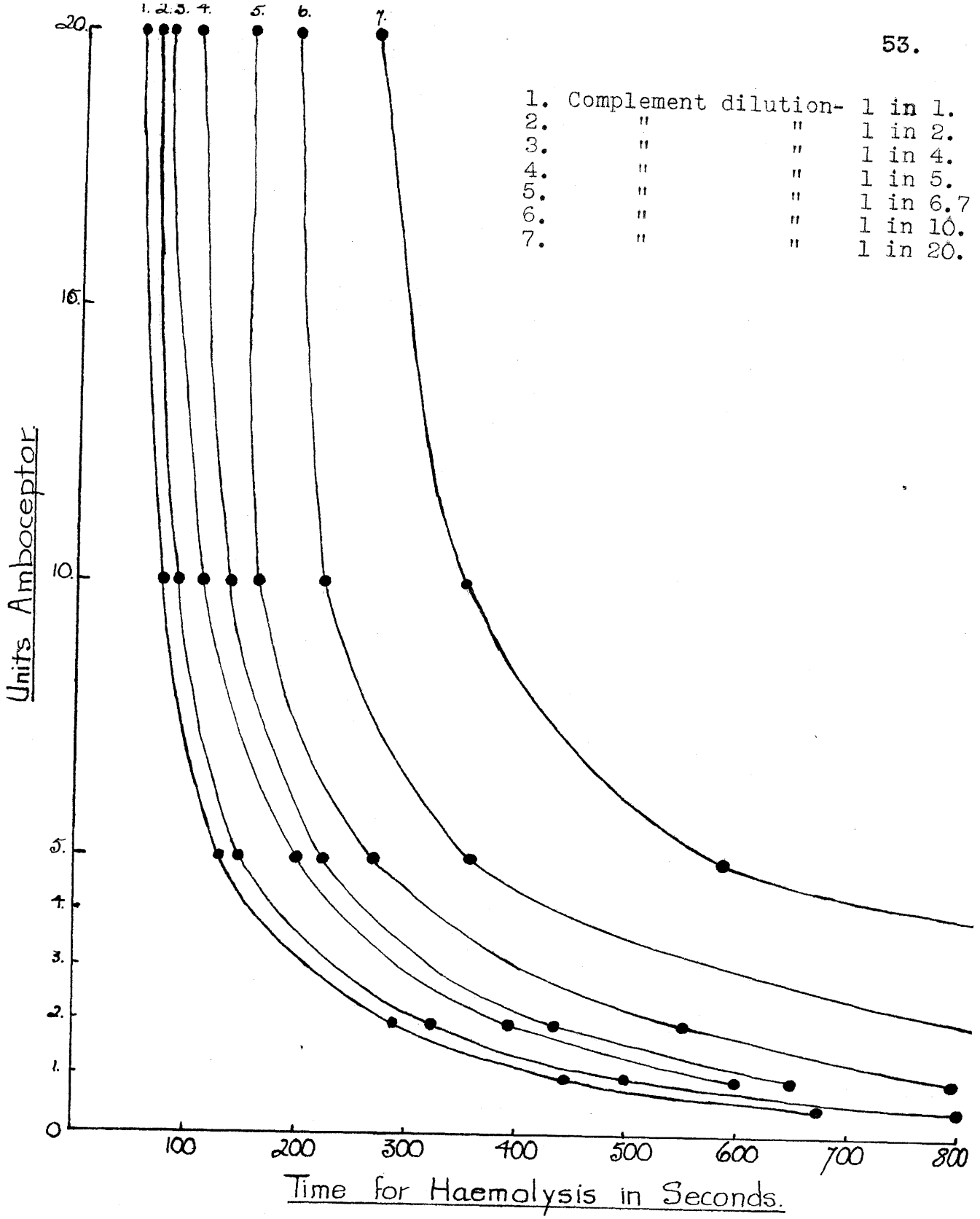

Fig.1. Time/ Amboceptor Dilution Curves for Guinea-pig Serum in Systems containing varying amounts of Complement.

this way. I have observed that highly sensitised corpuscles show a marked tendency to auto-agglutinate. This will reduce the surface area of the corpuscles to a considerable degree, and may in this way slow down amboceptor and complement adsorption by the cells.

If the various suspensions of sensitised cells are tested against diluted sera (complement), it is seen that an increase in the concentration of amboceptor will compensate for the decrease in complement concentration with the limits of ordinary experimental work. For example, a sensitisation of 20 units amboceptor and 1 in 20 complement yields the same haemolysis time as a sensitisation of 2 units amboceptor and undiluted complement. This relation of the amboceptor concentration to the amount of complement which has to be used to bring about haemolysis is often referred to as the reciprocal or interchangeable action of complement and amboceptor (Thiele and Embleton,1914; Eagle and Brewer, 1929; Ponder, 1934, 1936).

(iii) Amboceptor constant, Complement varying.

The dilution of the serum must be carried out under rigidly controlled conditions. The serum is kept in an ice-salt mixture (-5° to -10°C.). Ice-cold saline is run into small testtubes and serum measured into each so as

to give a range of dilutions of complement from 1 in 1 to 1 in 100. In the case of the higher dilutions they are prepared immediately prior to introduction into the haemolytic system, and are stood ten minutes to attain equilibrium (p. 45,46). The testtubes are kept in the ice-salt mixture. At this temperature it is found that there is no deterioration of complement up to six hours in serum dilutions up to 1 in 20 and stability from $\frac{1}{2}$ - 4 hours in greater dilutions. Duplicate estimations of the haemolysis time in the standard four volume system were made for each dilution employed and the results confirmed by preparing a fresh set of dilutions an hour later.

The results are graphed as a time - complement dilution curve (fig. 2). The curve thus obtained does not show the regular curve described by Ponder (1932,1934,1936), and it differs in several material respects. In the first place Ponder says that "the curve resembles a time-dilution curve for a simple typical haemolysin, for it passes very nearly, if not quite, through the origin, and proceeds rapidly to its asymptote." If we provisionally treat the curve as a typical time-dilution, and proceed to analyse it after the method of Ponder (1936a), it is found that the theoretical and experimental curves are not

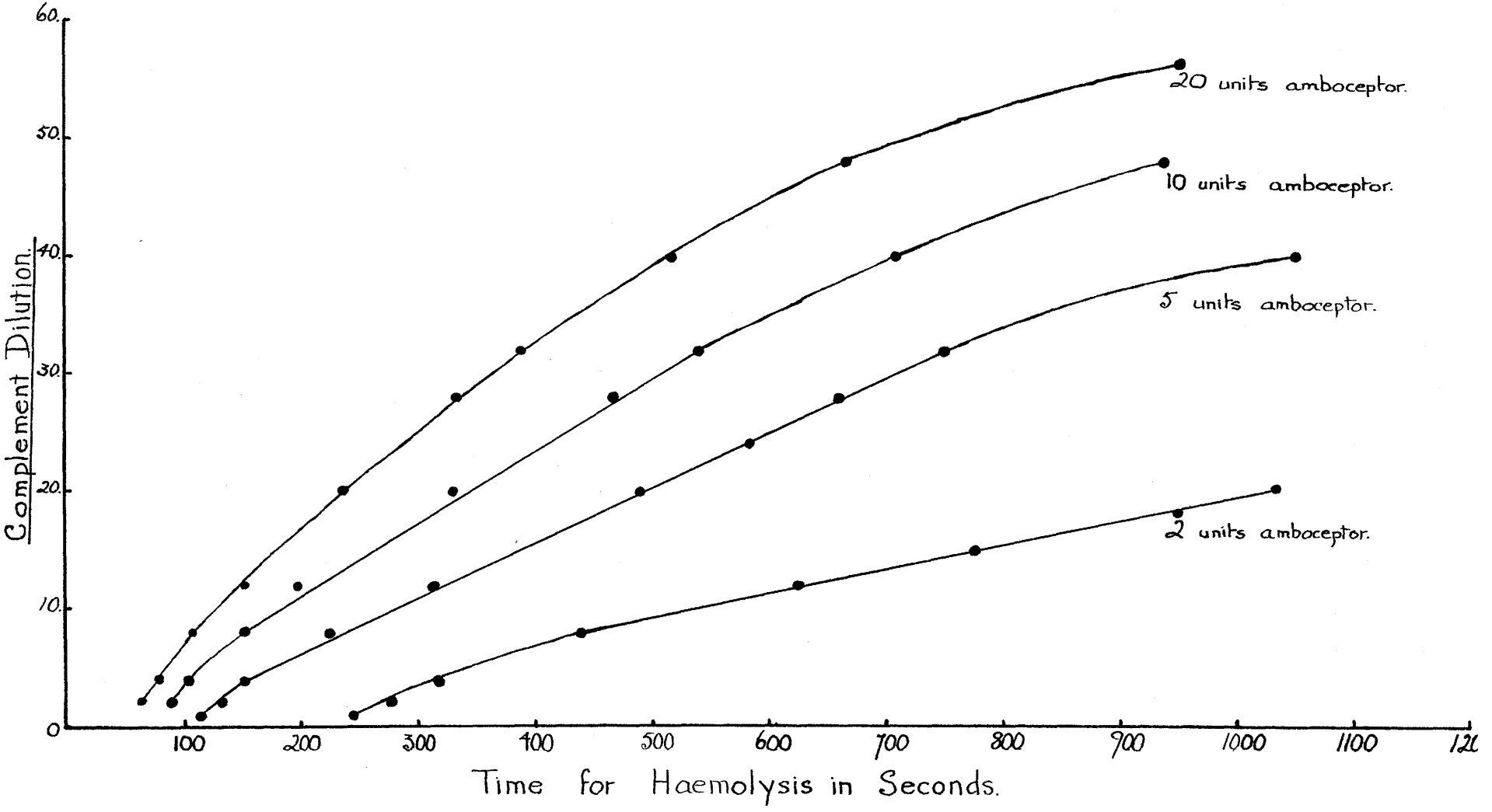

Fig.2. Time/Complement Dilution Curves For Guinea-pig Serum in Systems Containing Varying Amounts of Amboceptor.

in agreement, for while they agree near the origin and the asymptote, the middle portion of the curve was consistently found to be so flattened that, allowing for experimental error and allowing for the standard co-efficient of error, it appears justifiable to draw a straight line through the estimated points (fig. 2). Ponder, by taking a small number of observations for the time-dilution curve, has not paid sufficient attention to the lower part of the curve. Under experimental conditions it was found in the system employed that, when various high concentrations of amboceptor and complement were used, the time for haemolysis never fell below 40 seconds. Further, Ponder does not quote haemolysis times faster than 66 seconds. The failure of the curve to pass through the origin is due to two factors:- firstly, a well-defined "latent period" during which no haemolysis occurs at all, and secondly, an inhibitory influence exerted on complement by the high concentration of serum proteins which are introduced into systems containing only undiluted complement and/or very concentrated amboceptor. The fact that in vivo the system contains about 7% protein appears to indicate that the complement cannot be acting to the best of its ability under such conditions.

If the amount of haemolysis is traced in the amboceptor - complement haemolytic system from time to time,

an important relation is found between the amount of haemoglobin liberated in each successive unit of time. A series of tubes is prepared containing the following system:-

4.0 vols. sensitised sheep cell suspension
(5 units amboceptor)
1.0 vol. guinea-pig serum (diluted 1:10 to 1:50).

The haemolytic system is prepared from materials kept in an ice-salt mixture. The tubes are placed in a waterbath at 37°C., and every two minutes, a tube is removed and placed in the ice-salt mixture. Since complement is not bound at 0°C., the time taken for the system to be heated to 37°C from 0°C. and to be cooled back to 0°C neutralise each other, so that the time of action of the complement may be regarded as absolute. The tube is then centrifuged for 1 min. at 3,000 r.p.m. to throw down the unhaemolysed cells, and the supernatant fluid is treated with N/10 HCl to convert the haemoglobin liberated by haemolysis to the more stable acid haematin. A test volume of this fluid is compared in the colorimeter with prepared acid haematin standards from the same sample of corpuscles.

A protocol of one experiment is given in table 7, and a curve has been drawn from other results (fig.3). It is seen that there is an initial "latent period" during which there is no liberation of haemoglobin; this occupies less than a sixth of the time for complete haemolysis. The haemolytic

Haemolytic System:-

4.0 vols. 5% sheep cell suspension, sensitised 5 units amboceptor.

1.0 vol. 1:20 guinea-pig serum.

5.0 vols.

Colorimeter Test:- 4.0 vols. supernatant fluid + 1cc. N/10 HCl.

Prepared Acid Haematin Standards:- 12.5%, 25%, 50%, 75%, 100% haemolysis by laking the appropriate volume of unsensitised cells, and adding 1cc. N/10 HCl.

Standard	Test	% Haemolysis	Hence % Haemolysis of Test.
15.	0.496.	12.5.	6.2.
15.	0.896.	25	22.4.
15.	0.802.	50	41.0.
15.	1.168.	50	58.4.
15.	0.771.	100	77.1.
15.	0.949.	100	94.9.
15.	0.982.	100	98.2.
15.	0.994.	100	99.4.

Minutes after incubation.	% Haemolysis.	% of haemolysis in preceding two minutes.
2.	6.2.	6.2.
4.	22.4.	16.2.
6.	41.0.	18.6.
8.	58.4.	17.4.
10.	77.1.	18.7.
12.	94.9.	17.8.
14.	98.2.	3.3.
16.	99.4.	1.2.

Table 7. A Typical % Haemolysis Curve in the Amboceptor-Complement Haemolysis System Employed.

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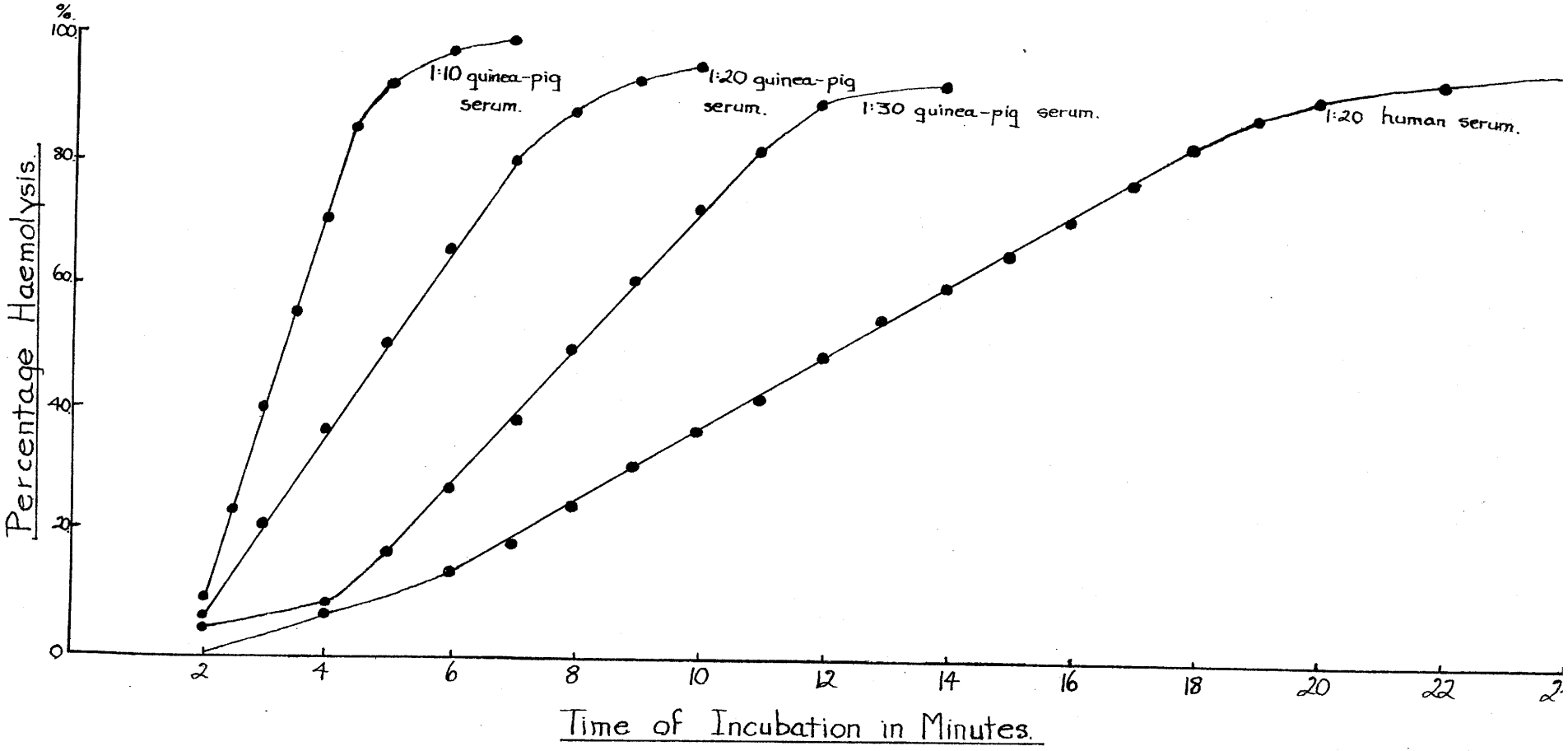

Fig. 3. Percentage Haemolysis Curves for Guinea-pig and Human Sera of Various Dilutions.

action of complement is then seen to proceed at a uniform rate, a uniform amount of haemoglobin being liberated in each successive unit of time. Towards the end of the haemolytic action the speed of the reaction slows down considerably.

A comparison of the percentage haemolysis curves obtained by this method with those obtained by Ponder using a different haemolytic system shows no quantitative differences.

(iv) Amboceptor and Complement varying.

In this series of experiments haemolytic systems were prepared in which the concentration of the complement was decreased as the concentration of the amboceptor was increased or decreased.

Various dilutions from a sample of fresh serum (1:1 to 1:60) were pipetted into a series of testtubes, and the equivalent of 5 units anti-sheep corpuscle serum was added to each dilution. The complement titre of the serum employed was 1:100, so that the volume of the 1:1 dilution (0.5 volumes ≒ .05 cc.) used contained 5 M.H.D. of complement. This was series 1.

To a second series of serum dilutions the sensitisation dose of amboceptor was doubled as each successive serum dilution was diluted by one-half. This

represented series 2.

To the original sample of serum amboceptor to the extent of 100, 400, and 1,000 units was added. From this serum similar dilutions were prepared, so that as the concentration of the complement was decreased so was there a diminution in the concentration of the amboceptor introduced into the system. This was series 3.

The time for haemolysis of these serum preparations was determined in the following haemolytic system:-

3.0 vols. 0.9% NaCl.
0.5 vols. guinea-pig serum.
0.5 vols. 3% sheep cell suspension.
4.0 vols. haemolytic system.

The observations are recorded in table 8, and have been analysed graphically in fig. 4. Compared with the time-dilution curve for the series containing a sensitising dose of five units amboceptor, the time-dilution curves for series 3 show that the degree of sensitisation has a pronounced influence on the time for haemolysis depending on the concentration of complement. Since the amboceptor does not produce haemolysis on its own account, it is apparent that the greater the concentration of the amboceptor introduced into the system the greater is the amount of complement absorbed by the cells in comparison with the amount introduced into the system. This is evident from the

Serum dilution.	Complement dose, M.H.D.	Antibody dose, M.H.D.	Haemolysis time in secs.	Antibody dose, M.H.D.	Haemolysis time in secs.	Antibody dose, M.H.D.	Haemolysis time in secs.	Antibody dose, M.H.D.	Haemolysis time in secs.
1:1.	5.00.	5.	302.	5.	295.	100.	108.	400.	72.
1:2.	2.50.	5.	325.	10.	275.	50.	137.	200.	94.
1:4.	1.25.	5.	361.	20.	264.	25.	207.	100.	145.
1:6.	0.83.	5.	408.	30.	260.	18.75.	275.	75.	188.
1:8.	0.63.	5.	440.	40.	250.	12.5.	340.	50.	228.
1:12.	0.42.	5.	548.	60.	283.	9.38.	490.	37.5.	310.
1:16.	0.31.	5.	930.	80.	355.	6.25.	867.	25.	375.
1:20.	0.25.	5.	2400.	100.	445.	5.0.	-	20.	500.
		SERIES I.		SERIES 2.		SERIES 3.		SERIES 3.	

Table 8. The Effect of Varying the Concentration of Amboceptor and Complement Introduced into a Haemolytic System.

