A Comparative Study of Complement in the Vertebrates.

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Summary of the Thesis

The present thesis consists in the main of a comparative study of complement, one of the phenomena in the field of immunology. As an introduction, a review of the past work on the general serology of the cold-blooded vertebrates is presented, supplemented briefly by observations in this laboratory. Following this the main body of the thesis is presented in several sections, the first of which covers the nature and activity of complement in the five classes of vertebrates as far as it has been studied. Original work is presented to show that the guinea pig, frog and carp have complements that are quite similar to each other in several properties, including the ways in which they may be specifically inactivated.

The extent of this similarity is further enlarged in the second section where it is shown for the first time that it is possible to recombine the components of complement among the three species and to obtain active mixtures in several cases.

Two short sections follow dealing with the relation of the above studies to the complement-deficient guinea pig and to the role of complement in combating disease, and the thesis is concluded with an appendix covering such matters as technique, original data and references.

In addition, a reprint is submitted on the relation of temperature to antibody formation in fish.

A Review of the Serology of Cold-Blooded Vertebrates

While the main body of this thesis has to do with a comparative study of complement, it is desirable to review first the available information on the serology of cold-blooded vertebrates, for this is of scattered and not very extensive occurrence. Metchnikoff and others of his time worked on these lower classes to some extent and as early as 1897 one can find reference to the production of agglutinins in the frog (Widal and Sicard). However, at that time, interest was centered upon the hematoxic effects of sera, particularly that of the eel and frog, where the presence of powerful hemolysins had been demonstrated; some of the early work on anti-toxins even including the production of antibodies against eel serum (Korsel 1898, Tschistovitch 1899). A review of most of this not too prolific literature is to be found in a paper by Nardi (1938). As these hemolysins have been shown to be due to the action of complement and antibody, they will be considered in more detail in the parts of this thesis on complement.

There are several papers showing that antibodies can be induced artificially in various cold-blooded vertebrates, these antibodies being similar to those induced in mammals as regards their specificity, relative heat, stability and so on. The first of these and only one known to me of induced antibodies in the reptiles is that of Gee and Smith (1941). They were able to obtain high titer agglutinins against Bacterium salmonicida in turtles, the sera of which previously was not able to react with these organisms. Additional and relatively

more work has been done on the amphibians, frogs being used to produce several kinds of hemagglutinins (Lazar 1904, Schwarzman 1927, Allen and McDaniel 1937, Wollman 1938) as well as at least two kinds of bacterial agglutinins, i.e. for Eberthella typhosa (Widal and Sicard 1897) and for Proteus hydrophilus (Kulp 1942). The most work has been done upon a variety of species of fish where several workers have been able to induce the formation of antibodies against such bacterial antigens as B. salmonicida (Smith 1940, Duff 1942), Vibrio (Bergman 1911, Aaser 1923, Nybelin 1935), Proteus piscidus versicolor (Babes and Riegler 1903), and Pseudomonas punctata (Plisyka 1939). This last author obtained antisera of high titer that was able to discriminate between various strains of the antigen. In addition to the bacterial antibodies, it has been possible to obtain agglutinins in carp against sea urchin sperm (Cushing 1942) and against sheep, carp and gold fish erythrocytes (Cushing unpublished), the antisheep sera being hemolytic in the presence of carp complement.

Natural antibodies also occur in the lower classes of vertebrates and to an extent that forces one to be careful to obtain control samples of serum from animals that are to be artificially immunized. Such antibodies are to be seen in the natural agglutinins for sea urchin sperm (Cushing 1942) in carp serum, and in the hemagglutinins that occur in almost any animal (including lobster, Tyler unpublished) one cares to investigate. Examples of these are discussed in the work of Toth (1932) on fish, in that of Bond (1939, 1940a, 1940b) on reptiles, and have been found in frogs and carp in this laboratory. These natural antibodies, as far as studied, are quite

comparable to the induced ones in that they are relatively heat stable, can act as hemolysins under the proper conditions, and are able to retain their properties for several months if kept in the cold. Their specificity has not been extensively studied, but the work of Bond indicates that it may be quite high. This author was able to show by absorptions that snakes (1939), turtles (1940a) and alligators (1940b) have agglutinins for type A, and for type B human erythrocytes, as well as a human species agglutinin, and that these may occur independently of each other in sera within the first two forms. In addition to these, various other hemagglutinins may or may not be present.

Further evidence of rather pronounced specificity is also seen in the western painted turtle where at least three main blood types and two sub-types have been found by Bond. Blood groups within snakes and alligators, however, were not found. In connection and in contradiction to Toth (1932) it may be said that the carp used in this laboratory had various iso-agglutinins in their sera. These unfortunately could not be worked out in detail as our population was not sufficiently heterogeneous, but they were well marked enough to warrant additional study.