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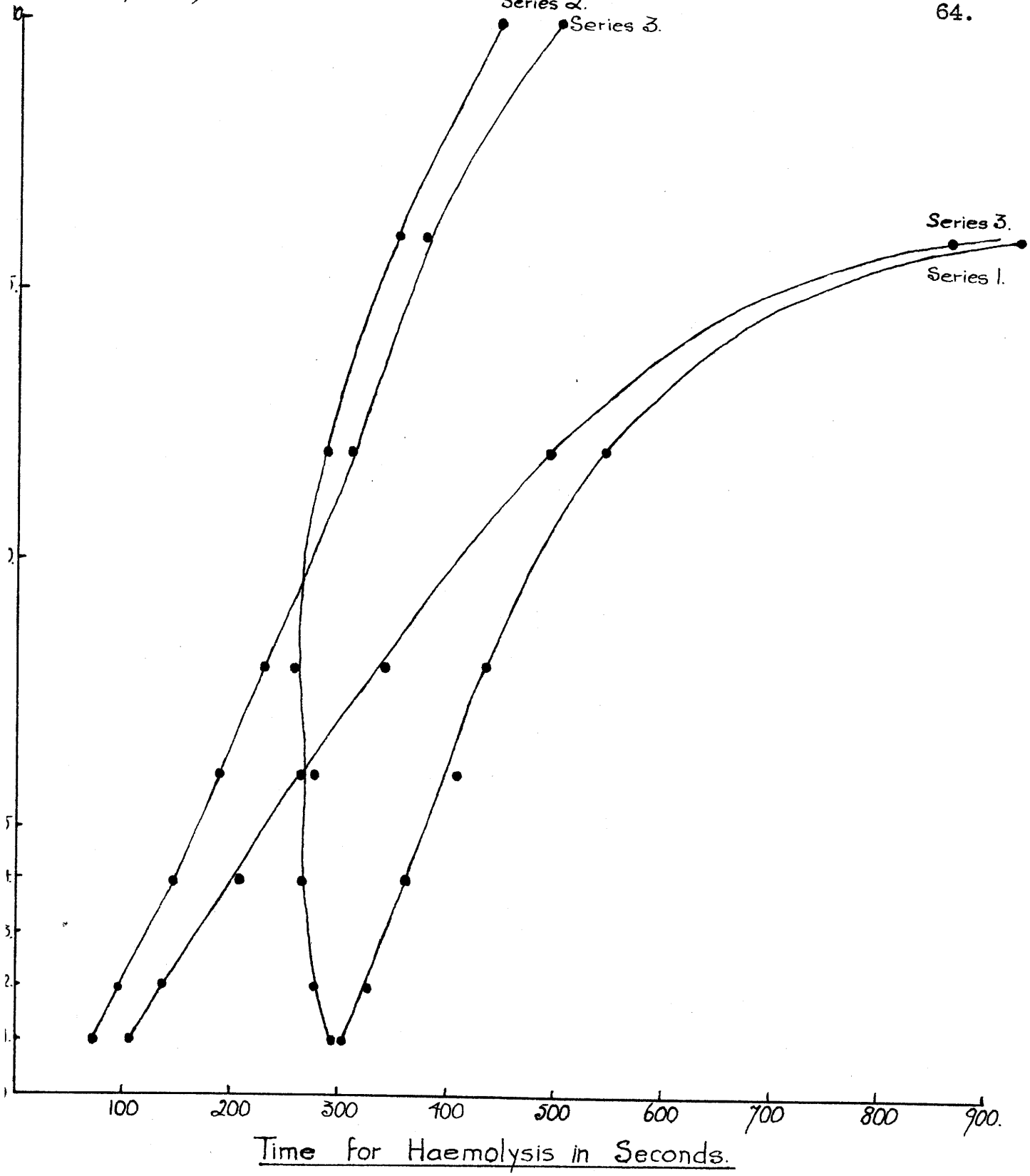

Fig.4. The Effect of varying the Concentration of Amboceptor and Complement introduced into a Haemolytic System.

results of series 2, where the increase in sensitisation permits of a greater proportion of the complement to be absorbed from the system, despite the fact that the concentration of complement is diminishing. Within these limits the time-dilution curve is therefore atypical, the slowing of haemolysis as the complement is diluted being slight, and there may be some acceleration of haemolysis. A point is reached when the maximum sensitisation does not produce a sufficient absorption of complement by the cells and haemolysis will be incomplete. Further, a very concentrated amboceptor tends to inhibit the absorption of complement due to the inhibitory effect exerted by the serum protein.

DISCUSSION.

The Relation between Amboceptor and Complement.

A constant concentration of amboceptor and complement will produce a constant time of haemolysis if the amount of antigen present in the system is not altered. As the amount of antigen is progressively increased, so the degree of haemolysis produced decreases in a regular manner. A stage is eventually reached when the dose of complement or amboceptor is less than one for that particular concentration of antigen, so that the resulting haemolysis is incomplete after one hour.

If the amount of amboceptor which is introduced into the haemolytic system is varied, it is found that the time for haemolysis is decreased as the amboceptor is increased. This relation holds within narrow limits of amboceptor concentration variation for any complement concentration, a time-amboceptor dilution curve being obtained of a hyperbolic character (fig. 1). In the presence of a constant complement an increase in the quantity of amboceptor introduced into the system does not lead to an accelerated haemolysis since the serum proteins introduced have an inhibitory influence on complement, while a decrease in the degree of sensitisation produces a subminimal amboceptor dose for haemolysis. The nature of this phenomenon can only be clearly understood from a study of the absorption of amboceptor and complement by the antigen. Ponder (1932, 1934, 1936), in a study of the kinetics of amboceptor-complement haemolysis, found that the amount of amboceptor removed by the cells was a constant fraction, within the limits in which experiment was possible, of the amount with which the cells were treated, i.e. the sensitising agent might be concentrated at the cell surface, but the quantity concentrated was a linear function of the amount introduced into the system, showing a constant value between 0.7 and 0.8. The actual amount of amboceptor adsorbed by the suspension is about three-quarters of that introduced into the system, (p. 98.) and is independent of the concentration of

the antigen, the degree of sensitisation, or the concentration of the complement.

Ponder further showed that the absorption of amboceptor was rapid and complete within thirty seconds, while the cells could apparently take up unlimited quantities of amboceptor. As the quantity of amboceptor became less, so the quantity of complement required to produce complete haemolysis became greater, and when the quantity of amboceptor was zero, the velocity constant approached zero, so that no haemolysis was possible even in the presence of an infinite concentration of complement. If, on the other hand, the concentration of amboceptor became very great, the quantity of complement required to bring about haemolysis tended towards a constant minimal value. This relation, in which a decrease in the concentration of either component is compensated for by an increase in the other, is illustrated in table 8, series 2, and can also be seen from an analysis of figure 1.

The question of the absorption of complement by the red cell has been very fully considered by Eagle (1929,b), Eagle and Brewer (1929), and Ponder (1932, 1934). These investigations have shown that sensitisation by amboceptor confers on the erythrocyte the property of absorbing complement, and that this absorption is a necessary preliminary to haemolysis. Complement they regard as a simple haemolysin,

which is absorbed by the cells in a constant fraction of the quantity initially introduced into the system in the presence of a constant amount of amboceptor. If the quantity of amboceptor present is great a large fraction of complement(.5) is absorbed independent of the complement concentration, while if the quantity of amboceptor is diminished a smaller proportion (·1) is absorbed. Absorption of a small fraction from a relatively concentrated complement due to a low sensitisation, and absorption of a large fraction from a relatively dilute complement due to a high degree of sensitisation, result in the absorption of a constant quantity and the transformation of a constant quantity in the production of the resultant haemolysis. The velocity of haemolysis is thus determined by the amount of complement so absorbed, and decreases in a regular manner as the concentration of the complement introduced into a system containing a constant amboceptor is diminished (fig.2). The time-dilution curve thus obtained differs from the curve published by Ponder in that it does not pass through the origin and its middle portion is flattened. Although the upper portion of the curve, if treated as a typical time-dilution curve according to the expression

$$dx/dt = k(C-x)^n$$

results in n = 1.0-1.1 in harmony with the results of Ponder, the lower portion of the curve does not fit.

(fig.3). There is an initial latent period during which no haemolysis occurs; it is partly taken up by the period during which absorption of the complement by the sensitised red cell occurs. Once haemolysis has commenced it proceeds at a uniform pace, the slowing down of the haemolytic process towards the end being due to the presence of red cells with a high resistance to haemolysis. Such curves do not fit in with the "monomolecular reactions" (law of mass action) postulated by Arrhenius(1915) for biological reactions; for such reactions the velocity of the reaction is

It seems that Ponder has not analysed the lower part of the curve in sufficient detail owing to the few experimental observations made.

The Nature of Complement Action.

Ponder's conclusion that a system containing cells, amboceptor and complement can be treated as a simple haemolysin, complement, after allowance is made for the degree to which it is absorbed at the cell interface, appears to require some amplification. Although the general reaction in a time-dilution curve is one of decreasing velocity, it was found that within a certain range, the reaction tended to be of constant velocity (fig.2).

If percentage haemolysis curves are constructed, a sigmoid or S-shaped curve is obtained under all conditions (fig.3). There is an initial latent period during which no haemolysis occurs; it is partly taken up by the period during which absorption of the complement by the sensitised red cell occurs. Once haemolysis has commenced it proceeds at a uniform pace, the slowing down of the haemolytic process towards the end being due to the presence of red cells with a high resistance to haemolysis. Such curves do not fit in with the "monomolecular reactions" (law of mass action) postulated by Arrhenius(1915) for biological reactions; for such reactions the velocity of the reaction is

determined by the concentration of unhaemolysed cells, and the curve is uniformly concave to the t- axis. Table 7 shows that the speed of the reaction is independent of the concentration of the residual, unhaemolysed corpuscles, once the complement absorbed is acting at full strength. This is characteristic of the action of many enzymes, and a further analogy to enzyme action is found in the observation that in low concentrations of complement the speed of the reaction is proportional to the amount of complement present (Osborn,1936,c).

The fact that complement does not haemolyse an unlimited quantity of corpuscles given infinite time, but appears to obey the law of definite proportions is not contrary to enzyme action, since many enzymes appear to obey the law of definite proportions. This is due to the production of products during the action which destroy the enzyme or complement (Northrop, 1922; Osborn, 1936c).

SECTION IV.

THE EFFECT OF VARIOUS PHYSICAL AND CHEMICAL AGENCIES ON THE COMPLEMENT ACTIVITY OF SERUM IN VITRO.

In the following series of experiments the behaviour of complement towards alterations in the osmotic pressure of the medium, variations of the tensions of the respiratory gases, and the addition of a series of non-hydrolysable salts to the haemolytic system, has been studied in vitro. These problems are fundamental to a study of complement action, since it has long been recognised that slight variation from the physical characteristics and chemical composition of the "normal" medium as represented by the blood produces marked variation in the complementary power. It was hoped that as a result of these experiments a means of artificially increasing the complement might be found, a measure which would prove to be of inestimable value, since complement is closely related to the general bodily resistance of the individual. In spite of the lability of complement and its ready tendency to undergo inhibition or inactivation, certain in vivo procedures are known which produce a marked increase in the complementary activity of the serum, e.g., stasis (Osborn, 1935), injection of adrenaline (Osborn, 1936b), ingestion of cod-liver oil (Osborn, 1933), and generalised thyroid and adrenal gland activity.

SECTION IVa.

THE INFLUENCE OF THE RESPIRATORY GASES ON HAEMOLYTIC COMPLEMENT.

Lumière and Grange (1928) have shown that definite differences exist between the coagulation times and complement activities of arterial and venous blood. Blood taken from the veins not only clots more rapidly but has a higher complement power, an observation which has been repeatedly confirmed. These investigators attribute this difference to the variation in carbonic acid content. Further, it has been suggested by Osborn (1935) that the rise in haemolytic complement produced by experimental stasis is due to an increased CO_2 tension in static blood. On the other hand Barbieri (1930) found that in CO_2 poisoning there was a slight drop in the complementary power of the serum, while Bauer (1930) found a low complement in diseases with an increased CO_2 content in the blood.

Hotta (1925) showed that treatment of serum with carbon dioxide in vitro increased the complement activity, while Fuchs (1930a) noted that bubbling carbon dioxide through plasma for six minutes increased its complement activity, while further bubbling for thirty-two minutes lowered the titre of haemolytic complement considerably. He further demonstrated that the increase in carbon

dioxide content of plasma raised the complement as long as the reaction of the medium remained alkaline or neutral to litmus; as soon as the reaction became acid the complementary activity of the serum was reduced below normal. Fuchs, therefore, attributed the changes in the complementary power of carbon dioxide treated serum as due to the effect of carbon dioxide acting through the hydrogen ion concentration. It has been accepted by most workers that the optimum pH for the action of haemolytic complement is at a slightly alkaline reaction corresponding with that of blood (Michaelis and Swirsky, 1909; Eagle and Brewer, 1929; Osborn, 1934).

Both Valley and McAlpine (1928) and Bauer (1929,1930) have shown that serum saturated with carbon dioxide has its complement reversibly inactivated and preserved from any subsequent deterioration for a period covering weeks. On allowing the carbon dioxide to escape, the serum regains its original complementary power. In a further paper Valley (1928) held that the preservative action of carbon dioxide in serum complement was due to the establishment of conditions which favoured reduction and prevented oxidation in the serum.

With reference to oxygen, the inactivation of complement produced by standing or shaking was attributed by early workers to an oxidative mechanism, while Valley (1928) showed that the removal of atmospheric oxygen completely or

partly from contact with the serum delayed the deterioration of complement. But we have the contrary observations of Schmidt (1913,1919), in an extensive investigation, that the destruction of complement was in no way related to the amount of oxygen present.

The lack of agreement in the literature on the effect of the respiratory gases on the haemolytic complement has led the writer to a re-analysis of the effects, covering a wide range of experimental conditions.

EXPERIMENTAL.

The Effect of Alveolar Air on Serum Complement.

About one cubic centimetre of fresh guinea-pig serum was placed in each of three long testtubes. Alveolar air collected in a Haldane respiratory bag (the carbon dioxide content was found to be 5.1 - 5.4 per cent. by means of Hill's catharometer method) was bubbled through the serum in the first tube, dry air was drawn through the second sample by means of a suction pump, while the remaining sample was left untreated at room temperature. At specified intervals of time samples of serum were withdrawn and their complement activity estimated by the immediate addition of 0.5 volumes guinea-pig serum to the following prepared system:-

3.0 volumes 0.9% NaCl.
0.5 volumes sensitised sheep cells (six units amboceptor).

4.0 volumes haemolytic system.

The haemolytic system was immediately incubated and the time for complete haemolysis recorded (table 9). The air treated serum showed no change in complement activity and was identical in this respect with the untreated serum. This was to be expected since the partial pressures of the gases present in serum kept at room temperature are in equilibrium with the tensions of the gases of the atmosphere. The introduction of nitrogen by air treatment into the serum is therefore without effect on the complement activity. The serum treated with alveolar air showed an immediate decrease in its complementary power, which was constant for the duration of the treatment. On stopping the treatment and allowing the tube to stand, it was found that the serum regained its original activity within a short period. From a study of the relative composition of atmospheric air and alveolar air it is apparent that either oxygen or carbon dioxide could be responsible for the observed effects, namely, the diminution in complementary activity could be ascribed to the decrease in oxygen tension or the increase in carbon dioxide tension in the serum.

The Effect of Oxygen on Serum Complement.

The haemolysis time of fresh guinea-pig serum in the standard four volume amboceptor-complement system was taken, and oxygen (a mixture of 7% oxygen and nitrogen) from a cylinder was bubbled through the serum in a long narrow

Details of Treatment.	Haemolysis Time in Seconds.		
	Sample A.	Sample B.	Sample C.
Before Treatment.	94.	99.	96.
Treatment.	Alveolar Air.	Atmospheric Air.	Untreated.
1 min.	153.	100.	100.
2 mins.	156.	105.	102.
5	160.	-	100.
10	151.	98	97.
20	158.	101.	103.
After Treatment.			
10 mins.	101.	100.	97.

Table 9. The Haemolysis Times of Sera treated with Alveolar Air and Atmospheric Air, compared with untreated serum.

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testtube at varying rates for varying periods of time. Estimations of complementary power were carried out at various intervals, and table 10 gives details of a typical result. The complementary activity of treated serum was similar with that of untreated and air-treated serum. Since the gas mixture may be regarded as equivalent to air with a very low content of oxygen, it may be concluded that a decrease in the oxygen tension of the serum has no effect on the complement. On the other hand, an increase in the oxygen tension of serum produced by passing pure oxygen through the serum produced a gradual diminution in its complementary power, which was not regained when the treatment was discontinued (table 10).

Irrespirable gases, e.g., nitrogen, hydrogen, hydrogen sulphide, were tested in similar manner, and were not found to produce any effect on the complementary power of sera (table 11).

The Effect of Carbon Dioxide on Serum Complement.

(i) The Effect of Bubbling Carbon Dioxide through Serum.

About a cubic centimetre of fresh guinea-pig serum was placed in a long narrow testtube and covered by a film of liquid paraffin to prevent the escape of the gas. Carbon dioxide from a Kipp's apparatus was led through concentrated sulphuric acid into the serum at the rate of 0.25 - 0.5 cc. per second, and after suitable intervals of time a sample

Details of Treatment.	Haemolysis Time in Seconds.			
	Sample A.	Sample B.	Sample C.	Sample D.
Before Treatment.	71.	68.	70.	71.
Treatment.	100% Oxygen.	7% Oxygen.	Air.	Untreated.
5 mins.	70.	70.	70.	71.
15 "	72.	74.	72.	70.
30 "	70.	69.	75.	73.
60 "	78.	73.	76.	71.
120 "	88.	76.	80.	78.
After Treatment.				
60 mins.	110.	75.	79.	80.

Table 10. The Effect of Oxygen and Air Treatment of Serum on the Complementary Activity.

Details of Treatment.	Haemolysis Time in Seconds.			
	Sample A.	Sample B.	Sample C.	Sample D.
Before Treatment.	80.	84.	81.	80.
Treatment.	Nitrogen.	Hydrogen.	Hydrogen. Sulphide.	Untreated.
1 min.	81.	82.	82.	81.
5 mins.	80.	80.	84.	82.
10. "	81.	80.	81.	80.
30. "	78.	83.	89.	81.
60. "	80.	85.	83.	83.

Table 11. The Effect of Irrespirable Gases on the in vitro Complementary Activity of Sera.

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was withdrawn and the speed of haemolysis estimated in the following system:-

3.0 vols. 0.9% NaCl.

0.5 vols. sensitised (6 units amboceptor) sheep erythrocytes, incubated at 37°C. for five minutes

+ 0.5 vols. carbon dioxide treated guinea-pig serum.

A representative result is given in table 12. The carbon dioxide treatment was found to produce a decrease in the complement concentration within a minute, and that this diminution remained at an unaltered level for long periods of time, being unchanged after eight hours. Slight turbidity was present in the serum after prolonged carbon dioxide treatment. The same serum stood at room temperature as a control showed no trace of turbidity or alteration in complement concentration for the duration of the test. On stopping the carbon dioxide treatment and standing the serum at room temperature, the original complementary activity of the serum was restored within a varying period of time. It was found that this time could be much reduced by shaking the serum; fine bubbles of gas were evolved, and the turbidity disappeared. The experiment shows that carbon dioxide produces a variable degree of inactivation of serum complement, which is reversible in character.

Many experiments were conducted over a wide range of conditions, and in no case did carbon dioxide produce a rise

Details of Treatment.	Haemolysis Time Of Carbon Dioxide treated Serum in seconds.	Haemolysis Time of Untreated serum in Seconds.
Before Carbon Dioxide.	116.	114.
Carbon Dioxide Treatment.		
30 secs.	184.	-
1 min.	216.	-
2 mins.	224.	-
5 mins.	223.	-
10 "	230.	-
20 "	230.	-
30 "	234.	116.
60 "	242.	112.
90 "	225.	115.
120 "	218.	113.
After Carbon Dioxide.		
10 "	186.	117.
20 "	170.	113.
30 "	135.	116.5.
40 "	121.	118.

Table 12. The Effect of Carbon Dioxide on the Complementary Activity of Guinea-pig Serum.

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in complement activity, as was claimed by Hotta (1925) and Fuchs (1930,a). Since the latter claimed that the complementary activity of plasma was increased significantly during the first six minutes of carbon dioxide treatment and attributed this to the fact that the pH was still on the alkaline side, this period was therefore closely investigated. The carbon dioxide was bubbled through a 5 c.c. sample of serum slowly and samples withdrawn at short intervals for pH and complement estimations. The pH was estimated by means of the quinhydrone electrode, while both the timing and colorimetric methods were utilised for the estimation of complement. In the colorimetric method the conditions are very similar to those employed by Fuchs (1930,a). The results show that in no instance was any increase in the complement activity found (tables 13, 14). Further, the inactivation of the serum complement did not bear any relation to the pH of the serum or haemolytic system; hence the effect of carbon dioxide cannot act through its alteration of pH.

Diluted sera were treated with carbon dioxide, and it was found that the greater the dilution of the serum the greater was the resultant inactivation and the longer the time taken for regaining the original activity on cessation of the carbon dioxide treatment (table 15). It is therefore difficult to explain Fuchs' results, since he employed plasma diluted 1 in 10 for his experiments.

Details of Treatment.	Haemolysis Time in Seconds.	pH of Serum.	pH of Haemo-lytic System.
Before Carbon Dioxide.	123.	7.3.	7.0.
Carbon Dioxide Treatment.			
15 secs.	177.	7.0	7.0
30 secs.	293.	6.8.	7.0.
1 min.	260.	-	-
2 mins.	270.	-	-
3 "	225.	6.2.	6.6.
4 "	220.	-	-
5 "	228.	-	-
6 "	231.	6.1.	6.6.
30 "	242.	5.8.	6.4.
After Carbon Dioxide.			
10 mins.	180.	6.8	6.8.
20 "	132.	7.2.	6.8.
Original Serum.	125	7.3.	6.9.

Table 13. The Effect of Carbon Dioxide Treatment of Serum for short Periods on the Complementary Activity and pH of Guinea-pig Serum.

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Colorimetric Method. 6/8/35.

Haemolytic System:-

4.0 vols. 3% sheep cell suspension, sensitised 5 units amboceptor.

1.0 vol. 1:20 guinea-pig serum.

5.0 vols.

Each sample, after carbon dioxide treatment, was incubated for 30 minutes at 37°C., and then centrifuged at 0°C.

Colorimetric Test:- 4.0 vols. supernatant fluid + 1c.c. H_2O.

Standard:- 50% Haemolysis - 1.6 vols. 3% sheep cell suspension haemolysed with 2.4 vols. H_2O + 1c.c. H_2O.

The standard was set at 15 on the colorimeter scale.

Details of Treatment	Test Reading.	Ratio, Av.	% of initial haemolysis
Before Treatment	10.2, 10.3.	1.464.	100.0
Carbon Dioxide Treatment.			
5 mins.	24.5, 24.6.	0.611.	41.7.
30 "	25.8, 26.0.	0.579.	39.6.
After Treatment.			
30 mins.	10.8, 10.9.	1.382.	94.4
Original serum (1 hr.).	10.4	1.442.	98.5.

Table 14. The Effect of Carbon Dioxide Treatment of Serum on the Degree of Inactivation Produced in its Complementary Power.

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Details of Treatment.	Haemolysis Time in Seconds.			
Guinea-pig Serum.	1:1.	1:5.	1:10.	1:20.
Before Carbon Dioxide.	61.	86.	115.	228.
Carbon Dioxide Treatment.				
2 mins.	122.	362.	850.	2,100.
5 "	125.	374.	910.	Incomplete, 60 mins.
15 "	130.	383.	880.	Incomplete, 60 "
30 "	120.	358.	846.	Incomplete, 60 "
After Carbon Dioxide.				
5 mins.	90.	338.	840.	-
10 "	62.	286.	760.	-
15 "	60.	252.	805.	-
20 "	-	217.	715.	-
25 "	-	228.	720.	-
30 "	-	136.	592.	-
35 "	-	110.	340.	-
40 "	-	94.	250.	-
45 "	-	87.	182.	286.
50 "	-	89.	156.	250.
55 "	-	-	152.	262.
60 "	-	-	128.	298.
Original Untreated Serum.	65.	92.	121.	242.

Table 15. The Effect of Carbon Dioxide Treatment on the Complement of Diluted Sera.

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It was also observed that, if diluted sera were used in the standard four volume haemolytic system in concentrations equivalent to the undiluted serum, there was little difference in the complementary activity of the various systems, but that on treatment with carbon dioxide, the diluted serum systems showed increasing inactivation of complement and a prolongation of the time required to restore to the serum its original complement power (table 16).

Diluted sera treated with carbon dioxide showed a definite turbidity. Since a method of fractionating complement consists in treating serum diluted 1 in 10 with carbon dioxide and obtaining a precipitate of globulin, this turbidity would appear to be of globulin character.

When carbon dioxide is bubbled through human whole blood (oxalated) the inactivation of the plasma is relatively slight. The carbon dioxide is passed in the usual manner through the blood, and samples are withdrawn under paraffin (to prevent the escape of carbon dioxide) for centrifuging. The sample is spun for 3 minutes at 3,000 r.p.m., and 0.5 volumes of plasma are added to 3.0 volumes 0.9% NaCl and 0.5 volumes semsitised corpuscles pre-incubated at 37°C. The haemolysis time is determined in the usual manner (table 17).

(ii) The Effect of Various Carbon Dioxide Tensions on Serum Complement.

Gas mixtures of carbon dioxide and hydrogen were made up in glass cylinders over water, the tensions of carbon

Haemolytic System Employed:-

(3.5 - x) vols. 0.9% NaCl.

0.5 vols. sensitised sheep corpuscles (3% suspension, sensitised 5 units amboceptor).

x vols. guinea-pig serum (diluted 1:1 to 1:7)

4.0 vols. standard haemolytic system.

Details of Treatment.	Haemolysis Time in Seconds.						
Vols. Guinea-pig Serum (x).	0.5 of 1:1.	1.0 of 1:2.	1.5 of 1:3.	2.0 of 1:4.	2.5 of 1:5.	3.0 of 1:6.	3.5 of 1:7.
Before Carbon Dioxide.	113.	118.	114.	110.	109.	106.	107.
Carbon Dioxide Treatment.							
1 min.	231.	302.	340.	410.	535.	710.	984.
5 mins.	244.	309.	348.	418.	550.	785.	990.
30 "	246.	317.	358.	426.	582.	816.	1260.
After Carbon Dioxide.							
10 mins.	167.	151.	-	189.	191.	253.	280.
20 "	121.	124.	120.	118.	131.	158.	165.
Original Sample (1 hour).	114.	119.	115.	112.	110.	107.	105.

Table 16. The Inactivating Effect of Carbon Dioxide on the same Complement concentration in Diluted Sera.

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30/7/35. Venous puncture 2 c.cs.:- Oxalate blood (0.2% sodium oxalate). Carbon dioxide was bubbled through the blood, and samples centrifuged under paraffin at intervals. The complementary activity of the plasma samples were tested in the following system:-

0.5 vols. 3% sheep cell suspension, sensitised 5 units amboceptor.

0.5 vols. human oxalate plasma.

3.0 vols. 0.9% NaCl.

4.0 vols.

Details of Treatment.	Haemolysis Time in Seconds.
Before treatment.	140, 137.
Carbon Dioxide treatment.	
30 secs.	143.
5 mins.	152.
15 "	161.
30 "	174.
40 "	192.
50 "	185.
60 "	179.

Table 17. The Effect of Bubbling Carbon Dioxide through whole Blood on the Complementary Activity of its Plasma.

-------oOo-------

dioxide ranging from 0 - 100 mms. Hg. The mixtures were analysed for their carbon dioxide content by means of a gas analysis apparatus.

A series of seven 250 c.c. tonometers was filled with the gas mixtures:- The tonometer tap at one end was closed, and the other end was connected to a suction pump. After two minutes' evacuation the tap was closed. The tonometer was then attached to the gas cylinder, and the gas allowed to pass through the tonometer for some time until any air remaining could be regarded as having been completely displaced. The open end was then attached to one limb of a mercury U-tube, and the pressure in the tonometer adjusted till it stood at barometric pressure. Both taps were then closed. One end of the tonometer was connected to a funnel containing fresh guinea-pig serum so that the glass tubing above the tap was occupied by serum. By opening the tap about one c.c. of serum was allowed to enter the tonometer, the pressure within the tonometer remaining unchanged.

The tonometers were placed in a waterbath at 18°C. and were rotated to equilibrate the serum. As a control serum was placed in a tonometer filled with air, and the same procedure was adopted. After twenty minutes' treatment the tonometer was removed, the serum withdrawn under paraffin, and the time of haemolysis estimated in the following system:-

3.0 vols. 0.9% NaCl.

0.5 vols. sensitised sheep red cells,
incubated for five minutes at 37°C.

+0.5 vols. guinea-pig serum.

As the carbon dioxide tension is increased from 0 - 60 mms. Hg there is an increasing inactivation of the complementary activity and a concurrent increase in acidity of the serum (table 18). But further increase in carbon dioxide tension is not accompanied by a greater inhibition of the serum complement, despite the fact that the pH of the serum is becoming more acid. Again we are forced to conclude that the action of carbon dioxide in the inactivation of complement does not result solely from the changes in pH which it produces in the serum.

(iii) Effect of Carbon Dioxide Treatment on Serum to which Bicarbonate or Lactate has been added.

To each of four tubes is added 1 volume of guinea-pig serum and one volume of isotonic saline, and equi-osmotic solutions of sodium bicarbonate, and potassium lactate respectively. The haemolysis times in the standard four volume system indicates that the addition of bicarbonate to the serum prior to its treatment with carbon dioxide diminishes the degree of inactivation that occurs in the ordinary course of events, while the addition of lactate accentuates the inhibition that is already present (table 19).

Serum equilibrated at 18°C. and barometric pressure, 630 mms. Hg.

CO_2 treated serum.	CO_2, %.	Tension, mms.Hg., 18°C. and A.P.	Haemolysis Time in seconds.	pH.
	0.0.	0.0.	78.	7.4.
	3.08.	19.4.	124.	7.2.
	6.24.	39.3.	151.	6.9.
	6.95.	43.8.	155.	6.6.
	8.66.	54.6.	156.	6.4.
	9.44.	59.5.	158.	6.4.
	13.02.	82.0.	157.	6.1.
	16.56.	104.3.	162.	5.9.
	47.70.	300.5.	160.	5.5.
Air treated serum.	0.0.	0.0.	80.	7.3.

Table 18. The Effect of various Carbon Dioxide Tensions on the Complementary Activity and pH of Guinea-pig Serum.

-------oOo------

Sample A-- Standard:- 1 vol. Guinea-pig Serum plus 1 vol. 0.9% NaCl.
Sample B-- Bicarbonate:- 1 vol. Guinea-pig Serum plus 1 vol. 1.31% $NaHCO_3$.
Sample C-- Bicarbonate:- 1 vol. Guinea-pig Serum plus 1 vol. 1.55% $KHCO_3$.
Sample D-- Lactate:- 1 vol. Guinea-pig Serum plus 1 vol. 2.01% Potassium Lactate.

Haemolytic System:-

0.5 vols. above mixture.
0.5 vols. sensitised sheep corpuscles (3% suspension, sensitised 5 units amboceptor).
3.0 vols. 0.9% NaCl.
4.0 vols. standard haemolytic system.

Details of Treatment.	Haemolytic Time in Seconds.			
	Sample A.	Sample B.	Sample C.	Sample D.
Before Carbon Dioxide.	115.	113.	110.	153.
Carbon Dioxide Treatment.				
1 min.	314.	161.	170.	684.
5 mins.	320.	171.	182.	675.
30 "	318.	148.	178.	712.
After Carbon Dioxide.				
10 mins.	122.	118.	115.	298.
Original Serum (45 mins.).	112.	114.	112.	158.

Table 19. The Effect of Carbon Dioxide on the Complementary Activity of Serum to which Bicarbonate or Lactate has been added.

-------oOo-------

(iv) Reversilibity of Carbon Dioxide Inactivation of Serum Complement.

If carbon dioxide treated serum is allowed to stand at room temperature open to the atmosphere, fine bubbles of gas are seen to be evolved, and the pH of the serum returns invariably to its original level of pH 7.2 - 7.4 from a former acidity of about pH 5.5. The complementary power of the serum is found to return to its original value, independent of the state of the serum, e.g., diluted serum, lactate or bicarbonate treated serum (tables 9,10,12, 13, 14, 15, 16, 19). The time required for the complement to return to its original value depends on the degree of inactivation, and is lessened by shaking the tube containing the serum.

(v) Properties of Carbon Dioxide Treated Serum.

Fuchs (1930a) found that in certain proportions carbon dioxide treated oxalate plasma enhanced the activity of fresh untreated oxalated plasma. He found that mixtures of 0.8 c.c. untreated and 0.2 c.c. carbon dioxide treated plasma and 0.6 c.c. untreated and 0.4 c.c. carbon dioxide treated plasma haemolysed 1.0 c.c. of a 5% cell suspension (1:600 amboceptor sensitisation) in 13 and 14 minutes respectively, while a cubic centimetre of untreated plasma took 24 minutes to produce complete haemolysis, and a mixture of 0.4 c.c. untreated and 0.6 c.c. carbon dioxide

treated plasma took 29 minutes. In my experiments I found that carbon dioxide treated serum did not increase the activity of untreated serum under any conditions, and that small amounts of carbon dioxide treated serum were just as inhibitory as large amounts in this respect (table 20).

Effect of Carbon Dioxide on Amboceptor.

The haemolysis time was taken in the standard four volume haemolytic system. Carbon dioxide was bubbled through the sensitised corpuscles, and the time for their haemolysis was found to be prolonged (table 21). After 30 minutes' treatment the suspension began to assume a brown colour due to the formation of acid haematin, and commenced to show incipient haemolysis.

The prolongation of the haemolysis time suggested that the carbon dioxide had some effect on the amboceptor. It was decided to investigate whether carbon dioxide influenced the absorption of amboceptor, a method described by Ponder (1932) being utilised. Various dilutions of amboceptor (1 in 20, 1 in 50, 1 in 100, 1 in 200) are made in normal saline, and 2 c.cs. of each dilution is pipetted into two sets of testtubes. The amboceptor dilutions in one series are covered with liquid paraffin and treated with carbon dioxide for 20 minutes, while the other series is stood at room temperature. Then 0.5 of a 3% sheep cell

(a)

Untreated serum, vols.	1.0.	0.8.	0.6.	0.4.	0.2.	0.0.
CO_2 treated serum 30 mins., vols.	0.0.	0.2.	0.4.	0.6.	0.8.	1.0.

+ 1.0 vol. 3% sheep cell suspension, sensitised 5 units amboceptor.

6.0 vols. 0.9% NaCl.

Haemolysis time in seconds.	72.	98.	132.	139.	141.	140.
pH.	7.3.	7.1.	6.8.	6.5.	6.1.	6.0.

(b) Fuchs, (1930,a).

Untreated plasma, ccs.	1.0.	0.8.	0.6.	0.4.	0.2.	0.0.
CO_2 treated plasma 40 mins., ccs.	0.0.	0.2.	0.4.	0.6.	0.8.	1.0.

+ 1.0 ccs. 5% sheep cell suspension, sensitised 1:600 amboceptor.

Haemolysis time in mins.	24.	13.	14.	29.	60.	60 incomplete
pH.	alkaline.	weakly alkaline.	neutral	neutral	weakly acid	acid.

Table 20. The Complementary Activity of Carbon Dioxide Treated Serum compared with Carbon Dioxide Treated Plasma.

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Haemolytic System employed:-

3.0 vols.	0.9% NaCl.
0.5 vols.	guinea-pig serum.
0.5 vols.	sensitised sheep suspension, sensitised 5 units amboceptor.
4.0 vols.	

The Haemolysis time in the above system was 60, 61 seconds. Carbon dioxide was bubbled through the sensitised corpuscles, and the time for haemolysis was estimated at intervals:-

Time of Treatment.	Haemolysis Time in Seconds.
5 mins.	105.
15 "	107.
30 "	108.

At this stage the suspension turned a brown colour, due to formation of acid haematin.

Table 21. The Effect of Carbon Dioxide Treatment of Sensitised Cell Suspension on the Speed of Haemolysis in an Amboceptor-Complement Haemolytic System.

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suspension is added to each, and the tubes are stood at room temperature for 10 minutes to ensure complete sensitisation. They are then centrifuged for 3 minutes at 3,000 r.p.m., and 1 c.c. of the supernatant fluid removed from each into another set of tubes. This set will be referred to as series 1 (carbon dioxide treated amboceptor) and series 2 (untreated amboceptor).

A further series of testtubes is prepared containing 2 c.cs. of the various dilutions of amboceptor and 0.5 c.c. normal saline, and 1 c.c. of the mixture is removed from each tube to constitute series 3. By this means the amboceptor has been diluted equally in all three series, but it differs in that in the first series the carbon dioxide treated amboceptor has been exposed to red cells, in the second series untreated amboceptor has been exposed to red cells, but the last series has not been treated with red cells.

Each tube is now treated with 0.5 c.c. 3% sheep cell suspension, and sensitisation allowed to proceed for 10 minutes at room temperature; then 0.5 c.c. 1/10 fresh guinea-pig serum is added to each tube, and the time for complete haemolysis observed. (table 22). We thus obtain three time-dilution curves, one of which shows the time t_1 taken by various known quantities of amboceptor a_1, acting in the presence of 1 in 10 complement, to bring about

Amboceptor Dilution.	Haemolysis Time in Seconds.			Concentration of Amboceptor.			Amount of Amboceptor Removed by Cells.		Absorption of Amboceptor by cells.	
	t_1.	t_2.	t_3.	a_1.	a_2.	a_3.	$a_1 - a_2$.	$a_1 - a_3$.	$\frac{a_1 - a_2}{a_1}$.	$\frac{a_1 - a_3}{a_1}$
1:20.	62.	171.	122.	0.1.	0.029.	0.043.	0.071.	0.057.	0.71.	0.57.
1:30.	85.	233.	159.	0.067.	0.022.	0.031.	0.045.	0.036.	0.67.	0.54.
1:40.	106.	343.	232.	0.05.	0.016.	0.022.	0.034.	0.028.	0.68.	0.56.
1:50.	130.	508.	345.	0.04.	0.012.	0.016.	0.028.	0.022.	0.70.	0.55.
1:60.	150.	785.	410.	0.033.	0.009.	0.014.	0.024.	0.019.	0.73.	0.57.
1:80.	200.			0.025.						
1:100.	258.			0.02.						
1:150.	440.			0.013.						
1:200.	650.			0.01.						
1:250.	1030.			0.008.						

Table 22. The Absorption of Carbon Dioxide Treated Amboceptor by Sheep Cell Suspension.

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haemolysis, the second of which shows the time t_2 taken by similar amboceptor concentrations which have been in contact with cells, to produce complete haemolysis in the presence of the same amount of complement, and the last of which gives the time t_3 taken by carbon dioxide amboceptor which has been in contact with cells, to produce complete haemolysis. From the data of the first curve we can read off a_2 and a_3, the amount of amboceptor present in tube series 2 and 3 which have been in contact with the cells. It will be seen that the concentration of amboceptor in the carbon dioxide treated series is greater than in the untreated series. The amount of amboceptor removed by the cells is represented by $a_1 - a_2$ and $a_1 - a_3$ for the untreated and treated series respectively, and the ratio of these values to the quantity of amboceptor introduced into the system is an index of the degree of absorption of amboceptor by cells.

The ratio of amboceptor absorbed to amboceptor introduced into the system has a value of 0.7, which is close to the value of 0.75 given by Ponder (1932). In the case of carbon dioxide treated amboceptor the ratio was found to be 0.55; in other words, the carbon dioxide decreased the normal absorption of amboceptor.

DISCUSSION.

The experiments described showed that both the respiratory gases possessed an influence on the complementary activity of serum, while the treatment of serum with other gases, e.g., nitrogen, hydrogen, hydrogen sulphide, did not affect the serum complement. In the case of oxygen it has been shown that the effect of a gas mixture (7% oxygen and 93% hydrogen) and of atmospheric air passed through concentrated sulphuric acid and sodium hydroxide (21% oxygen and 79% nitrogen) on the complement power of serum did not differ from untreated serum or serum treated with an inert gas. The treatment of serum with pure oxygen caused a slow deterioration in the complementary power, which did not halt on the cessation of the treatment. This is in agreement with the observation of Valley and McAlpine (1928) that the removal of atmospheric oxygen from serum delays the deterioration of complement.

The effect produced by treating serum with carbon dioxide on its complementary activity is clear-cut and unmistakable. The action of the gas is to produce an immediate inactivation of the serum complement. The inactivation is such that it decreases the speed of haemolysis by about one-half, and in no case is there

an increase in the speed of haemolysis. The degree of inactivation is dependent on several factors, among which the concentration of the complement, the dilution of the serum, and the tension of carbon dioxide in the serum are outstanding. The degree of inactivation is less in a "fast" complement than in a "slow" serum; in other words, the higher the complement titre the less will be the degree of inactivation. Further, when sera are diluted, the greater the dilution the greater is the inactivation of the serum complement; this has been shown quantitatively by the use of equal absolute quantities of complement in the diluted serum haemolytic systems.

The effect of bubbling carbon dioxide through serum resulted in a steady inactivation level of the complement being attained within one minute; and this level was maintained in these experiments for some length of time. Valley and McAlpine (1928) found that serum kept in a carbon dioxide atmosphere maintained a constant degree of inactivation for several weeks.

The minimum carbon dioxide tension required to attain this "inactivation level" of the serum complement was found to be in the neighbourhood of 40 - 50 mms. Hg. Between this tension and a nil carbon dioxide tension the degree of inactivation was lessened in proportion.

Since the estimation of complement concentration of a serum is made when the partial pressures of the gases in

the serum are in equilibrium with the atmosphere so that the tension of carbon dioxide is about 0.25 mms. Hg., it is apparent that in the circulating blood the complement is partially inactivated in consequence of a carbon dioxide tension of 40 - 60 mms. Hg. As the carbon dioxide tensions of arterial and venous blood are 40 and 46 mms. Hg. respectively, it is clear that the serum must be estimated under these tensions of carbon dioxide for "true" complement activity. As it is known that venous blood has a higher serum complement concentration than arterial blood, it may be supposed that in the circulating blood the complement activity is of the same order both in the arterial and venous systems.

Hence the conclusion of Lumière and Grange (1928), that the enhanced serum complement activity of venous blood is due to an increased carbonic acid content as compared with arterial blood, is based on an incorrect interpretation of results. In a similar manner the increased complementary activity of static blood (Osborn, 1935) cannot be attributed to an increase in carbon dioxide tension of the blood (since the maximum inactivation is produced by a tension of 40 mms. Hg. or more).

The inactivation of serum complement by carbon dioxide treatment is reversible in nature. On opening the container or shaking, the gas escapes from the serum, and the complement regains its former activity. The time

required for the serum to regain its original activity is dependent on the degree of inactivation, and is speeded up by shaking. Bauer (1930) found that serum treated with carbon dioxide could be reactivated in this way with no alteration in complement activity after 8 days, while Valley and Mc Alpine (1928) found that only after two months under carbon dioxide did the serum begin to lose its original activity. This reversible inactivation is therefore similar in character to the complement inactivation exhibited by treating serum with hypertonic saline or salts. It therefore provides an excellent method for the conservation of complement (Osborn, 1936c).

The addition of bicarbonate to a serum prior to its treatment with carbon dioxide leads to a diminished inactivation of complement. This is borne out by the observations of Michaelis and Swirsky (1910) and Sherwood (1917) that injection of bicarbonate into the blood causes a rise in blood complement, and that the action of bicarbonate in vitro accelerates the haemolytic speed of complement (Piettre and Chrètien, 1927; Bernstein, 1937). On the other hand, it has been advanced by Andreesco (1930) that the anti-complementary nature of sera is due to a pronounced hyper-alkalinity; the indication is that excess bicarbonate determines this special property. This is contra-indicated by the observations of Hilgers and Zain (1928). It would seem that the ratio of the fixed carbon dioxide to the free carbon dioxide determines the

degree of complement inactivation; the less the free carbon dioxide content and/or the greater the fixed carbon dioxide the less the resulting inactivation of complement.

The addition of lactate to serum prior to carbon dioxide treatment caused an inactivation of the complement which was greater than that normally found. This is due to increased acidity bringing the pH of the serum to or on the acid side of the iso-electric point of the serum proteins, and thus causing an irreversible inactivation of the complement. In vivo the effect of lactic acid is to favour an increase in complement activity (Sherwood, 1917), who ascribes this phenomenon to the fact that the body can oxidise organic acids to carbonates and bicarbonates, which would account for the similarity of action between lactic aicd and sodium bicarbonate.

The method by which carbon dioxide produces a reversible inactivation of complement has been variously attributed to a pH effect, inactivation of midstuk or endstuk, or to the establishment of conditions favouring reduction and inhibiting oxidation. Fuchs (1930a) considered that carbon dioxide acted on complement through its effect on the pH of serum. In a serum on the alkaline side of litmus, the complementary activity increased during carbon dioxide treatment till the pH of the serum was neutral to litmus(pH 4.5 - 8.3); as the reaction of the serum became more acid due to prolonged carbon dioxide treatment, the

complementary activity was diminished. Fuchs did not realise the reversible nature of the inactivation, while his method of pH determination was decidedly crude.

The results presented in the experiments show a totally different state of affairs. The complementary power of the serum is immediately decreased on carbon dioxide treatment; this occurred while the reaction of the serum was still alkaline, and while the pH continued to increase with further carbon dioxide treatment, there was no change in the complementary power of the treated serum. The maximum acidity reached in the experiments was pH 5.8, 5.5, and 5.7, a concentration of hydrogen ions which will be seen to be most significant. On allowing the carbon dioxide to escape, the pH of the serum became more alkaline, and was invariably restored to its original value of pH 7.2 - 7.4. The restoration of the complementary power to its original activity bore no relation to alterations in the pH of the serum.