In concluding this review of serological phenomena in the cold-blooded vertebrates, some miscellaneous papers might be mentioned, the first by Goodner (1926) showing that, while intact frog and frog alimentary smooth muscle did not give anaphylatic reactions, excised heart tissue showed an abrupt decrease in rate and strength of contraction upon contact with specific sensitizing antigen. Similar results were obtained

with turtle heart by Downs (1928), who was able to demonstrate both active and passive anaphylaxis, finding that chicken but not rabbit antiserum was effective in the latter case.

Another paper to be mentioned is that of Phisalix (1926) which states that eels are naturally immune to the rabies virus and that their serum has anti-rabid factors. This recalls the early demonstrations (Zinsser and Bayne-Jones/939) that cold-blooded animals are often naturally immune to the diseases of warm-blooded ones, a field that has been but little investigated. In connection with immunity, the work of Nigrelli (1937) showed that certain marine fishes were able to acquire immunity to a nematode parasitic upon them.

The last paper to be discussed is that of Ishihara and Misao (1928) which is reported by Cumley and Irwin (1941) as showing that the sera of the carp, crucian and goldfish contain species specific substances antigenic in the rabbit, and that the sera of hybrids among these fish possessed some of these antigenic units from each of their parents. This is of interest in that it parallels the studies of Irwin and Cumley (1942) on the inheritance of serum antigens in the Columbidae, and suggests that serum differences as well as cellular ones may be useful material for the study of problems in fish populations.

In summarizing, it may be said that, as far as studied, the serological properties of cold-blooded animals closely resemble those of the warm-blooded ones, and it seems very probable that the same immunological principles apply for both. This probability is further increased by the similarity of the complements in the two groups, as will be seen in the sections to follow.

A Comparative Study of Complement

I. Introduction

Complement, while difficult to define precisely, is the general term applied to a specific property of fresh, normal serum; namely, its ability to bring about the destruction of such proteinaceous materials as erythrocytes and bacteria through lysis, phagocytosis or other means, when these materials have been combined with their specific antibody. This destructive activity of serum is apparently a function of certain specific serum proteins that are not increased by immunization, and has long been studied and utilized as one of the chief phenomena of immunology.

Quite early in these studies the guinea pig was found to be the source of a complement that was very active with many of the better known antigen-antibody complexes and since then this animal has furnished almost all of the material that has been investigated. While the complements of several other species have been studied, such as those of the common domestic animals and of man, the general tendency has been to ignore such problems as might be concerned with the source of complement or its extent as a general phenomenon and to treat its activity as essentially non-specific and not subject to the usual biological influences of variability, specificity and so on. Although such a point of view has been far from unfruitful, it has at times led to some misinterpretations of observations and, in any event, can be supplemented by as full a knowledge as possible of the general biological properties of complement without suffering any practical set-backs.

The purpose of the thesis presented here has been to make a comparative survey of complement in the vertebrates, emphasizing particularly that of the carp, frog and guinea pig, in order to learn something of the extent and degree of similarity of complement in this phylum. As such a comparison must, in last analysis, attritute its findings to the action of evolution, the evidence that the variations noted are due to heritable factors will be considered at this time. Evidence of a general sort is to be found in the field of systematic serology where the proteins of many species have been compared immunologically and found to differ from each other roughly in inverse proportion to the degree of relationship of the forms compared. This subject has most recently been reviewed by Boyden (1942) and the reader is referred to his paper for details. Of particular interest are the several cases where the whole sera of various animals have been compared for, as will be described in more detail, the action of complement depends upon certain specific serum proteins. These comparisons agree with other work in supporting the observation that in general the proteins of closely related forms are antigenically more alike than are those of distantly related ones, and that these antigenic properties are consistently characteristic of any particular species.

The best evidence that the specific nature of serum proteins is genetically determined has been given by Cumley, Irwin and Cole (1942) through their work on hybrids between various dove and pigeon species. They have shown quite conclusively that the species-specificity of the serum proteins

that they were working with "falls within the pattern of Mendelian inheritance" and that therefore it may be assumed to be gene controlled. In this connection it is interesting to note that the genes involved are not correlated with those responsible for the species-specificity of the erythrocyte antigens, the hereditary nature of which has also been firmly established by these authors.

A recent paper by Cumley and Irwin (1943) states that it has been possible to show individual antigenic differences within the serum of a single species by injecting human serum into rabbits. These differences are not obviously correlated with blood types, themselves an additional demonstration of the fact that individual as well as specific variations exist in serum proteins just as in all other biological properties of organisms.

A last case should be cited as the most convincing evidence that the complementing activity of serum proteins is under genic control and therefore as subject to the influences of mutation and selection as any other heritable character. This is the occurrence of the complement-deficient guinea pig, established as a strain by Downing and first reported by Moore in 1919 as being caused by a simple, recessive mutation that reduced the complementing power of the homozygotes to 1/100 of normal, besides affecting them in other ways. Although the stock was once represented by hundreds of individuals, it has since been lost and further work with it has become impossible. However, several things were learned from it and these will be reviewed in a later section.

In summarizing, it may be said that the evidence presented above would seem to establish with great probability the fact that the differences in the complementing activity of the different sera that are to be discussed are genetically determined and therefore products of evolutionary activity, a fact that will eventually be of value in drawing the final conclusions as to the origin and function of complement.