The effect of pH on haemolytic complement has been studied by various workers, notably Eagle and Brewer (1929) and Osborn (1935). The latter found that the optimal pH for the action of haemolytic complement was at the pH of circulating blood, namely, pH 7.5, and that the complementary activity was diminished on the acid and alkaline ranges of this optimum. Eagle and Brewer concluded that the optimal

pH lay between 6.5 - 8.0, that on the acid side of pH 6.5 complement was inhibited, and that on the acid side of pH 5.3 irreversible inactivation occurred, which was complete at pH 4.8.

When the point of maximum acidity was reached in the carbon dioxide treatment of serum a turbidity was often observed, and was due apparently to the serum proteins precipitating out of solution. The iso-electric point of serum globulin is pH 5.3; at pH 7.3 - 7.5 globulin would ionise as a sodium salt, and the degree of ionisation decreases as the iso-electric point is approached, with a proportionate greater tendency for the globulin to separate from solution. The similarity between the results of Eagle and Brewer (1929) and the turbidity obtained at pH 5.5 - 5.8 after carbon dioxide treatment of serum is therefore striking. In the case of serum albumin the iso-electric point is pH 4.5, and at the maximum point of acidity this protein would not precipitate. Thus the carbon dioxide treatment of serum causes a precipitation of the serum globulins.

The cause of the complement inactivation due to carbon dioxide treatment of serum may therefore be ascribed to:-

(i) rise in the albumin:globulin ratio.

(ii) precipitation of complement (that is, complement is of globulin nature, or it is carried down with the globulins).

Alterations in the albumin:globulin ratio influences the complementary activity of serum considerably. Plant (1933) and Terry (1935) found that alternate freezing and thawing of guinea-pig serum caused a separation into two layers, the upper layer having a complement titre of 1 in 150 and the lower layer a titre of 1 in 5. The loss of complementary activity in the upper layer was associated with a change in the albumin:globulin ratio, consisting in a relative decrease in albumin and an increase in globulin content. Hence the decreased complementary activity of carbon dioxide treated serum is not related to changes produced in the albumin or globulin content of the serum.

The knowledge that complement is of protein nature or is closely associated with the serum proteins by physical forces leads to the conclusion that the action of carbon dioxide is due to a direct precipitation of the protein. It is well-known that the passage of carbon dioxide through serum diluted 1 in 10 with water leads to a protein precipitate, which is of euglobulin nature and constitutes the midstuk of complement (Liefmann, 1909). The inactivation of serum complement treated with carbon dioxide would therefore seem to be due to a precipitation of the midstuk, while Fuchs (1930b) attributes it to the carbon dioxide adsorbing the midstuk to a greater or lesser degree.

It appears that the presence of carbon dioxide in a

complement serum allows the midstuk to be bound, but prevents the action of the endstuk, i.e., the sensitised corpuscles become "persensitised". This is seen from the fact that, if sensitised corpuscles are incubated with serum in a medium saturated with carbon dioxide, and if they are then separated by centrifuging, they will be found to be susceptible to haemolysis by endstuk alone, and the supernatant fluid will be found to have lost its complement activity.

The carbon dioxide inactivation of complement is therefore due to a precipitation of the globulin fraction of the serum, carrying with it the midstuk of complement. The degree of precipitation will increase enormously as the iso-electric point of globulin is approached in the serum, and will be complete at that point. Since the pH of carbon dioxide treated serum never attains the iso-electric point of globulin, complete precipitation does not occur. A portion of the midstuk is therefore bound to the sensitised corpuscles, so that haemolysis occurs. The concentration of the endstuk present in the serum does not compensate for the diminution in the amount of active midstuk, so that the activity of the serum complement on the sensitised corpuscles is naturally lessened.

The action of carbon dioxide on amboceptor was found to cause a diminution in the amount of amboceptor which was

absorbed by the cells. From the experiments of Diacono (1933), who showed that 80% of the amboceptor in an anti-sheep cell serum could be recovered in the globulin fraction precipitated by carbon dioxide treatment, it is evident that globulin precipitation by carbon dioxide decreases the quantity of amboceptor available for absorption by the cell suspension.

SECTION IVb.

THE INFLUENCE OF OSMOTIC PRESSURE ON HAEMOLYTIC COMPLEMENT.

Previous workers have pointed out the necessity of avoiding the effects due to hypotonicity (Sachs and Teruuchi, 1907; Guggenheimer, 1910; Sachs and Altmann,1917; Neter, 1931) or hypertonicity (Nolf, 1900; Hektoen and Ruediger, 1904; Manwaring, 1904; Topley, 1915) in amboceptor-complement haemolytic systems. Despite this, there has been much discussion regarding the strength of saline solution which is to be reckoned as isotonic with that of blood (Osborn,1936c).

The osmotic pressure of blood has received much attention lately, due to the application of Hill's thermopile to vapour pressure methods and the Beckmann and Heidenhain thermometers to depression of freezing point determinations. Collip (1920) observed the depression of freezing point of sheep's corpuscles to be .582°C. in one case and .538°C. in another, while Bugansky and Tangl (1898) found three samples of sheep corpuscles to have Δ of .567°C., .665°C., and .618°C. respectively. Starling (1936) states that the depression of freezing point of mammalian blood is about .53°C., while Margaria (1930) found the vapour pressure to be equal to that of .945% NaCl (Δ = .553°C.) for men, and .927% NaCl (Δ = .543°C.) for women. He found that the

osmotic pressure was remarkably constant under normal conditions, and stated that the osmotic pressure did not alter appreciably on haemolysis.

A curve of the concentration of saline/depression of freezing point for NaCl constructed from data obtained in this laboratory is given in fig. 5. It is seen that the depression of freezing point of .9% NaCl is .54°C., while that of .85% NaCl is .50°C. These results are to be compared with the nomogram (fig. 5a) which have been constructed from the tables of Landolt-Börnstein and the formula of Stadie and Sunderman (1931). Here the depression of freezing point for a .9% NaCl solution is .528°C. and .498°C. respectively.

The sum total of the evidence points to the fact that .9% NaCl is nearer than .85% NaCl to the average osmotic pressure of mammalian blood. On the basis of this it was decided to use 0.9% NaCl solution as the normal isotonic solution, and it is difficult to know why 0.85% saline is more popular than 0.9% NaCl among bacteriologists (Muir,1931).

EXPERIMENTAL.

A series of saline solutions were made up varying from 0.5 - 2.0 per cent sodium chloride. Duplicate estimations of the depression of freezing point were carried out, and the

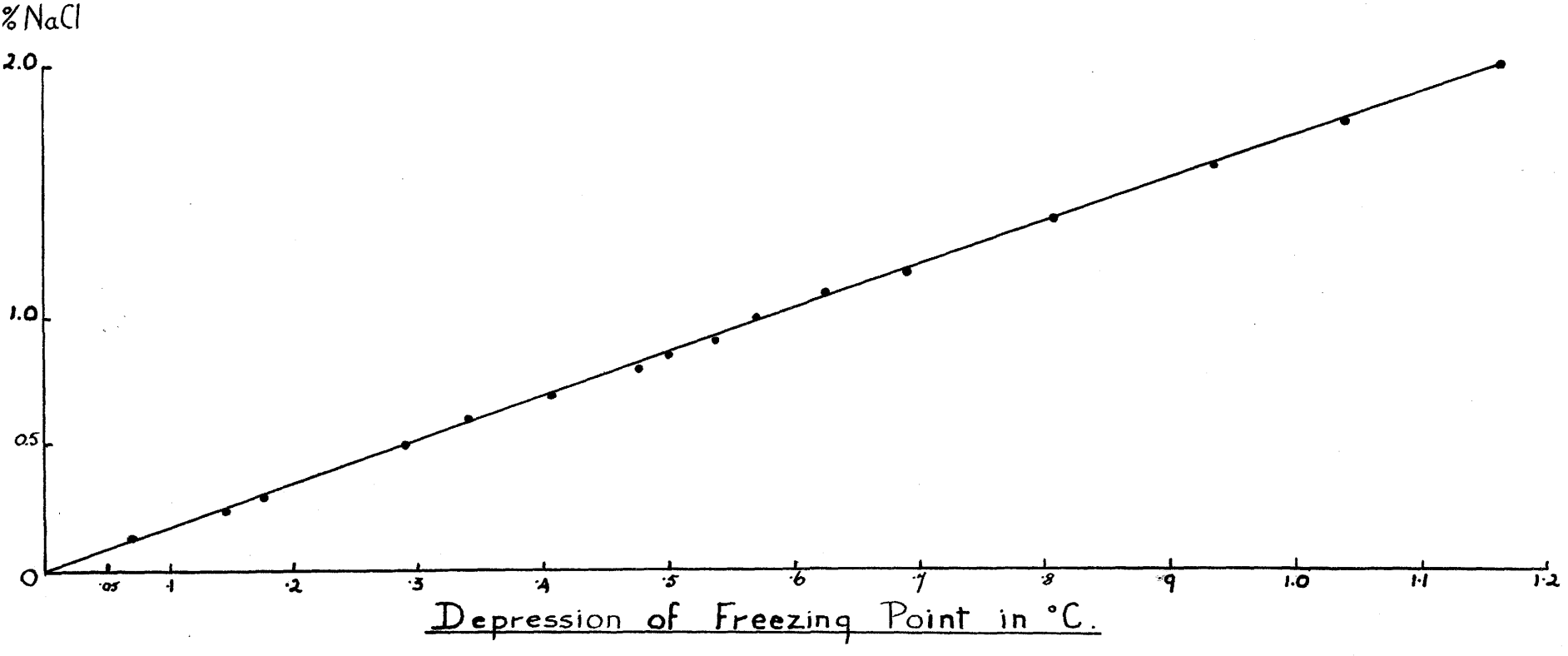

Fig. 5. Freezing Point Depression/Concentration of Salt Curve for Sodium Chloride.

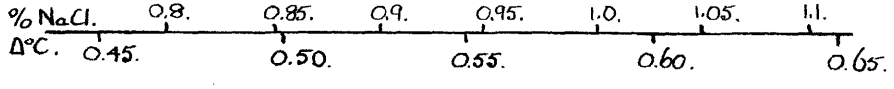

Fig. 5a. Nomogram Showing the Relation between the Concentration of a NaCl solution expressed in grms. Salt/100 grms. Water, and the corresponding depression of Freezing Point.

results are compared with those calculated from the formulae of Stadie and Landolt-Börnstein (figs. 5, 5a).

The effect of hypotonicity and hypertonicity was tested in the standard amboceptor complement haemolytic system consisting of:-

3.0 volumes saline (0.5 - 2.0% NaCl).
0.5 volumes guinea-pig serum.
0.5 volumes sensitised sheep corpuscles (6 M.H.D. amboceptor).
4.0 volumes.

The time for complete haemolysis was estimated, and expressed as a percentage of the time required for complete haemolysis in a system containing 0.9% NaCl (table 23). In this set of experiments the sensitised corpuscles have been suspended in normal saline.

In subsequent experiments it was decided to suspend the corpuscles in the saline which was used in making up the system in order to bring the osmotic pressure relations of the system as close to the strength of saline employed as possible. Examination of these suspensions by centrifuging showed that in no case did a trace of haemolysis occur due to osmotic phenomena. In these systems, therefore, the constant osmotic pressure of guinea-pig sera could produce only slight variation in the range of systems with different

Saline Concentration.%.	Δ °C.	Haemolysis Time in Seconds.	Haemolytic Speed as % of speed in isotonic saline.
0.5.	0.292.	35.	143.
0.6.	0.341.	42.	119.
0.7.	0.400.	45.	111.
0.8.	0.475.	48.	104.
0.85.	0.500.	49.	102.
0.9.	0.540.	50.	100.
0.95.	0.560.	51.	98.
1.0.	0.592.	52.	96.
1.1.	0.627.	54.	92.6.
1.2.	0.712.	56.	89.3.
1.4.	0.810.	58.	86.
1.6.	0.932.	61.	82.
1.8.	1.050.	66.	75.7.
2.0.	1.163.	72.	69.4.

Table 23. The Effect of Hypotonic and Hypertonic Saline Solutions on the Complementary Activity of Guinea-pig Sera.

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Concentration of Sodium chloride in system, %.	Haemolysis Time, in Seconds.	Haemolytic speed as % of speed in isotonic saline.
0.5.	48.	125.
0.6.	50.	120.
0.7.	53.	115.
0.8.	57.	105.3.
0.85.	59.	101.7.
0.9.	60.	100.
0.95.	60.	100.
1.0.	63.	95.2.
1.1.	65.	92.3.
1.2.	66.	91.
1.4.	69.	87.
1.6.	72.	83.3.
1.8.	76.	79.
2.0.	80.	75.

Table 24. The Effect of Hypotonic and Hypertonic Saline Concentrations in the Haemolytic System on the Speed of Haemolysis by Complement.

-----oOo-----

osmotic pressures. The results obtained by this system is similar to table 23 (see table 24).

It is seen that in hypotonic systems the complementary activity of serum is increased in proportion to the degree of hypotonicity and therefore to the osmotic pressure of the solution, since the depression of freezing point of sodium chloride in low concentrations (0.1 - 0.6 M or 0.5 - 3.0%) is a linear function of its concentration.

The increased haemolysis in hypotonic solutions by complement would not appear to be related to osmotic haemolysis, since no haemolysis occurred in sensitised red cells suspended in saline concentrations down to 0.5%, and the maximum concentration for the fragility of red cells is 0.4 - 0.45% in the case of sodium chloride (Williams, 1931).

The complement shows a progressive decrease in hypertonic solutions. The process is actually one of reversible inactivation, as can be shown by the following experiment. A sample of serum has its complement estimated and is subsequently treated with sodium chloride until the serum has a 5% NaCl concentration. The speed of haemolysis is prolonged, but when the serum is diluted with distilled water to bring the NaCl concentration to 0.9%, the speed of haemolysis is the same as the speed of haemolysis of the same

serum diluted to the same degree. The increased osmotic pressure preserves the complement for considerable periods, as in the case of increased carbon dioxide tension. On the other hand, complement in hypertonic solution is unstable (Sachs and Teruuchi, 1907; Sachs and Bolkowska, 1910). Markl (1902) found that an irreversible inactivation of complement occurred when a high concentration of sodium chloride or other salt was employed. He attributed this to a failure of the sensitised corpuscles to bind complement, since the combination between the antigen and amboceptor was not affected. Dungern and Hirschfeld (1911) reported that highly sensitised corpuscles became persensitised in 4% sodium chloride solutions, but that the endstuk fraction failed to function.

The influence of hypotonic and hypertonic solutions on diluted complement has been studied. The serum is diluted with the requisite saline used in each individual system, in order to keep the osmotic pressure of the system at the figure stated. The haemolysis times are recorded in table 25. The effect of diluting the serum is to accentuate the inhibition seen in hypertonic solutions, and to increase the time for haemolysis relative to the isotonic saline in hypotonic solutions.

The effect of variation in the sensitisation of the r.b.c. was also noted. The system employed was:-

Concentration of NaCl in System.	Dilution of Serum.											
	1:1.		1:3.		1:4.		1:6.		1:8.		1:16.	
	Haem. Time.	%initial Speed.	Haem. Time.	%initial Speed.	Haem. Time.	%initial Speed.	Haem. Time.	%initial Speed.	Haem. Time.	%initial Speed.	Haem. Time.	%initial Speed.
0.5%	39s.	130.0	43s.	128.0	44s.	141.0	51s.	143.1	55s.	165.4	104s.	178.8
0.6	42	119.0	48	115.0	-	-	58	126.0	64	142.2	128	145.3
0.7	44	113.5	50.5	109.0.	55	112.7	64	114.0	70	130.0	145	121.3
0.8	48	104.0	52	105.8	-	-	65	112.3	80	113.7	168	110.8
0.85	50	100.0	54	101.8	59	105.0	68	107.2	85	107.0	178	104.5
0.9	50	100.0	55	100.0	62	100.0	73	100.0	91	100.0	186	100.0
0.95	51	98.0	57	96.5	63	98.4	82.5	88.5	97	93.8	200	93.0
1.0	53	94.3	58	95.0	66	94.0	90	81.1	105	86.6	210	87.6
1.1	54	92.6	61	90.0	69	90.0	97	75.2	127	71.7	238	78.1
1.2	58	86.2	61	90.0	-	-	106	68.8	152	60.0	260	71.6
1.4	60	83.3	64	85.9	-	-	120	60.8	180	50.5	330	56.4
1.6	60	83.3	68	81.0	80	77.5	138	52.9	212	43.0	455	40.9

Table 25. The Effect of Varying the Dilution of Serum on the Speed of Haemolysis in Hypotonic and Hypertonic Amboceptor-Complement Haemolytic Systems.

-----oOo-----

3.0 vols. saline (0.5 - 3% NaCl)

0.5 vols. g.p. serum

0.5 " sensitised corps. in 0.5 - 3% NaCl (sensitisation varied from 2 - 400 M.H.D. amboceptor).

4.0 volumes

The first series consisted of the time for haemolysis of complement in the various saline concentrations against sensitised corpuscles treated with 5 units amboceptor. This was used as a standard of reference, and further series were tested with sensitised corpuscles treated with $\frac{1}{2}$,2,50, and 100 unit amboceptor. The results are presented in table 26. It is seen that the inhibition of complement haemolysis seen in hypotonic solutions is diminished by an increased sensitisation, and that the acceleration of complement haemolysis in hypotonic solutions is diminished by a decreased sensitisation.

It was further decided to investigate whether other chlorides would produce the same effects of osmotic pressure. A series of concentrations of monovalent chlorides, e.g. NaCl, KCl, LiCl, was made up so that they had the same range of osmotic pressures. The haemolysis time was determined in the following system:-

Concentration of NaCl in System.	Units Amboceptor									
	0.5.		2.0.		5.0.		50.		100.	
	Haem. Time.	%initial Speed.	Haem. Time.	%initial Speed.	Haem. Time.	%initial Speed.	Haem. Time.	%initial Speed.	Haem. Time.	%initial Speed.
0.5%	295s.	117.0	113s.	113.3	53s.	121.6	40s.	125.0	36s.	122.2
0.6.	307	112.4	-	-	58	112.1	-	-	38	116.0
0.7.	318	108.5	120	106.6	59	110.2	-	-	40	110.0
0.8.	333	103.9	125	102.4	60	108.3	47	106.4	42	104.8
0.85.	340	101.5	-	-	62	104.9	49	102.0	44	100.0
0.9	345	100.0	128	100.0	65	100.0	50	100.0	44	100.0
0.95.	350	98.6	129	99.2	65	100.0	50	100.0	45	98.2
1.0.	360	96.0	134	95.5	65	100.0	51	98.0	47	93.0
1.2.	388	88.9	142	90.1	66	98.5	51	98.0	46	95.6
1.4.	410	84.1	148	86.5	70	92.9	52	96.0	49	90.0
1.6.	430	80.2	152	84.2	78	83.3	54	92.6	48	91.7

Table 26. The Effect of Variation in the Degree of Sensitisation of the Sheep Red Cell on the Speed of Haemolysis in Hypotonic and Hypertonic Haemolytic Systems.

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3.0 vols. chloride solution (depression of freezing point 0.4 - 0.7°C.)

0.5 vols. fresh guinea-pig serum.

0.5 vols. sensitised sheep corpuscles (5 units amboceptor).

4.0 vols. haemolytic system.

It was found that while the use of LiCl in the isotonic system produced an increased speed of haemolysis compared with the sodium chloride or potassium chloride systems, the speed of haemolysis in the LiCl system was found to show an enhanced acceleration and decreased inhibition in iso-osmotic hypotonic and hypertonic solutions respectively (table 27). When the fragility of the sheep erythrocytes was tested with the above chlorides, it was found that the erythrocytes were more fragile in the lithium chloride solution than in the sodium chloride or potassium chloride.

DISCUSSION.

The osmotic pressure of blood was found to correspond to a solution of 0.9% saline, and the haemolytic system was regarded as isotonic and of a standard osmotic pressure when this diluent was employed.

The effect of a decreased osmotic pressure in the haemolytic system was to produce an increased speed of haemolysis by complement compared with the time for haemolysis

Concentration of chloride in System.	LiCl		NaCl		KCl.	
	Haemolysis time in Seconds.	Haemolytic speed as % of speed in isotonic chloride.	Haemolysis time in Seconds.	Haemolytic speed as % of speed in isotonic chloride.	Haemolysis time in Seconds.	Haemolytic speed as % of speed in isotonic chloride.
0.5%	42	190.5	64	134.4	75	132.0
0.6	50	160.0	70	123.0	81	121.0
0.7	60	133.3	77	111.7	86	114.0
0.8	72	111.0	82	105.0	92	106.5
0.85	77	104.0	-	-	-	-
0.9	80	100.0	86	100.0	98	100.0
0.95	82	97.5	86	100.0	-	-
1.0	83	96.4	88	97.7	106	92.4
1.2	85	94.1	94	91.5	117	83.8
1.4	88	90.9	100	86.0	127	77.2
1.6	91	88.0	105	81.9	138	71.0

Table 27. The Effect of Hypotonic and Hypertonic Chloride Solutions on the Speed of Haemolysis (Haemolytic Complement Activity).

----oOo----

in an isotonic system. The increased speed of haemolysis is enhanced in the presence of a diluted complement or by the use of a lithium chloride solution, and is retarded by a low degree of sensitisation or the use of a potassium chloride system. The question arises as to whether osmotic haemolysis plays any part in the increased speed of haemolysis. Although the sheep cell suspension when suspended in the various hypotonic solutions showed no visible sign of haemolysis, Ponder and Marsland (1935) and Daland and Worthby (1935) have shown that in osmotic haemolysis the cell may swell considerably before any liberation of the haemoglobin occurs. The increase in the volume of the red cell is progressive until the point of minimum fragility of the cells is reached. It would therefore appear that the swollen cell is less resistant to the action of complement, and therefore undergoes haemolysis with increasing readiness as the solution becomes more hypotonic. This is confirmed since haemolysis by complement is more rapid in hypotonic lithium chloride solution than in hypotonic sodium or potassium chloride solutions, due to the fact that sheep cells are more fragile in the former solution than in the latter.

An increased osmotic pressure in the system produces a reversible inactivation of complement. It is therefore a

means of preserving complement, but the complement is unstable and if the hypertonicity is unduly high, irreversible inactivation occurs due to the failure of the corpuscles to bind the endstuk. The degree of inhibition is increased by dilution of the complement serum in the system, and decreased by an increased sensitisation. The effects observed in the case of the chloride systems are due simply to the fact that the sodium chloride and potassium chloride are more inhibitory in these concentrations than the solution of lithium chloride (p. 138).

The degree of sensitisation plays an important rôle in determining the effects observed. If the degree of sensitisation is low, the absorption of the complement by the sensitised cells will be diminished; as a sequel the increase in speed of haemolysis in hypotonic solutions is small compared with the increased haemolysis time of normal sensitised corpuscles, while the inhibition in hypertonic solutions does not vary from the normal. With a high degree of sensitisation a higher proportion of the complement introduced into the system is absorbed, with the result that the inhibition observed in hypertonic solutions is diminished. This is in explanation of Topley's observation (1915) that "if the concentration of the antibody be markedly increased, it is possible, up to a certain point, to counteract the

effect of increased salt concentrations; and if the salt concentration be decreased, a decreasing concentration of antibody serves to produce the union of cells and complement."

SECTION IVc.

THE INFLUENCE OF NEUTRAL INORGANIC SALTS ON THE SPEED OF COMPLEMENT ACTION.

The effect of various salts on complement activity has received much attention since Hektoen (1903) pointed out that inorganic salts, such as calcium chloride, barium chloride, magnesium chloride, sodium sulphate, sodium oxalate, and sodium tartrate, inhibited the amboceptor-complement haemolytic system when present in certain concentrations.

Wright and MacCallum (1922) have made a complete survey of the inhibition of complement activity by salts. Unfortunately their results are vitiated since the salts selected show considerable variations in pH; that there is an optimal pH for haemolysis by complement has been shown by Osborn (1934) and others. It is therefore possible that some of the effects which they observed were due to variations in hydrogen-ion concentration as well as to the specific action of the salts they used.

Eagle and Brewer (1929) found that salts with divalent kations were more inhibitory than salts with monovalent kations. These workers described a peculiar three-fold effect occurring as a result of adding electrolytes to mixtures of complement and sensitised corpuscles suspended in glucose

solution. Low concentrations of electrolyte produced inhibition, concentrations above this gave optimum conditions for haemolysis, while still greater concentrations resulted again in inhibition. This last effect is a good example of the well-known inhibition produced by salts in hypertonic solution. The use of a 5% glucose solution as a diluent accounts for the apparent anticomplementary effect of small amounts of electrolytes, this, in reality, being due to the known inhibitory effect of glucose (Marks,1911,a; Sachs and Teruuchi, 1907; Ponder, 1934), which is only counteracted by the electrolyte when it reaches a sufficiently high concentration. Altogether these results do not provide an uncomplicated study of salt action. Later, Gordon and Thompson (1933) studied the effect of strictly neutral salts of sodium and potassium on complement action. They employed twice normal salt solutions, and their resulting systems were not isotonic with blood, but showed varying degrees of hypertonicity. It has been shown that the osmotic pressure of the medium has an appreciable influence on haemolysis by complement (tables 23 - 7).

It is apparent that the study of complement inhibition by salt action has not been sufficiently systematic from the physico-chemical viewpoint. It was therefore decided to investigate the effect of salt solutions on

complement, paying particular attention to the osmotic pressure and pH of the solutions. Further, the serial tube method of estimation used by these workers is, owing to the stepped results that it yields, less accurate than the timing and colorimetric methods to be employed. In short, the measurement of the speed of complement action and the progress of haemolysis by complement for specified time enable a much finer differentiation of inhibitory effects to be obtained than is possible with the older methods of estimating the smallest amount of salt which caused complete inhibition of complement, or that amount which allowed a trace of haemolysis to occur (Wright and MacCallum, 1922; Gordon and Thompson, 1933), or the minimal haemolytic dose of complement required for complete haemolysis (Eagle and Brewer, 1929).

EXPERIMENTAL.

In this study it was essential to exclude all other factors which might influence the action of complement. Ammonium salts were excluded since they destroy the fourth component of complement (Gordon, Whitehead, and Wormall, 1926), and they produce haemolysis even in isotonic solutions. This is due to the fact that these salts hydrolyse in solution to yield ammonia, which penetrates the cell

membrane readily causing osmotic attraction of water (Jacobs, 1926). Other salts, e.g., sodium citrate, potassium citrate, ferric chloride and borates were not employed since they were haemolytic to sensitised or unsensitised erythrocytes in the absence of complement.

In order to avoid the effects due to hypotonicity or hypertonicity (Section IVb), it was important to use solutions isotonic with blood. A solution of 0.9% sodium chloride was taken as isotonic with blood (p. 110), and the salt solutions were made up in strength to correspond with the normality of 0.9% NaCl, viz. 0.154 N. The depression of freezing point of this standard isotonic solution was found to be 0.545°C. By addition of the salt or dilution with distilled water the concentration of the salt solutions was adjusted to give freezing point determinations between 0.540°C. and 0.565°C. From table 23 it is apparent that variations in tonicity equivalent to freezing point determinationsbetween 0.540°C. and 0.565°C. have a negligible effect on the speed of complement action. The concentration, normality, and freezing point depression of the solutions used are given in table 28.

The hydrogen ion concentration of these isotonic solutions was determined by Gillespie's colorimetric drop method or by means of the quinhydrone electrode. Only

Potassium salts.	Concentration,%	Normality	$\triangle^{o}C.$	pH.
1. KBr.	1.72.	0.145N.	0.540.	7.1.
2. KNO_3.	1.70.	0.168	0.554.	6.9.
3. KCl.	1.13.	0.154.	0.562.	6.8.
4. KI.	2.54.	0.153.	0.559.	7.0.
5. KCNS.	1.51.	0.158.	0.557.	7.6.
6. K_2SO_4.	2.15.	0.247.	0.546.	7.7.
7. K oxalate.	2.24.	0.249.	0.556.	7.6.
Sodium salts.				
1. NaBr. 2 aq.	1.97.	0.142.	0.550.	6.8.
2. $NaNO_3$.	1.25.	0.147.	0.554.	6.6.
3. NaCl.	0.90.	0.154.	0.545.	6.8.
4. NaI. 2 aq.	2.15.	0.116.	0.550.	6.8.
5. NaCNS.	1.55	0.191.	0.552.	7.2.
6. Na_2SO_4. 10 aq.	3.65.	0.227.	0.560.	7.0.
7. Na oxalate.	1.71.	0.255.	0.555.	7.8.
Chlorides.				
1. LiCl. 2 aq.	0.63.	0.080.	0.545.	6.7.
2. NaCl.	0.90.	0.154.	0.545.	6.8.
3. KCl.	1.13.	0.154.	0.562.	6.8.
4. $MgCl_2$. 6 aq.	2.84.	0.280.	0.543.	6.9.
5. $BaCl_2$. 2 aq.	2.32.	0.222.	0.540.	7.7.
6. $CaCl_2$.	1.80.	0.324.	0.556.	7.9.
7. $SrCl_2$. 6 aq.	2.76.	0.207.	0.565.	6.8.

Table 28. The Concentration, Normality, Depression of Freezing Point and pH of the Neutral Salts Studied.

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those solutions having a pH of 6.6 - 7.9 were employed; within this range of pH the speed of Haemolysis is optional (Eagle and Brewer, 1929; Osborn 1934). As a result salts such as caesium chloride (pH 5.0), rubidium chloride (pH 5.3), beryllium chloride (pH 3.8), KCN (pH 11.0), trivalent salts, phosphates, borates, acetates, and citrates were discarded. It was considered inadvisable to adjust the pH of these solutions, since this would lead to complicating salt effects and to a lowering in concentration of the salt in question. Further, we have the observation of Ehrlich and Sachs (1902) and Piettre and Chrètien (1927) that the addition of NaOH increased the complementary activity of the haemolytic system, whilst the addition of acid diminished complement activity. The study of complement inhibition has therefore been confined to the following series of neutral salts listed in table 28.

These series of salt solutions were tested for their effect on the speed of haemolysis in a standard amboceptor-complement haemolytic system when added in increasing

quantity and for their influence on the degree of haemolysis produced by complement in a standard time.

(i) Speed of Haemolysis.

The time for complete haemolysis was estimated in a system consisting of equal volumes of fresh guinea-pig serum as the source of complement and a 3% sheep cell suspension sensitised with 5 units anti-sheep cell serum as the antigen-amboceptor complex, acting in the presence of increasing amounts of the salt solution. In carrying out the estimation the following volumes were drawn up in a graduated micropipette (1 volume is equivalent to 0.1cc.) with a bubble of air between each:-

3-x vols. 0.9% NaCl.
x vols. salt solution (x was varied from 0-1.0 by 0.2 volume intervals).
0.5 vols. fresh guinea-pig serum.
0.5 vols. 3% sheep cell suspension, sensitised five units amboceptor.
4.0 vols. haemolytic system.

The measured volumes were expelled into a Dreyer agglutination tube, incubated at 37°C. in a waterbath, and the time for haemolysis noted in each case. The speed of haemolysis is expressed as a percentage of the haemolysis time in the system initially observed (i.e., in the absence of foreign

salts). The details of the results obtained for one salt solution are given in table 29.

It was found that the initial haemolysis time in the above system varied appreciably for different samples of serum, due to differences in the absolute amount of complement present in the measured volume of serum from time to time. In order that the absolute amount of complement might be approximately constant throughout all experiments, only those guinea-pig sera which gave complete haemolysis in 70 - 90 seconds in the absence of foreign salt, were employed. As a result the percentage haemolysis speeds for each salt-concentration lie within close limits, and must be regarded as quantitatively accurate for the concentration of complement, viz. 5-6 units.

Since the total volume of the haemolytic system was constant and the volumes of serum and sensitised sheep cell suspension were constant, the only variable factors were the saline and the substance under investigation. The variation in the volume of saline present in the system can be ignored as having any effect on the haemolytic reaction, since the absolute amount of sodium chloride present, if re-calculated as a normality concentration, does not fall outside the zone for optimal complement fixation (Eagle and Brewer, 1929 , fig.6).

Vols. Isotonic Salt Solution.	Haemolysis Time in Secs.	% of initial Speed.	Haemolysis Time in Secs.	% of initial Speed	Haemolysis Time in Secs.	% of initial Speed.	Haemolysis Time in Secs	% of initial Speed.	Aver. Speed.
0.0	71.	100.0.	78.	100.0.	73.	100.0.	92.	100.0.	100.0.
0.2	77.5	95.9.	80.	97.5.	74.	98.6.	93.5.	98.4.	97.6.
0.4	79.	92.0.	84.5.	92.3.	77.	94.8.	98.5.	93.4.	93.1.
0.6.	84.	84.5.	88.	88.8.	79.	92.4.	104.5.	90.1.	88.7.
0.8.	85.	83.5.	-	-	83.	87.9.	107.	86.0.	85.8.
1.0.	86.	82.6.	98.	79.6.	85.	85.9.	108.	85.2.	83.3

Table 29. The Effect of Increasing Concentrations of NaCNS on the Speed of Haemolysis by Complement.

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The mean speed of haemolysis for each salt, expressed as a percentage of the initial observation, was plotted against the concentration of the salt (figs. 6,7,8). As the concentration of the salt was increased, so was the speed of haemolysis increased or decreased in a regular manner. The salts at any concentration produced changes in the complementary activity which fell into an anion and kation series of increasing inhibition of complement:-

Na salts:- $Br = NO_3 < Cl < I < CNS < SO_4 <$ oxalate.

K salts:- $Br < NO_3 = Cl < I < CNS = SO_4 <$ oxalate.

The potassium series of salts were found to be consistently more inhibitory than the sodium series.

Chlorides:- $Li < Na < K < Mg < Ba < Ca < Sr$.

In the anion series, due to the small range of inhibition between the different salts, there was occasionally a little variation in results. For example, the effect of sodium bromide was usually found to be equal to that of sodium nitrate, but occasionally appeared to be less inhibitory. The accuracy of the experiment hardly allowed one to say definitely whether bromide was equal in effect to nitrate or less inhibitory. However, on no occasion was either salt found to be as inhibitory as chloride.

(ii) Degree of Haemolysis

The degree of haemolysis was estimated at specified

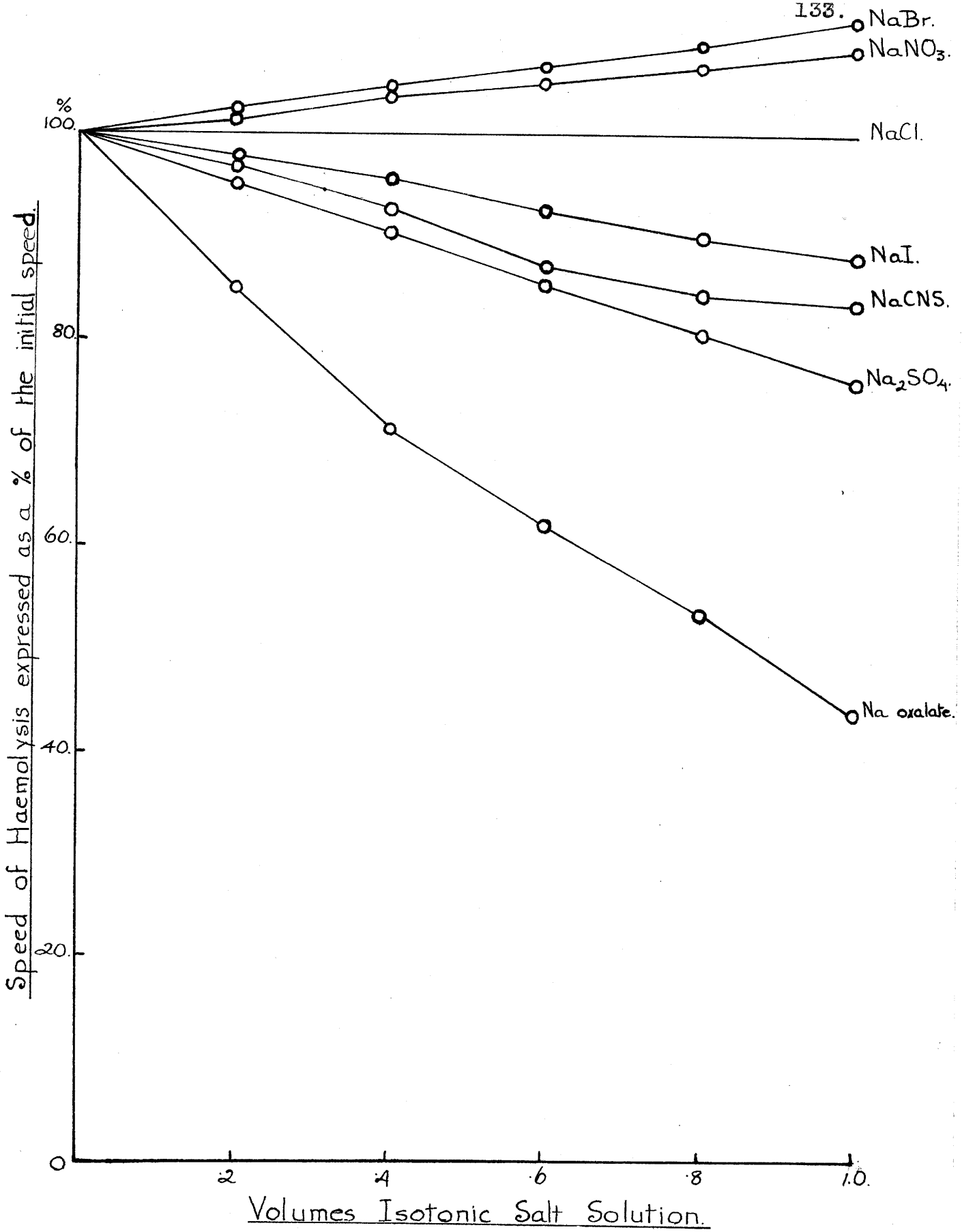

Fig.6. The Effect of Isotonic Sodium Salt Solutions on the Speed of Complement Action.

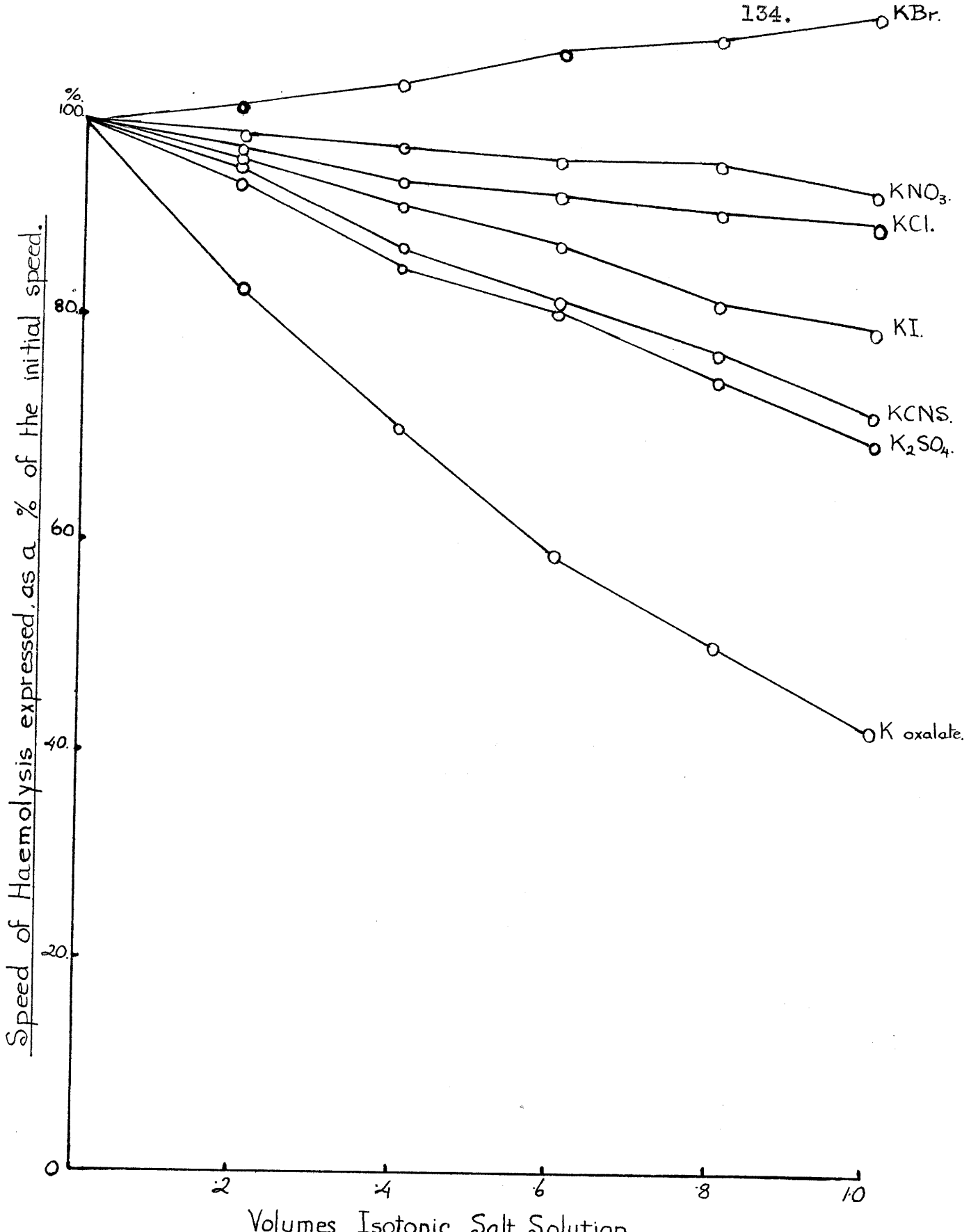

Fig.7. The Effect of Isotonic Potassium Salt Solutions on the Speed of Complement Action.

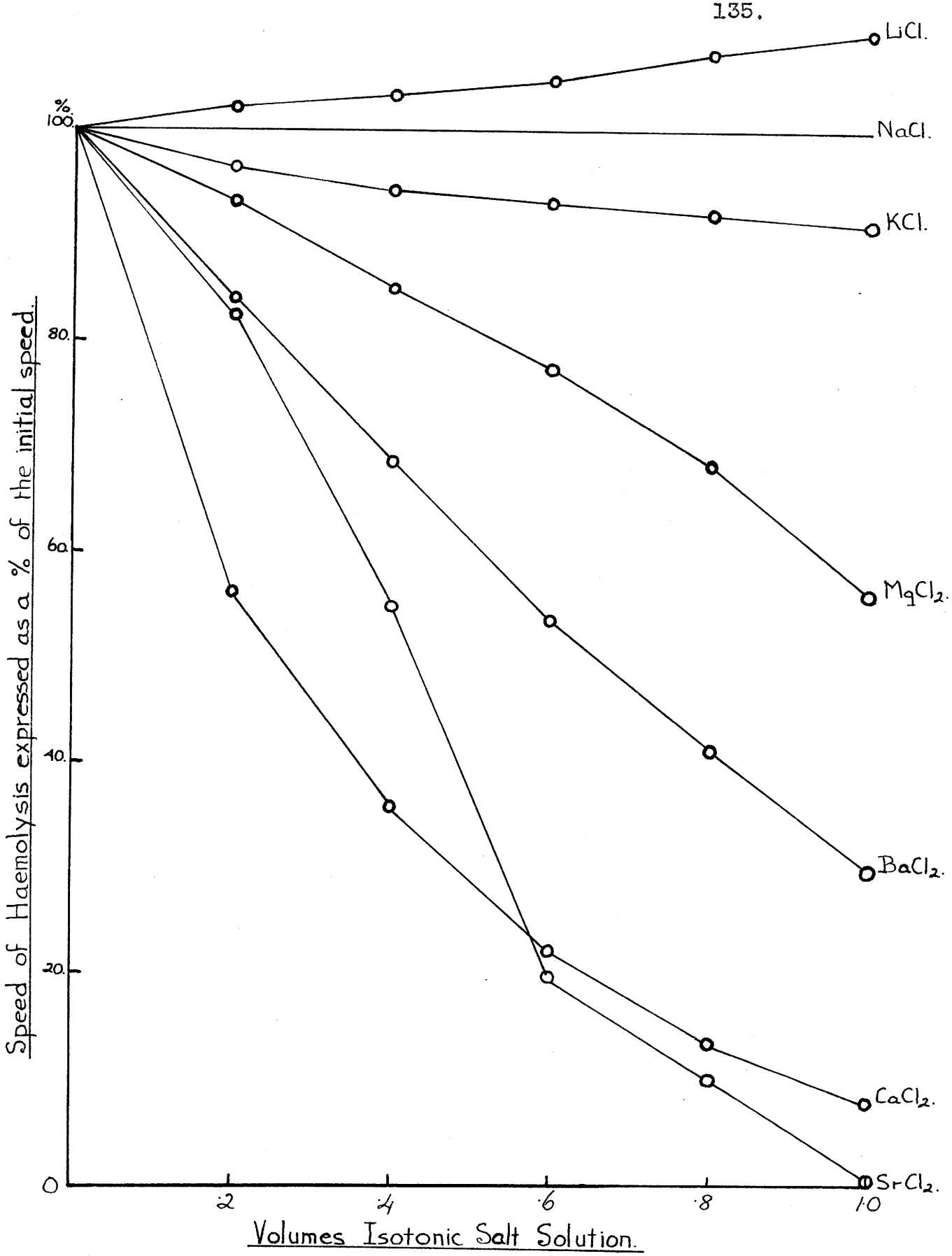

Fig.8. The Effect of Isotonic Monovalent and Divalent Chloride Solutions on the Speed of Complement Action.

intervals of time in a standard system containing sensitised cells, complement, and normal saline (0.9% NaCl) and compared with the percentage haemolysis in similar systems in which the saline is replaced by iso-osmotic salt solutions. (Table 30). The progress of haemolysis was followed in systems consisting of:-

3.0 vols. 5% sheep cell suspension, sensitised 5 units amboceptor.
1.0 vol. fresh guinea-pig serum, diluted 1:20.
1.0 vol. isotonic salt solution.
5.0 vols. (equivalent to 0.5 cc.) haemolytic system.

A series of Dreyer agglutination tubes containing the same salt system was incubated at 37°C., and every two minutes a tube was removed into an ice-salt mixture to prevent any subsequent binding of complement. The tube was rapidly centrifuged, 1 minute at 3,000 r.p.m., in order to throw down the unhaemolysed cells. Four volumes of the supernatant fluid was treated with 1.6 cc. N/10 HCl, stood for 15 minutes to stabilise the colour of the acid haematin, and was then compared in a colorimeter against a series of standard percentage haemolysis solutions prepared by laking an appropriate volume of the sheep cell suspension with the correct volume of distilled water and adding the

29/11/35. Colorimetric Method:- the complete series of neutral salts was tested for its effect on the % haemolysis curve.

Haemolytic System:-

3.0 vols. 5% sheep cell suspension, sensitised 5 units amboceptor.
1.0 vol. guinea-pig serum.
1.0 vol. isotonic salt solution.
5.0 vols.

Colorimeter Test:- 4.0 vols. supernatant fluid + 1.6 ccs. N/10 HCl.

Acid Haematin Standards:-
1. 25% haemolysis- 0.8 vols. sheep cells + 3.2 vols. H_2O + 1.6 cc. N/10 HCl.
2. 50% haemolysis- 1.6 vols. sheep cells + 2.4 vols. H_2O + 1.6 cc. N/10 HCl.
3. 75% haemolysis- 2.4 vols. sheep cells + 1.6 vols. H_2O + 1.6 cc. N/10 HCl.
4. 100% haemolysis- 3.2 vols. sheep cells + .8 vols. H_2O + 1.6 cc. N/10 HCl.

The standard was set at 15 on the colorimeter scale.

Protocol of some results:-

Period of Incubation in Minutes	NaCl.				$BaCl_2$.			
	Standard.	Test	Test Ratio.	Test Haem.	Standard.	Test.	Test Ratio.	Test Haem.
2.	1.	-	.3	7.5%	1.	-	.16.	4.0%.
4.	1.	16.7	.898	22.5	1.	-	.32.	8.0
6.	2.	17.9	.838	41.9	1.	25	.60.	15.0
8.	2.	13.2	1.136	56.8	1.	15	1.00.	25.0
10.	3.	14.6	1.031	77.3	1.	11.5	1.303	32.6
12.	4.	15.8	.949	94.9	2.	18.7	.802.	40.1
20.					2.	11.5	1.303.	65.2
30.					3.	15.8	.952.	71.4
60.					4.	16.7	.898.	89.8

Table 30. The Effect of Isotonic Salt Solutions on the Degree of Haemolysis in an Amboceptor-Complement Haemolytic System.

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same volume of N/10 HCl. (table 30).

The results have been presented graphically in figs. 9, 10, 11. It is seen that the haemolysis produced by each salt proceeds at a regular uniform speed, and that the anions and kations arrange themselves in the following order of increasing inhibition of complement at any stage in the haemolytic process:-

Anions:- Na —— Br = NO_3 < Cl < I < CNS < SO_4 < oxalate.

K —— Br = NO_3 < Cl = I < CNS < SO_4 < oxalate.

Kations:- Cl —— Li < Na < K < Mg < Ba < Ca < Sr.

The variations observed in the latent period during which no haemolysis occurs would indicate that the various salts produce their effect by affecting the absorption of complement, since this latent period is due primarily to the time taken by the sensitised cells to absorb complement.

DISCUSSION.

By taking great care to preserve the correct osmotic pressure and optimal pH in the haemolytic systems, and by using methods giving a considerable degree of accuracy, the anion and kation series for isotonic solutions in order of increasing inhibition of complement is:-

Anions:- Br = NO_3 < Cl < I < CNS ⩽ SO_4 < oxalate.

Kations:- Li < Na < K < Mg < Ba < Ca < Sr.

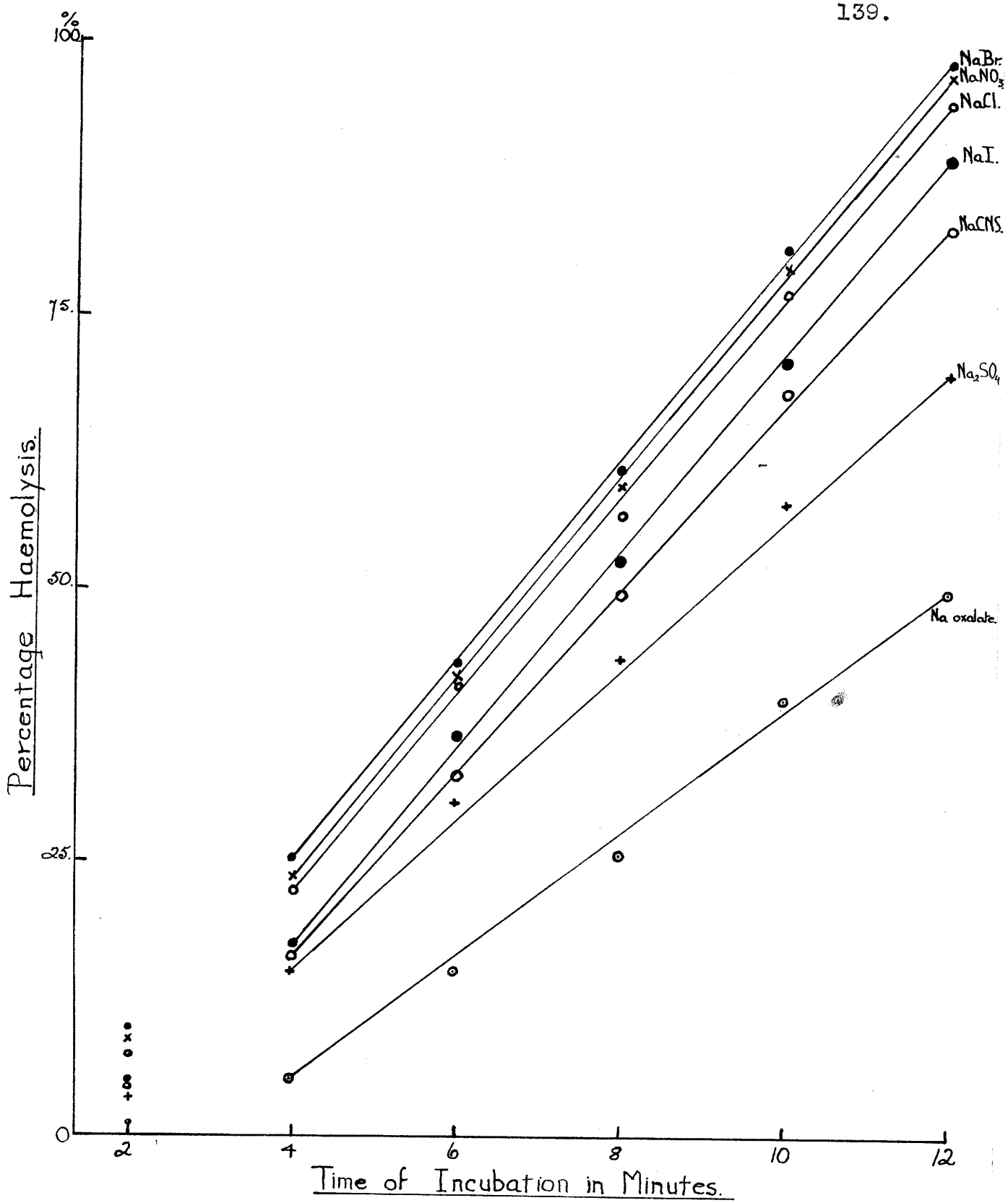

Fig.9. The Effect of Isotonic Sodium Salt Solutions on the Percentage Haemolysis Curve in an Amboceptor-Complement Haemolytic System.

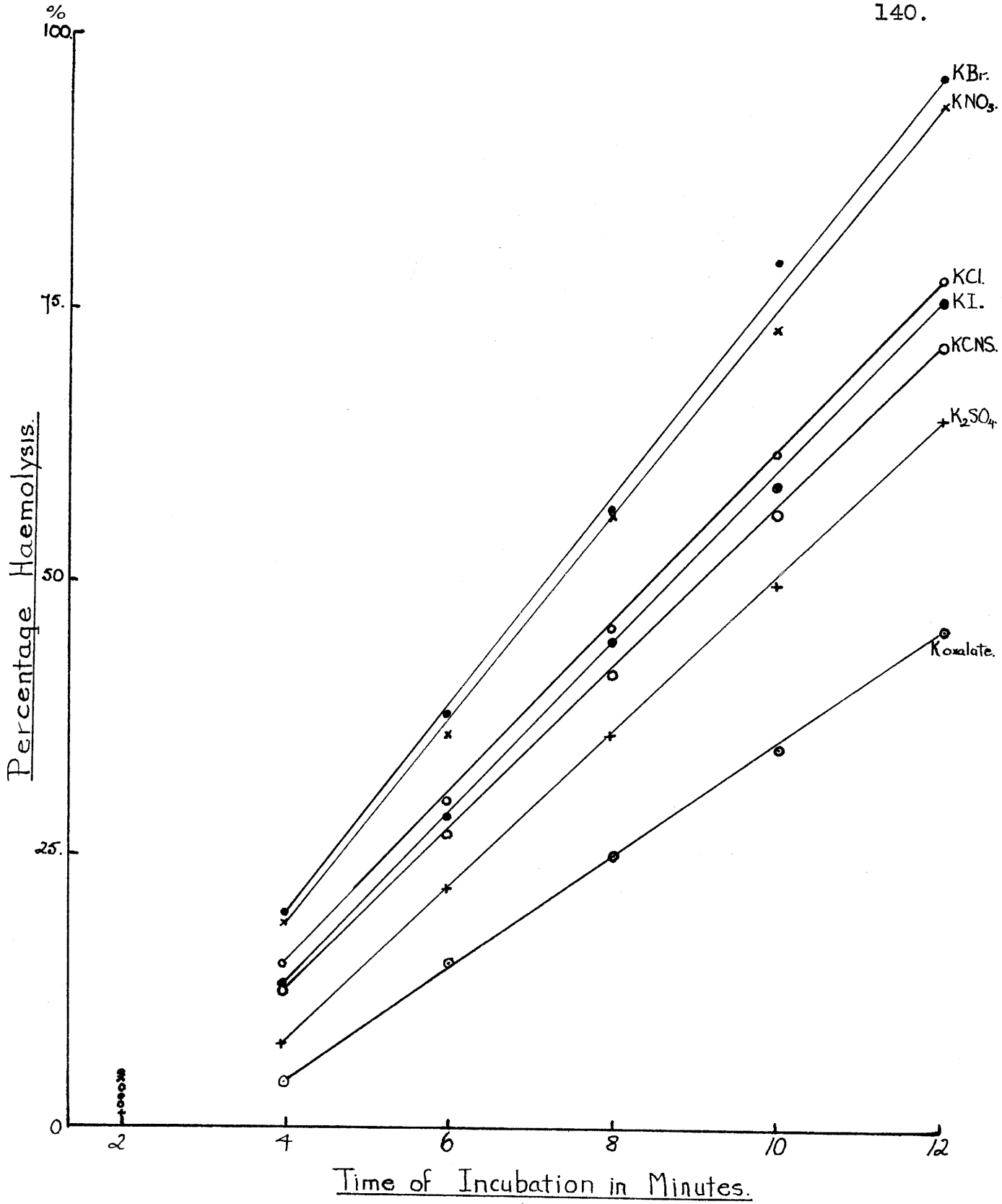

Fig.10. The Effect of Isotonic Potassium Salt Solutions on the Percentage Haemolysis Curve in an Amboceptor-Complement Haemolytic System.

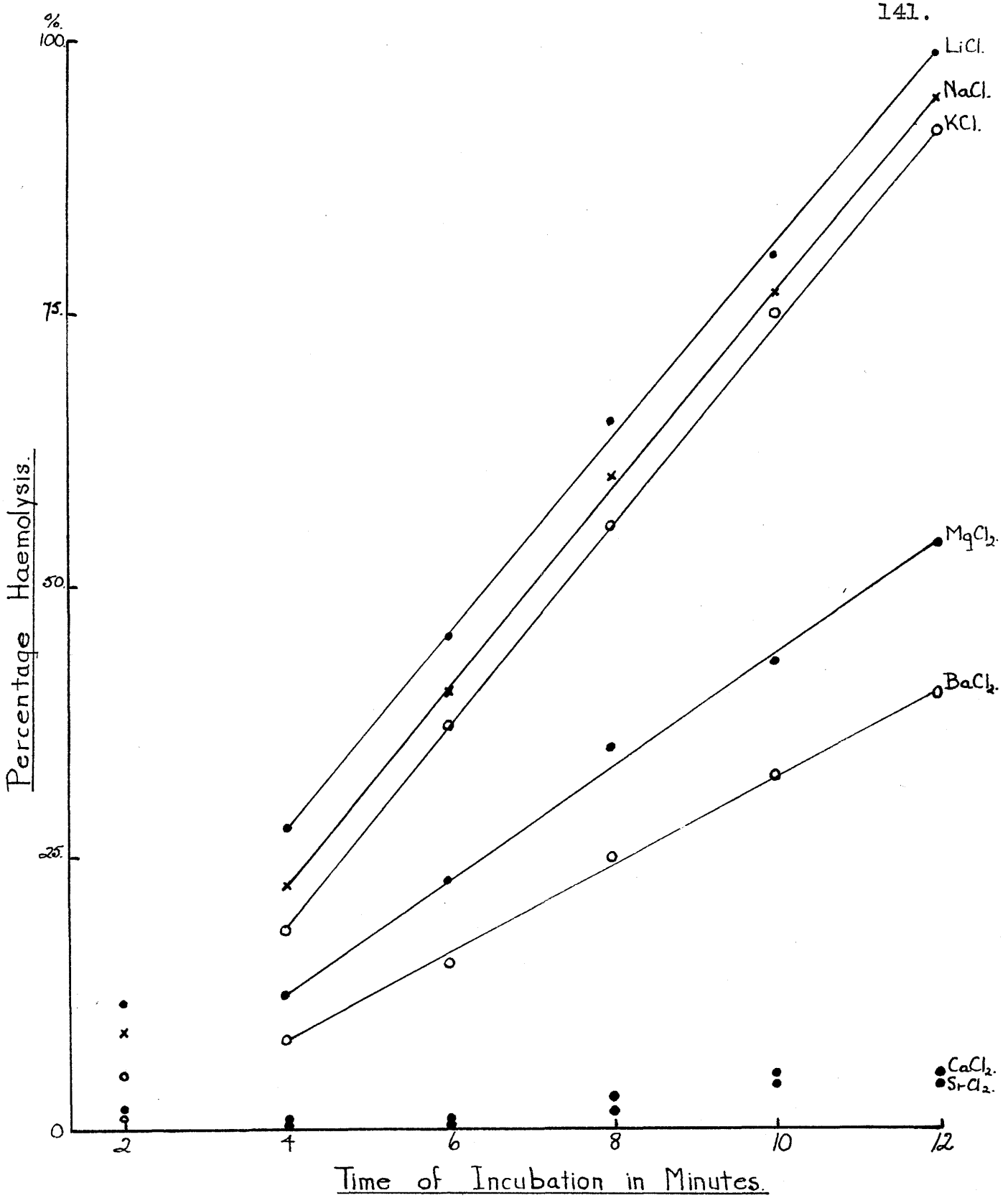

Fig.11. The Effect of Monovalent and Divalent Chloride Salt Solutions on the Percentage Haemolysis Curve in an Amboceptor-Complement Haemolytic System.

The divalent kation is far more inhibitory than the monovalent ion; it was not found possible to study the effect of trivalent ions, since their acidity completely inactivated the complement. However, in concentrations insufficient to produce a high acidity in the haemolytic system, they produced appreciable inhibition of complement. The strongly inhibitory character of magnesium, barium, calcium and strontium calls to mind their anticoagulant property, and the close identity held to exist between the midstuk of complement and prothrombin (Fuchs, 1929,a,b; 1930b; 1933; Barcroft, Quick, and Stanley-Brown, 1934; Quick, 1935).

The anion series for complement inhibition given by Gordon and Thompson (1933) for normality solutions,

$Cl < SO_4/2 < NO_3 = Br < I < CNS$,

is, with the exception of the sulphate iron, the same as the lyotrope (Hofmeister) series for the effect of anions on the properties of protein solutions:-

$SO_4/2 < Cl < Br = NO_3 < I < CNS$. (Höber,1908; Lillie,1910, 1911; Trondle, 1918; Northrop and Kunitz, 1925; Stiasny, das Gupta and Tresser, 1925).

$K < Na < Li < Mg < Ba < Ca$. (Lillie,1909; Trondle,1918.)

The variations in these experiments from the experimental Hofmeister series are not significant and may be ascribed to the fact that the author used isotonic solutions in preference to

normality solutions, since differences in ion effects do not remain quite the same at all concentrations of the salts.

On the basis of their results Gordon and Thompson (1933) have drawn an analogy between the inhibitory action of neutral salts on complement and their parallel action on the dispersion of gelatin (Northrop and Kunitz, 1925; Stiasny, das Gupta, and Tresser, 1925). They postulate, therefore, that complement activity is associated with a particular state of aggregation of the serum proteins.

The ultimate effect is, however, produced by the salts causing variations in the absorption of complement by the sensitised cells. This is seen from the range in the "latent period" in the percentage haemolysis curves, a period which is due to the absorption of complement by the antigen-antibody complex. It is in confirmation of the results of Eagle and Brewer (1929), who found that complement fixation was an essential preliminary to haemolysis, and that the influence of electrolytes was due primarily to the effects produced upon the fixation (absorption) of complement by the sensitised cell.

SECTION IV d.

THE EFFECT OF VARIOUS ANTI-COAGULANTS, CHEMICAL SUBSTANCES AND ENDOCRINE PRINCIPLES ON HAEMOLYTIC COMPLEMENT.

Manwaring (1904) and Hektoen and Ruediger (1904) showed that small doses of M/8 solutions of many salts prevented the haemolysis of red cells and lysis of bacteria by various sera, and concluded that the antilytic activity was the result of the action of these salts on complement. Bordet and Gay (1908), Gengou (1908) and Tokunaga (1928) have pointed out that sodium citrate inhibits complement in vitro, and this has been confirmed for various chemical substances possessing anti-coagulant properties. Isotonic solutions of sodium and potassium oxalate, citrate and fluoride were introduced into a haemolytic system in increasing amounts:-

3 - x vols. 0.9% NaCl.
x vols. salt solution (x varies from 0.0 - 1.0 vol. by 0.2 vol.intervals).
0.5 vols. sensitised corpuscles, 5 units amboceptor.
0.5 vols. guinea-pig serum.

It is seen from table 31 that these substances possess a high degree of inhibition against complement in the following order of increasing inhibition:-

Vols. Isotonic Solution in System.	Haemolytic Speed Expressed as % of Speed with no Added Salt Solution.					
	Sodium Oxalate.	Potassium Oxalate.	Sodium Citrate.	Potassium Citrate.	Sodium Fluoride.	Potassium Fluoride.
0.0	100.0	100.0	100.0	100.0	100.0	100.0
0.02	-	-	94.0	90.0	91.0	89.5
0.04	-	-	88.2	84.0	84.2	82.0
0.06	-	-	84.1	80.0	78.0	77.2
0.08	-	-	80.2	76.0	70.6	70.0
0.1	93.0	92.0	76.4	72.2	64.0	60.5
0.2	85.0	84.6	58.4	57.0	31.0	28.4
0.4	71.5	71.5	36.8	35.2	8.8	6.5
0.6	62.2	59.5	23.0	22.0	3.0	1.3
0.8	53.5	51.0	10.0	9.3	0.0	0.6
1.0	44.0	43.0	5.4	3.6	0.0	0.0

Table 31. The Effect of Isotonic Solutions of various Chemical Anti-coagulants on the Speed of Complement Action.

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oxalate < citrate < fluoride, the potassium salt being more inhibitory than the sodium. The inhibitory action of the oxalate, citrate and fluoride ions on complement is as yet unexplained, since these ions produce a precipitation of the calcium ions in the blood in insoluble form while the removal of the diffusible calcium was found to have no effect on the complementary activity of the serum (Gordon, Whitehead and Wormall, 1926).

Heparin.

Tests were carried out on the anti-coagulant heparin for its effect on complement activity with a view to its use in the preparation of plasma samples, since the chemical anti-coagulants usually employed, viz., oxalate, citrate, fluoride, have been shown to be highly inhibitory. The effect of adding 1:1,000 heparin in normal saline to the standard haemolytic system used in the experiments was found to be only slightly inhibitory in contrast to the strong inhibition produced by the chemical anti-coagulants (table 32).

The anti-complementary action of heparin is said to be due to the inactivation of the third component of complement (Ecker and Gross, 1929), while Fuchs (1930, c) regards heparin, i.e., anti-prothrombin (on his hypothesis of the identity of prothrombin with the midstuk of complement)

Vols. Heparin Solution in System.	.001 % Heparin.		.01% Heparin.		.05% Heparin.	
	Haemolysis Time in seconds.	% of Initial Speed.	Haemolysis Time in Seconds.	% of Initial Speed.	Haemolysis Time in Seconds.	% of Initial Speed.
0.0	66.	100.	62.	100.	81.	100.
0.2	71.	93.	69.	90.	87.	93.
0.4	73.5	90.	72.	86.1.	93.	87.
0.6.	78.5.	84.	76.	81.5.	100.	81.
0.8.	81.5.	81.	84.	73.6.	108.	75.
1.0.	80.	82.5.	82.	75.6.	128.5.	63.

Table 32. The Effect of Heparin Solutions on the Speed of Complement Action.

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as having a direct inactivating effect on the midstuk. Kowarzyk(1931), on the other hand, attributes its action to the fact that it prevents the binding of complement.

Cyanide.

The effect of cyanide on the action of complement is of interest, since this chemical acts as an enzyme poison. The effect of sodium or potassium cyanide on complement haemolysis was strongly inhibitory. Although the isotonic cyanide solution is strongly alkaline (pH 11.0), complement is completely inhibited by 0.01 c.c. KCN in a 0.4 c.c. haemolytic system, a concentration of cyanide that will not materially alter the pH of the standard haemolytic system (table 33). Randall (1933) found that sodium cyanide inhibited complement in 1/6 by volume of a 1% solution, and that the effect is produced on the complement, the antigen-amboceptor combination being unaffected.

Bicarbonates.

The injection of bicarbonates into the bloodstream was found to produce an increase in complement activity (Michaelis and Świrsky, 1910; Sherwood, 1917). The addition of an isotonic solution to an amboceptor complement system was found to produce an increase in the speed of haemolysis (table 34). It will also be recalled that addition of bicarbonate to carbon dioxide treated serum diminished the inactivation of the complement.

Isotonic Solution:- KCN-0.96%; 0.156N; Δ °C. 0.546; pH 11.0.

Vols. Isotonic Solution in System.	Haemolysis Time in Seconds.	% of Initial Speed.	Haemolysis Time in Seconds.	% of Initial Speed.	Haemolysis Time in Seconds.	% of Initial Speed.
0.00.	82.	100.0.	90.	100.0.	52.	100.0
0.02.	106.	77.5.	142.	62.7.	102.	51.0
0.04.	183.	44.8.	215.	41.8.	296.	17.6.
0.06.	430.	19.1.	394.	22.6.	600.	8.6.
0.08.	6300.	1.3.	956.	9.4.	850.	6.1.
0.10.	No haem.	0.	1250.	7.2.	1150.	4.5.
0.20.	"	0.	1432.	6.3.	No haem.	0.
0.40.	"	0.	No haem.	0.	1800.	2.9.
0.60.	"	0.	"	0.	No haem.	0.
0.80.	"	0.	"	0.	"	0.
1.00.	"	0.	"	0.	"	0.

Table 33. The Effect of Potassium Cyanide on the Speed of Complement Action.

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Isotonic Solution:- $NaHCO_3$ - 1.29%; 0.154N; $\Delta^{\circ}C$ 0.541; pH 8.3.
$KHCO_3$ - 1.55%; 0.157N; $\Delta^{\circ}C$ 0.548; pH 8.0.

Vols. Isotonic Solution in System.	$NaHCO_3$.		$KHCO_3$.	
	Haemolysis Time in Seconds.	% of Initial Speed.	Haemolysis Time in Seconds.	% of Initial Speed.
0.0.	78.	100.0	79.	100.0
0.2.	76.	102.6.	77.	102.6.
0.4.	77.5.	100.6.	78.	101.3.
0.6.	76.	102.6.	77.	102.6.
0.8.	75.	104.0	78.	101.3.
1.0.	75.	104.0	74.	106.8.

Table 34. The Effect of Isotonic Bicarbonate Solutions on the Speed of Complement Action.

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Endocrine Principles.

Adrenaline, pituitrin and thyroxine were tested for their effect on complement in vitro.

Adrenaline was tested in the form of its hydrochloride in 1:1,000 and 1:10,000 solution in the following amboceptor-complement haemolytic system:-

3 - x vols. 0.9% NaCl.
x vols. adrenaline solution (x ranges from 0.0 - 1.0 vol).
0.5 vols. 3% sheep cell suspension, sensitised 5 units amboceptor.
0.5 vols. guinea-pig serum.
4.0 vols. haemolytic system.

The 1:1,000 adrenaline solution was found to be extremely inhibitory, while the more dilute strength (1:10,000) was slightly inhibitory (table 35). In no case was adrenaline found to have an accelerating action on the speed of haemolysis by complement in vitro. This is in contrast to the finding that the injection of adrenaline, 1 c.c. of 1:1,000 subcutaneously or intraperitoneally or 1 c.c. of 1:10,000 intravenously into rabbits, cats and rats increased the complementary power of their blood (Osborn, 1936b), and this was due to an increase in the actual haemolytic complement activity and not to any increase in the naturally occurring amboceptors of the serum. Larger injections

Vols. Solution.	Adrenalin HCl. 1:1,000.		Adrenalin HCl. 1:10,000.		Adrenalin HCl. 1:10,000.	
	Haemolysis Time in Seconds.	% of Initial Speed.	Haemolysis Time in Seconds.	% of Initial Speed.	Haemolysis Time in Seconds.	% of Initial Speed.
0.0.	90.	100.0.	75.	100.0.	56.	100.0.
0.2.	192.5.	46.7.	76.	98.7.	61.	91.8.
0.4.	373.	24.1.	81.	92.6.	67.	83.6.
0.6.	2100.	4.3.	85.	88.2.	72.	77.7.
0.8.	No haem.	0.	88.	85.2.	76.	73.7.
1.0.	"	0.	91.	82.4.	80.	70.0.
3.0.	"	0.	162.	46.3.	120.	46.6

Table 35. The Effect of Adrenalin Hydrochloride on the Speed of Haemolysis in a Standard Amboceptor Complement System.

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produced a distinct fall in complement activity. It is therefore hardly likely that the presence of adrenaline itself in the blood caused the observed increase in complementary activity. Osborn regards it as a further protective mechanism of the sympathetic whereby an animal anticipates bacterial infection of a wound by increasing the defences of the blood.

The effect of pituitrin extract on the speed of haemolysis by complement was found to be entirely different. When it was added to a 3% suspension of sheep cells, either unsensitised or sensitised, it produced instantaneous haemolysis. When serum was added to the system the time for haemolysis was prolonged in the presence of low concentrations of pituitrin, but had no effect when the pituitrin was present in higher concentrations (table 36).

The effect of extracts of the thyroid gland was found to have an acceleratory action on the speed of haemolysis by complement (table 37). Fassin (1907) found that the injection or ingestion of thyroid gland increased the haemolytic action of serum, and that thyroidectomy decreased the haemolytic activity of complement. Müller(1910) confirmed this relationship between the thyroid gland and complement, and found that the naturally occurring amboceptor was also considerably increased on thyroid administration.

Pituitrin (Pituitary Posterior Lobe Extract) 10 units per mil.

.2 vols. pituitrin, .5 vols. sensitised corpuscles, 3.3 vols. saline - immediate haemolysis.

.4 vols. pituitrin, .5 vols. sensitised corpuscles 3.1 vols. saline - immediate haemolysis.

.6 vols. pituitrin, .5 vols. sensitised corpuscles, 2.9 vols. saline - immediate haemolysis.

.8 vols. pituitrin, .5 vols. sensitised corpuscles, 2.7 vols. saline - immediate haemolysis.

1.0 vol. pituitrin, .5 vols. sensitised corpuscles, 2.5 vols. saline - immediate haemolysis.

0.0 vols. pituitrin, .5 vols. sensitised corpuscles, 3.0 vols. saline, .5 vols. guinea-pig serum - complete haemolysis in 92 seconds.

0.2 vols. pituitrin, .5 vols. sensitised corpuscles, 2.8 vols. saline, .5 vols. guinea-pig serum - incomplete haemolysis.

0.4 vols. pituitrin, .5 vols. sensitised corpuscles, 2.6 vols. saline, .5 vols. guinea-pig serum - complete haemolysis in 50 seconds.

0.6 vols. pituitrin, .5 vols. sensitised corpuscles, 2.4 vols. saline, .5 vols. guinea-pig serum - complete haemolysis in 10 seconds.

1.0 vol. pituitrin, .5 vols. sensitised corpuscles, 2.0 vols. saline, .5 vols. guinea-pig serum - immediate haemolysis.

Table 36. The Effect of Pituitrin on Sensitised Corpuscles and on the speed of Haemolysis in an Amboceptor - Complement Haemolytic System.

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Vols. Reagent.	Pituitrin. 1:1.	Pituitrin. 1:5.	Desiccated Thyroid Gland. 0.1%.	
	Haemolysis Time in Seconds.	Haemolysis Time in Seconds.	Haemolysis Time in Seconds.	% of Initial Speed.
0.0.	74.	75.	78.	100.0.
0.2.	incomplete.	115.	76.	102.6.
0.4.	"	194.	77.	101.3.
0.6.	immediate.	immediate.	73.	106.8.
0.8.	"	"	72.	108.3.
1.0.	"	"	70.	111.4.

Table 37. The Effect of Pituitrin and Desiccated Thyroid Gland on the Speed of Haemolysis in an Amboceptor-Complement Haemolytic System.

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SECTION IVe.

SUMMARY.

Complement was found to be extremely sensitive to the alterations in the physical and/or chemical composition of the medium. There is inhibition of complement, as judged by the speed of haemolysis in a standard amboceptor-complement haemolytic system, on standing at room temperature, heating, shaking, addition of certain salts and organic substances, the presence of carbon dioxide in the system, increase of osmotic pressure, and the presence of haemoglobin and serum proteins. The inactivation is reversible in the case of increased osmotic pressure of the solution or in solutions maintained under carbon dioxide, and the complement may be maintained under such conditions for several months without deterioration. On bringing the solution to isotonicity or allowing the gas to escape, the complementary activity of the serum is restored to its original power. These techniques are therefore employed to preserve the complement in the serum.

From the observations of in vitro effects on complement it must be concluded that complement is acting under adverse conditions in the blood due to the presence of the following inhibitory factors:- a body temperature of 37°C., a high

concentration of serum proteins, the presence of a tension of carbon dioxide of 40-46 mms. Hg., and the many inhibitory chemical and organic substances which form the various constituents of the blood serum or plasma. It is therefore not surprising that the methods of increasing the complementary power of blood are limited, viz., injection of bicarbonate, adrenaline or thyroxine; ingestion of large quantities of cod-liver oil (vitamins A and D).

In vitro the only methods of enhancing the complementary activity of serum was through the addition of bicarbonate, thyroxine, or the addition of certain neutral salts, e.g., bromides, nitrates and lithium chloride. The increased speed of haemolysis by complement in hypotonic solutions is accounted for by the lowered resistance of the red cells to haemolysis.

SECTION V.

THE HAEMOLYTIC COMPLEMENT OF NORMAL SERA.

During the investigation into the properties of amboceptor-complement haemolytic systems it was observed that the concentration of complement in a serum varies considerably from species to species. From experiments in this laboratory and the observations of Capart (1928), Cruz and Penna (1930) and Schattenberg and Harris (1934) it appears that, for sensitised sheep corpuscles, the complementary power of the sera of various species can be arranged in the following descending order:-

guinea-pig > human = rat > rabbit = cat.

(a) THE NORMAL COMPLEMENT CONCENTRATION OF GUINEA-PIGS.

The concentration of serum complement in guinea-pigs was estimated over a period of several years (1932-6). The blood was obtained by the method described on p. 41 , a fresh animal being employed for each day's experiment. The complementary activity was estimated in a four volume system containing 0.5 volumes guinea-pig serum and 0.5 volumes sensitised sheep corpuscles by the timing method. The average determination of the speed of haemolysis for each day's serum has been used in drawing up the frequency table (table 38). The mean haemolytic speed of 238 observations is 78.2. seconds.

Haemolysis Time. Class Range.	Class Frequency.
40–49 secs.	13
50–59	49
60–69	35
70–79	34
80–89	41
90–99	34
100–109	7
110–119	11
120–129	8
130–139	4
140–149	2
	238

Table 38. The Frequency Distribution for the Time of Haemolysis of Guinea-pig Sera.

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The standard deviation is 28.4 seconds, and it will be seen that the majority of the observations show a haemolytic speed between 50 - 99 seconds.

Time dilution curves for guinea-pig serum in the standard four volume system show that the asymptote is reached when the serum is diluted 1 in 60 to 1 in 70.

(b) THE NORMAL COMPLEMENT CONCENTRATION IN EUROPEANS AND BANTU.

Observations carried out on the serum of human subjects, both European and Bantu, are reported below with a view to ascertaining its "normal" complement concentration. A portion of the results has already been presented (Bernstein, Maingard, and Osborn, 1935). The factors causing variations in the normal complement are also discussed, and these are correlated with the factors causing variation in in vitro experiments.

Experimental.

Estimations were carried out on the blood serum of 120 medical students and 45 Bantu subjects during the summer months of January - March, 1934. Healthy students between the ages of 18-22 were selected at random, while the Bantu cases were hospital boys and convalescent cases from the surgical wards. Samples were taken in the fore-noon in order

to avoid any variations due to the effect of food (Bernstein, 1936), body temperature (Hadjapoulos and Burbank, 1928), or diurnal fluctuations (Hadjapoulos and Burbank, 1928; Cadham, 1926).

A standard technique of drawing blood was found necessary for constant results. The finger was cleansed with ether and allowed to dry completely. It was essential to avoid any ether haemolysis of the red blood cells (observations showed that haemoglobin-stained serum had a diminished complementary activity compared with clear serum from the same individual). The finger was then coated with paraffin in order to avoid any contamination of the blood by the skin surface. After passive hyperaemia for a minute the blood was drawn by needle puncture. This method was found to give the most consistent results. A protocol of one of the experiments carried out on this point of technique is given in table 39. It was further found that the complement activity was very constant independent of the finger chosen or the depth of the puncture.

About $\frac{1}{2}$ - 1 cc. of the freely flowing blood was collected in a Wrights' tube and sealed off in a flame. The blood was allowed to clot at room temperature (15 - 22°C.) for 15 minutes, then placed in a refrigerator for one hour (temperature kept below 8°C.) and the serum was finally

Blood samples were taken by means of various techniques from a subject during a period of twenty minutes and complement estimations carried out in the following haemolytic system:-

3.0 vols. 0.9% NaCl
0.5 vols. sheep corpuscles, sensitised 5 units amboceptor.
0.5 vols. serum.
4.0 vols. haemolytic system.

Sample.	Haemolysis Time in Seconds.			
	Ether & Paraffin.	Ether.	Iodine.	Paraffin.
Left hand finger 1.	108.	-	107.	-
" " " 2.	105.	-	112.	-
" " " 3.	107.	Hb-stained	-	-
" " " 4.	-	110.	-	109.
" " " 5.	-	139.	-	110.
Right hand finger 1.	107.	112.	-	-
" " " 2.	110.	121.	-	-
" " " 3.	-	-	118.	104.
" " " 4.	-	-	98.	105.
" " " 5.	-	-	106.	111.
Venous puncture lt. arm.	-	105.	-	-
" " rt. arm.	-	109.	-	-

Table 39. The effect of various techniques for drawing blood on the haemolytic complement activity of the serum expressed as a time for haemolysis in the standard four volume system.

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separated by centrifuging at 3,000 r.p.m. for five minutes.

Though human serum contains naturally occurring antibodies, it was found on analysis that the amount of the naturally occurring antibodies was of the same order as that of the artificial sensitisation, viz: about 6 M.H.D. It was therefore not considered essential to remove the naturally occurring antibodies, but to estimate the haemolytic complement concentration of the "crude" serum. Nor was it found feasible to correct for the variation in the "susceptibility to haemolysis" of the corpuscles employed as antigen.

Immediate estimations in duplicate were made by the timing method of the time for complete haemolysis in an amboceptor-complement system composed of:-

3.0 volumes 0.9% NaCl.

0.5 volumes human serum

0.5 volumes sensitised sheep corpuscles (a 3% suspension sensitised with 6 M.H.D. amboceptor).

Since the time for complete haemolysis of a fixed volume (number) of sheep corpuscles is inversely proportional to the amount of complement that has been used up in the lytic process, the time is hence a function of the complement concentration of the serum.

The results have been analysed in the form of a

frequency table (table 40). The observations on the 120 European subjects yielded a mean haemolytic speed of 102.5 seconds, and only 17 results lay without the range of 70-129 seconds. This indicated a low degree of dispersion, the standard deviation being 21.0 seconds. A comparison with estimations on Bantu subjects yielded the interesting fact that there is no significant difference in blood complement.(Tbl.41) The mean haemolysis time for 45 observations was 97.3 seconds, with a standard deviation of 30.6 seconds. Similar findings have been discussed in an earlier paper (Bernstein, Maingard and Osborn, 1935.)

Though there are variations in the blood complement from individual to individual, it is found that the concentration of haemolytic complement in the same individual does not vary appreciably from day to day. Table 42, records results obtained for the complement concentration of "crude" serum of subjects over a varying period of time. The daily variations in the susceptibility to haemolysis of the sensitised corpuscles was estimated against a standard preserved serum. By making an allowance for this the haemolytic speed could be corrected. These results show that the complement concentration is not liable to sudden change. Further no evidence of seasonal change was found both in experiments on humans and experimental animals.

Haemolysis Time.

Class Range.	Class Frequency.
50–69 secs.	5
70–89	27
90–109	51
110–129	25
130–149	9
150–169	3
	120

Table 40. Frequency Table Showing Distribution of the Speed of Haemolysis (Haemolytic Complement Concentration) of the "crude" serum of 120 Europeans.

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Haemolysis Time.

Class Range.	Class Frequency.
50–69 secs.	5
70–89	18
90–109	10
110–129	6
130–149	6
	45

Table 41. Frequency Table showing Distribution of the Speed of Haemolysis (Haemolytic Complement Concentration) of "Crude" Serum of 45 Bantu.

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Date.	Conserved Serum Haemolysis Time.	J.F.M. serum Haemolysis Time.	R.E.B. serum Haemolysis Time.
4/1/34.		75	95
5/1/34.		72	80
8/1/34.		97	
9/1/34.			93
30/1/34.		86	107
31/1/34.		89	
15/3/34.	150	68	
16/3/34.	121	75	92
17/3/34.	129	76	
18/3/34.	127	69	
20/3/34.	180	85	102
21/3/34.		93	
24/3/34.		76	
25/3/34.	179	81	
28/3/34.	181	89	108
29/3/34.	188	93	110
31/3/34.	109	69	
3/4/34.	168	99	100
4/4/34.		91	105
5/4/34.	157	95	102
6/4/34.	154	90	95
7/4/34.		87	
10/4/34.			96

Table 42. Daily Estimations of the Haemolytic Complement (Expressed as Speed of Haemolysis) of Two Subjects.

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Experiments were carried out to see whether there was any diurnal variation in the complement of individuals. Hadjapoulos and Burbank (1928) have shown that the complement titre is lowest during the resting period (.04 cc.) and rises steadily during the day until a maximum is reached between the hours of 4 - 6 p.m., when the titre is .025 cc. They have correlated this with similar variations in body temperature and metabolism. Gunn (1914), Cadham (1926) and others have also commented on the fact that the titre of a subject's serum tends to be higher in the afternoon than in the forenoon.

Hourly estimations of the complement concentration between the hours of 8 a.m. - 6 p.m. were made on different subjects. The results (table 43) show that the general tendency is towards an increase in the blood complement during this period with a fall after the lunch period. This effect resulting on the ingestion of food seemed to be worthy of further analysis since it was not noted by these workers. This fall was confirmed by subsequent experiments (p. 177).

Owing to the time required to prepare the serum and the precautions necessary to prevent its deterioration, attempts were made to apply the colorimetric method to the estimation of complement in whole blood, but this is not successful as yet.

Time.	Haemolysis Time in Seconds.		
	R.E.B.	J.F.M.	J.S.
8 a.m.	96.	82.	68.
9 a.m.	98.	80.	70.
10 a.m.	95.	85.	65.
11 a.m.	111.	81.	66.
12 a.m.	100.	86.	-
1 p.m.	97.	85.	66.
Lunch.			
2 p.m.	146.	94.	89.
3 p.m.	-	90.	70.
4 p.m.	112.	80.	74.
5 p.m.	100.	80.	62.
6 p.m.	90.	74.	58.

Table 43. The Diurnal Variation of the Haemolytic Complement Concentration (Speed of Haemolysis) of Human Subjects.

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DISCUSSION.

Haemolytic complement is a normal constituent of blood, though the factors governing its concentration are as yet imperfectly understood. This is partly due to the fact that the origin of complement within the body is obscure. It has been variously attributed to the leucocytes (Metchnikoff, 1899; Gengou, 1901), liver (Ehrlich and Morgenroth, 1901; Olsen 1922), reticulo-endothelial system (Landsberger, 1930) and various other sites.

Hadjapoulos and Burbank (1928) have postulated that complement is a catabolic product which is used in preventing and fighting disease. If it is a catabolite, then the complementary titre represents the balance between production and destruction. They have found that the complement titre runs parallel with the body temperature, CO_2 elimination, and caloric output in conditions of exercise, fever, and diurnal variation. A high complement was also found in cases of pathological high metabolism, viz. hyperthyroidism, while a low complement was found in cases of hypothyroidism. This is supported by the work of Anderson and Fraser (1934), who showed that a caloric deficiency produced a decrease in serum complement and in the natural haemolytic activity of sheep's whole serum to rabbit's r.b.c.

This is an attractive supposition. It has been seen

that the blood complement concentration of an individual is rather constant from day to day, but shows a definite diurnal variation corresponding with temperature and metabolic changes in the body. But it does not explain the fall in complement activity following the taking of food. Here another factor is at play, namely, the effect of sympathetic dormancy or the shift of the blood enzymes to the alimentary tract (Section Vc.)

Regarding the variation between species it is seen that, both by the time-dilution curves and the time for haemolysis in a standard system, guinea-pig sera are definitely faster than those of human, notwithstanding the presence of naturally occurring antibodies in the latter. Both Fränkel (1911) and Braun (1911) found that the midstuk fraction of complement was present in fairly constant amount in the various species and that the endstuk (albumin fraction) was responsible for the observed variations.

The influence of age, diet and muscular exercise on the haemolytic complement is important, from its general relationship to bodily resistance. The whole subject has been adequately reviewed by Osborn (1936,c).

(c) THE INFLUENCE OF THE INGESTION OF FOOD ON THE HAEMOLYTIC COMPLEMENT OF HUMAN BEINGS.

Ehrlich (1906) conceived the mechanism of immunity as a special application of the normal enzyme system of the body and this point of view was shared by most of the older workers (Metchnikoff, Bordet). Walker (1905-6) brought forward the view that "enzymes are double bodies, consisting of a specific amboceptor or go-between, and a non-specific active kinase ,or complement which can be obtained from many different sources. And it is suggested that the bacteriolysis of micro-organisms and similar phenomena are special cases of a general physiological reaction typified in the action of normal ferments." The evidence of Neuberg and Reicher (1907), Liefmann (1912), Thiele and Embleton (1913), and Sachs and Nathan (1914) was also in favour of regarding the action of complement as enzymic in nature, while it was observed that the inactivation of complement by shaking (Jacoby and Schütze,1910), radiations (Brooks, 1918), increased temperature and various other physical influences ran parallel with the effects on fermentative processes under similar conditions.

More recently Fuchs (1931) has presented evidence in support of a relationship between enzymes and complement. He has shown that the midstuk of complement, which he and his co-workers (Fuchs,1929,a,b; 1930,b: 1931; Fuchs and

Falkenhausen, 1927) regard as identical with the enzyme prothrombin of blood clotting, is able to activate a thermostable substance ("amylase-amboceptor") in heat-inactivated amylase. Further experiments have shown that the complement activity of a serum is reduced on treating it with a fine suspension of starch. On this supposition it might be expected that, under certain conditions, a change in some of the agents of immunity might be found during the digestion of food. During digestion midstuk would be withdrawn from the blood to provide the basis of the digestive enzymes in the alimentary canal and the body cells. In this way since complement is made up of a number of different components, and if the concentration of blood complement, as measured by haemolysis of red blood cells, is the concentration of the least concentrated of the components, then only variations due to this limiting factor will be manifested.

That ingestion of food produces a lowered complement has been experimentally shown by Kolmer, Matsunami, and Trist (1919) for guinea-pigs; they state that blood taken after a 24-hour fast is richer in complement than blood taken after a meal. Similarly Osborn (1931) found a variation in the complement level of rats following the ingestion of a basal diet. Twenty-three experiments were performed, eleven cases of which showed no definite change in complement level. In the other cases the change was of an inconstant nature. It

was decided, therefore, to investigate what effect food produced in the haemolytic complement of human beings (see also Bernstein, 1936).

EXPERIMENTAL

The experiment was carried out on 36 healthy medical students. Samples of blood were taken before the meal (1 p.m.), and an hour later (2 p.m.). The meal consisted of a three-course lunch of fairly constant composition. In each series of observations about $\frac{1}{2}$ cc. of blood was withdrawn from the finger-tip into a Wright's tube, which was immediately sealed off in a flame. The sample was then centrifuged at 3,000 r.p.m. for five minutes, and the supernatant serum was allowed to stand on ice.

Estimations were made one and four hours after sampling of the time for complete haemolysis at 37°C. in a haemolytic system containing standard volumes of sensitised sheep corpuscles and serum (p. 26). Since the time taken for the haemolysis of a fixed volume of sensitised corpuscles is inversely proportional to the amount of complement which has been used to bring about haemolysis, the speed of haemolysis is a function of the complement concentration of the serum.

The complementary activity of the serum after the meal was expressed as a percentage of the concentration before the meal:-

$$\frac{\text{Time for complete haemolysis before meal}}{\text{Time for complete haemolysis after meal}} \times \frac{100}{1}$$

$$= \frac{\text{Speed of haemolysis after meal}}{\text{Speed of haemolysis before meal}} \times \frac{100}{1}$$

$$= \frac{\text{Complementary activity after meal}}{\text{Complementary activity before meal}} \times \frac{100}{1}$$

A protocol of one experiment is set out in table 44. The results have been expressed in the form of a frequency table, showing the distribution of the complement concentration after a meal expressed as a percentage of the initial concentration (table 45). From this it will be seen that of the 36 cases studied ten showed no change outside the limits of accuracy of the experiment, while 24 showed a decreased complement after the meal. The statistical analysis of the above frequency table gave the following results:-

Mean complement conc. before meal is	100.
Mean complement conc. after meal (m)	90.59
Difference	9.41.
Standard deviation of m =	11.76
Standard error of m =	1.96.
m =	90.59 ± 1.96.

Hence the mean complement concentration after the meal differs from 100 by 4.8 times its standard error. Such a difference is probably significant.

Protocol of one experiment:-

Subject:- S.J.L.

Date:- 20/3/34.

	1 p.m. Blood.	2 p.m.Blood.
Vols. 0.9% NaCl.	3.0	3.0
Vols. serum.	0.5	0.5
Vols. sensitised sheep corpuscles. (3% suspension, sensitised with 5 units anti-sheep serum).	0.5	0.5
Haemolysis time in secs. (1 hr.after sampling).	90	120
Haemolysis time in secs. (4 hrs. after sampling).	94	110
Haemolysis speed after meal as % of initial speed.	-	$\frac{90 \times 100}{120}$
		75.0%
Haemolytic speed after meal as % of initial speed.	-	$\frac{94 \times 100}{110.}$
		85.4%
Mean haemolytic speed after meal.	=	80.2%

Table 44. Complement Concentration of Human Serum After a Meal.

Mean haemolytic speed as % of initial speed.	Class frequency.
45-54.	1.
55-64.	1.
65-74.	0.
75-84.	9.
85-94.	13.
95-104.	10.
105-114.	0.
115-124.	1.
125-134.	0.
135-144.	1.

Table 45. Frequency distribution of the complementary activity of sera from human subjects after a meal, expressed as a % of the initial complement concentration.

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In a further series of observations on two subjects (R.E.B.; J.F.M.) daily estimations were made before and after lunch covering a period of three weeks. The results have been summarised in table 46. In 22 out of 26 observations the complementary activity was decreased after the meal. The mean complement concentration after the meal was 89.8% and 95.6% respectively of the initial concentration, and differed from 100 by 3.1 and 1.7 times their standard errors. These differences are probably significant.

Thus the complement reaction of the individual to food does not alter materially from day to day, so that the results obtained for the 36 cases may be regarded as significant.

DISCUSSION.

Sufficient data have been presented to show that there is a significant fall in the complementary activity of human sera after a meal. This effect appears to be specifically due to the ingestion of food, since observations by the author, Cadham (1926), Hadjapoulos and Burbank (1928) and others have shown that there is a slight rise in complement during the day.

The effect of absence of food has yielded contradictory results. Zilva (1919) found no lowering of complement after animals had been kept on a quantitatively restricted diet for 6 months, while Koch and Smith (1924) found a drop in

Subject.	Date.	Haemolysis Time in Seconds.				Haemolytic Speed expressed as a % of initial speed.		
		1 hr. after sampling.		4 hrs. after sampling.		1 hr.	4 hrs.	Mean.
		1 p.m	2 p.m.	1 p.m.	2 p.m.			
J.F.M.	16/3/34.	76.	78.	-	-	97.4	-	97.4
	17/3/34.	79.	90	-	-	85.6	-	85.6
	18/3/34.	69	78.5	-	-	87.9	-	87.9
	20/3/34.	85	94	-	-	90.4	-	90.4
	21/3/34.	93	104	-	-	89.4	-	89.4
	24/3/34.	76	74	-	-	102.7	-	102.7
	25/3/34.	81	90	-	-	90.0	-	90.0
	28/3/34.	140	125	89	77	112.0	113.0	112.5
	29/3/34.	94	99	123	135	95.0	91.1	93.0
	31/3/34.	69	72	-	-	95.8	-	95.8
	3/4/34.	100	89	123	95	120.2	128.0	120.2
	4/4/34.	91	97	96	104	91.7	90.2	90.9
	5/4/34.	94	96	95	97	97.9	97.9	97.9
	6/4/34.	90	93	101	120	96.7	84.0	90.3
	7/4/34.	87	89	-	-	97.8	97.8	97.8
	8/4/34.	110.	125	-	-	88.0	-	88.0
								95.6 Av.
R.E.B.	20/3/34.	102	116	-	117	87.9	-	87.9
	28/3/34.	108	110	113	-	97.3	-	97.3
	29/3/34.	110	121	162	166	90.9	97.6	94.2
	30/3/34.	-	-	108	130	83.1	-	83.1
	3/4/34.	116	115	100	120	99.2	83.3	91.2
	4/4/34.	105	121	114	131	86.8	87.0	86.6
	5/4/34.	110	100	102	103	110.0	99.0	104.5
	6/4/34.	95	102	111	114	93.1	97.3	95.2
	9/4/34.	123	134	-	-	91.8	-	91.8
	10/4/34.	-	146	-	-	65.7	-	65.7
								89.77Av.

Subject.	Observations.	Cases of lowered complement after meal.	Mean after meal as % of initial speed.	Extreme Values	Standard deviation.
R.E.B.	10	9	89.8±3.29	104.5:65.7	10.42
J.F.M.	16	13	95.6±2.61	120.2:85.6	10.43

Table 46. Analysis of the Haemolytic Complement After a Meal in Two Subjects Over a Period of Time.

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complementary power during starvation.

Since complement is a complex of parts acting in sequence, we are unable to state which component is or may be the limiting factor in any one reaction. From Fuchs' demonstration (1931) that enzymes appear to use a complement it would seem that the midstuk component (which is regarded as possessing enzymic properties) might be removed from the bloodstream by the intestinal epithelium in order to activate the digestive enzymes. In this way the complementary activity of serum after a meal would be lowered. Such a state of affairs would be remarkable since all cases of inhibition of complement have been found to be due so far to a failure to bind the endstuk, while the midstuk has been bound (viz. the corpuscles have been "persensitised").

The influence of factors which might complicate results has not been studied, e.g. the effect of the alkaline tide in the blood, the shift of the blood to the internal viscera, and the alteration in the hormonal and chemical constituents of the blood. During digestion there is a resting secretion of adrenaline and a minimal adrenal activity. Cannon (1929) has shown that adrenaline causes a more rapid coagulation, and Fuchs (1929, a,b; 1930 a,b,) has shown that there is a direct relation between the coagulative and complementary powers of blood. A more direct association is the observation that adrenaline, on injection in suitable

doses into rabbits, rats and cats, increases the complementary activity of blood (Osborn,1936,b). An overdose of adrenaline produces a fall in complement, and this is in keeping with the observation (p. 151) that adrenaline in vitro has an inhibitory effect on complement.

There is, therefore, a possibility that sympathetic dormancy and minimal adrenal activity during digestion may explain the fall in blood complement, though we have no proof that lack of adrenaline in vivo decreases blood complement.

SECTION VI.

GENERAL DISCUSSION AND CONCLUSIONS.

The ultimate destination in the study of complement is the discovery of the constitution of complement, the mechanism of its action, and the possibility that an increased blood complement concentration is effective in increasing the general bodily resistance to infection. It is now fifty years since the substance now known as complement was discovered, and so fragmentary is our present knowledge on these aspects of the subject that in a recent review (Osborn, 1936c) the author reported that the investigation of complement had almost fallen into disrepute as a subject for research. The reason why there has been little advance in our knowledge of complement, despite a voluminous literature, can be ascribed to three factors:- firstly, the lack of knowledge regarding the manner in which many of the constituents of serum, particularly protein, are held in solution and the effect of factors producing variations in their concentration; secondly, our inadequate explanations for the phenomenon of lysis; and lastly, the fact that only within recent years have improvements been made in our inefficient methods of complement estimation.

It is therefore considered desirable to put forward a hypothesis of the nature and action of complement, based

on a study of literature and on the results of several years' work and outlook on the subject. The question "What is complement?" and "How does it act?" cannot be answered, as yet, on the basis of experimental data obtained, but depends on the interpretation of such data and the elimination of the more remote possibilities.

The idea that the complementary activity of serum is but a manifestation of some physical characteristic of the serum, e.g., surface tension, colloidal dispersion (Traube, 1908; Liefmann, Cohn and Orloff, 1912; Schmidt, 1913; Gordon and Thompson, 1933), may be regarded as unproven, since the analogous reactions are based on isolated observations. Further, the general properties of complement, e.g., inactivation on standing, shaking or heating, and variations in activity produced by alterations in the pH, osmotic pressure, and various chemical agencies, do not indicate a physical phenomenon, but rather an entity of very labile character.

Studies on the composition of complement have shown that it is a complex of parts which must act in a definite sequence to produce lysis of cells. Fractionation of the serum proteins with distilled water, carbon dioxide, hydrochloric acid, or ammonium sulphate produces a globulin precipitate soluble in normal saline, while the albumin

fraction remains in solution. The midstuk component of complement is resident in the globulin precipitate while the endstuk is associated with the albumin fraction. The question arises as to whether these components of complement are protein in nature or whether they are present in association with the serum proteins by chemical (prosthetic group) or physical means (adsorption). The numerous papers of Fuchs and his co-workers have shown the close identity between midstuk and prothrombin, a globulin clotting enzyme. Midstuk is therefore regarded by Fuchs as a substance of globulin character and possessing enzymic properties. This is confirmed by the fact that the action of complement on r.b.c.(substrate) has certain characteristics of enzyme action, and the many labile properties of complement can only be explained on the supposition of an enzyme nature.

Besides these two fractions in association with the serum proteins, two other components have been shown to be necessary for complement action. They are thermostable and have been postulated because of their inactivation by specific methods. In the chain reaction which is supposed to occur when complement acts on sensitised red corpuscles the fourth component reacts first and produces its effect before midstuk can be bound. This component, which is inactivated by treatment with ether, chloroform, ammonium and cadmium compounds, appears to be of lipoid character or is

associated with the lipoid constituents of the serum (Toda and Misuse, 1933). The oft-quoted statement that it is associated with the endstuk fraction is confusing and inaccurate, since it merely indicates that the fourth component is not precipitated with the globulin fraction by the usual techniques. There is further no evidence to suggest that the fourth component is present in combination with the albumin fraction containing the endstuk (the observations of Guggenheimer, 1911, Toda and Misuse, 1933, and Tokano, 1936 that ether and chloroform treatment of serum specifically inactivates the fourth component is against any lipoprotein complex).

Sensitised cells, which have been acted on by the fourth component and midstuk, and which will undergo haemolysis by the aid of the third component and endstuk, are said to be "persensitised". It has been put forward that the third component is not a chemical entity, because it cannot be shown to be used up in the reaction nor can it be shown to be bound to the corpuscles. Some particular physico-chemical state is suggested, but why yeast cells and cobra venom should destroy this state is not apparent.

In our present state of knowledge we can only say that complement is associated with the lipoid and protein constituents of the serum and possesses enzyme properties. The fact that its activity depends on a sequence of reactions

means that the net result will be a function of the concentration of each component. The interrelation of each component to the subsequent complement activity has never been critically analysed, but it appears that complement activity is proportional to the sum of the concentrations of the individual components above the minimal threshold concentration of each component required to produce complete haemolysis of a standard sensitised suspension in infinite time. Hence an increase or decrease in any one component results in an increased or decreased complement activity respectively. Further, a decrease in the concentration of one component (midstuk or endstuk) can, within limits, be compensated by an increased concentration of the other. This is referred to as the reciprocal or interchangeable action of midstuk and endstuk. However, it has been found that the endstuk must be present in a relatively high proportion, while midstuk could be reduced considerably in the mixture and still produce haemolysis. This is in favour of the enzyme concept of midstuk, and the fact that midstuk (or complement) does not obey the typical laws of enzyme action is due to the production of products during the course of haemolysis which destroy complement. The observation of Terry (1935) that large amounts of globulin fraction lowered the complement titre considerably is due to the inhibitory effect of serum proteins on complement. It would appear that endstuk is the

limiting factor in complement reactions; this is amply comfirmed by the fact that in all cases of inactivation or decrease in complement activity, apart from those produced by precipitation of the serum globulins, the sensitised corpuscles are "persensitised", i.e., the midstuk has been bound, but the endstuk has been prevented from playing its part.

The mechanism of the absorption of complement has been the subject of much controversy ever since Ehrlich postulated a side-chain action and Bordet suggested an adsorption process. Because of its extremely accurate experimental procedure, the recent work of Ponder (1932, 1934, 1936) probably supplies the correct explanation of this phenomenon. This investigator found that the amount of amboceptor absorbed by the sheep erythrocytes was a constant fraction of that introduced into the system; that sensitation conferred on the red cell the property of absorbing complement; and that the greater the quantity of amboceptor absorbed the greater was the proportion of complement absorbed from any complement concentration in the system.

Once the complement has been absorbed, the subsequent events leading to the liberation of haemoglobin from the envelope of the red cell are obscure. The strong evidence in favour of complement possessing enzymatic properties leads one to the assumption that the

actual change produced by haemolytic complement is a digestion of the red cell envelope. Since Liefmann and Cohn (1910) showed that more serum is required for the haemolysis of a given volume of sensitised corpuscles if it is added in repeated small volumes than if it is all added at once, it is apparent that the complement can digest a limited quantity of sensitised corpuscles, due to the formation of products during the haemolytic process which inactivate complement (the met-haemolytic reaction of Basil and Suzuki). The destruction of complement only commences after the first trace of haemolysis has occurred, and this is correlated with the known fact that free haemoglobin has a markedly inhibitory effect on complement.

If the progress of haemolysis is traced, it is found that, if the concentration of sensitised corpuscles is high compared with the complement, the speed of the reaction is independent of the concentration of the residual, unhaemolysed corpuscles, so that a uniform quantity of haemoglobin is set free in each succeeding interval of time. Further, Osborn (1936c) has shown that in low concentrations of complement the speed of haemolysis is proportional to the amount of complement present. In these respects the action of complement is characteristic of many enzymes.

Fuchs (1930d) has interpreted his results from the following viewpoint. He regards complement as present in circulation in combination with a substance termed "antiprothrombin"; as the corpuscles are haemolysed, they liberate cytozyme which neutralises the antiprothrombin, so that the greater the degree of haemolysis the greater the amount of complement that can be absorbed by the sensitised corpuscles. The velocity of reaction would therefore be slow at first and gradually become faster, and such a state of affairs was found by Fuchs (1930,e; p.309). However, if Fuchs would have centrifuged his systems and estimated the liberated haemoglobin by colorimetric methods, he would have obtained an S-shaped curve concave to the t(time) axis (fig.3) and not a convex curve. In other words, the actual degree of haemolysis is far greater than that estimated by visual inspection, and on this basis alone Fuchs' curve is rendered useless for interpretation.

The mechanism of digestion is obscure, and though lipolytic or proteolytic activity is advanced, there are as many negative results as positive. A possibility that has not been explored is that complement, by virtue of its concentration (adsorption) at the cell interface, produces some alteration in the properties or permeability of the red cell membrane, causing a"leakage" of osmotically

active substances with resultant haemolysis. The red cell stromata after complement haemolysis have properties differing from those normally met with, and apparently the effect of complement has been to increase their permeability.

SECTION VII.

SUMMARY.

1. The present-day concept of haemolytic complement has been shortly reviewed.
2. The methods of estimating haemolytic complement have been described. It is concluded that the method estimating the titre of complement is far inferior to the methods estimating the time for complete haemolysis and the degree of haemolysis in constant time. The present methods of estimation are inadequate, inasmuch as they do not give any information as to the concentration of the component elements of complement taking part in the "chain reaction", while the products of haemolysis are destructive to complement. The latter effect could be overcome by taking the time for the first trace of haemolysis.
3. The time-dilution and percentage haemolysis graphs for amboceptor-complement haemolytic systems are found to be similar to those for simple lytic systems. The fundamental processes underlying the absorption of amboceptor and complement by an antigen, viz., sheep erythrocytes, are described.
4. It is found that complement resembles an enzyme in the nature of its action.

5. The fact that the serum complement of guinea-pigs is greater than that of human beings is confirmed by statistical analysis, and that many influences produce variations in the normal concentration of complement, prominent among which is a fall in complementary activity on the ingestion of food.

6. It is found that the treatment of serum with pure oxygen facilitates deterioration of the complement, while the removal of atmospheric oxygen delays the process of inactivation. The treatment of serum with carbon dioxide produces a reversible inactivation of the complement, and this bears no relation to the concomitant alterations in the pH of the serum. The degree of inactivation is about 40-60% of the original complementary power for undiluted sera, and is increased as the serum dilution is increased. The mechanism of inactivation of complement produced by carbon dioxide treatment of serum appears to be due to a precipitation of the globulins of the serum, resulting im a decreased concentration of active midstuk in the system. The amboceptors, since they are of globulin nature, will also be precipitated out of solution to a greater or lesser degree, so that the absorption of amboceptor by red cell suspensions is diminished in the presence of carbon dioxide. As a consequence the

absorption of complement by the sensitised cell is decreased with a resultant lowered complement activity.

7. It is found that a 0.9% sodium chloride solution is isotonic with mammalian blood. The increasing speed of haemolysis exhibited in hypotonic amboceptor-complement systems is ascribed to an increased susceptibility to haemolysis of red cells in solutions of decreasing osmotic pressure; this finds confirmation in the parallel action of various chlorides on the fragility point of blood corpuscles and on complementary activity. In hypertonic solutions there is reversible inactivation of complement.

8. The effect of isotonic neutral salt solutions on complement is complex, yielding a modified Hofmeister series. The differences produced by the salts appear to be caused by variations produced in the absorption of complement.

9. It is concluded that the concentration of endstuk has an important influence on the resulting complement activity, and that the following factors inhibiting complement allow the midstuk of complement but not the endstuk to be bound:-

low temperatures.
hypertonic solutions.
absence of inorganic salts.
presence of sugars.
acid reaction of system.
carbon dioxide excess.

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