II. Accounts of the Complement of Various Species Representing the Five Classes of Vertebrates.

A. Mammalia

Introduction

The guinea pig will be presented first and in some detail as it is the form from which most of our knowledge of complement has been derived. In order to facilitate comparison, the various points of interest will be discussed under subheadings that will be repeated as far as possible for the different species to follow. As the chief objective of this section is a comparative study, only those points that bear upon this objective will be taken up here, and other points, such as the relation of complement to prothrombin, the history of its discovery, its origin in the body and so on will be omitted. The more pertinent of these will be discussed in later sections and should further information than this be desired, the reader is referred to the work of Osborn (1937), and of Ecker and Pillemer (1942).

Unless otherwise mentioned, the various data presented in this section refer to the hemolytic effects of complement. In the case of the guinea pig, the procedure followed by most investigators has been to use a saline suspension of sheep erythrocytes sensitized with from one to four hemolytic doses of rabbit antibody as test antigen for hemolytic activity. The final dilution of the guinea pig serum used in fractionation experiments has usually been one part of serum to ten of physiological saline, although in some cases I have used

dilutions as low as one to two. Experimental details will be presented with the text in whatever summarized form that seems necessary, and in the appendix in original form along with the descriptions of the various techniques used.

The Guinea Pig (Cavia porcellus)

General comments: The complement of this species, as said before, has been studied so extensively that it must be taken as an arbitrary standard with which other forms are to be compared. The complementing power of the fresh serum is known to be due to at least four distinct components that are identified by the specific ways in which they can be inactivated or separated. The existence of other components has been claimed, but these claims have, to date, been based upon insufficient evidence (Ecker and Pillemer 1942). The technique of demonstrating the components known at present has been to inactivate samples of the same serum in two or more specific ways, as for example by heating and by treating with yeast, and then to mix these inactive samples and show that the mixture has hemolytic activity. It is important to realize that one must consider the specificity of any one technique as relative in that, while it will remove one component more or less completely, the remaining components are apt to be affected to some degree. This partially accounts for the fact that recombined fractions usually have lost from one fourth to one half of their original activity. However, in properly conducted experiments, this non-specific inactivation is of little consequence for it is usually slight in comparison with the

specific effects. To date very little work has been done in clarifying the role of these non-specific effects, but eventually it may be possible to overcome them by refining the various techniques.

The terminology that has recently been proposed for the four components by Pillemer and Ecker (1941) and that is also used by Heidelberger will be employed below. The new terms are designed to replace previous ones long in use, but held by these authorities to be misleading as to the true nature of the substances involved. They do have the advantages of simplicity and uniformity, especially when using tabulated data.

Characteristic Properties of the Components

C'1 (formerly called the mid-piece, mid-stuk, or globulin fraction of complement and discovered by Ferrata, 1907): This fraction of complement is characterized by being associated with those serum globulins that are precipitated by the carbon-dioxide, hydrochloric acid, distilled water or other specific treatments of whole serum (see Ecker and Pillemer 1942, and appendix of this thesis for details). It is further characterized by being more heat labile than the third and fourth components, losing its activity when heated to fifty-four degrees for thirty minutes. It also looses its activity when kept in physiological saline for a few hours, although remaining active for several days if kept suspended in distilled water (Ecker and Pillemer, 1942).

The results of many workers agree in confirming the fact that this fraction must combine first with the antigen-antibody

complex, if complementary activity is to be demonstrated (Osborn 1937). C'2 (end-piece) will combine in its absence, but the subsequent addition of C'1 will not result in hemolysis. It is also most generally agreed that, within certain limits, optimum hemolysis or fixation is obtained when C'1 and C'2 are present in equal amounts, an excess of one or the other inhibiting the reaction to some extent.

Recently Pillemer, Ecker, Oncley and Cohn (1941) have obtained C'1, as well as C'2 and C'4, in a highly purified state and their findings are to be seen summarized in table 1. . This table is largely self-explanitory; however, it is interesting to note in passing that the definite characterization of these components should be a final blow to those who have contended that complementary activity is due, not to specific serum proteins, but to a specific physical state of normal serum.

C'2 (formerly called the end-piece, end-stuk or albumin fraction of complement and discovered by Ferrata 1907): This component differs from C'1 in that it is associated with those serum proteins that are not precipitated by the various techniques noted under C'1, and that it remains stable in physiological saline for several days. Like C'1 it is inactivated when heated at fifty-four degrees for thirty minutes.

As pointed out above, while C'2 is capable of being fixed by the antigen-antibody complex in the absence of C'1, hemolysis will occur only if C'1 has been fixed first.

This second component of complement has been shown (table 1.) to be intimately associated with C'4 and present

Table 1.

Characterization of the Mid-Piece and the End-Piece and the Fourth Component of Complement. (table from Pillemer, Ecker, Oncley, and Cohen 1941.)

Electrophoretic	Euglobulin (mid-piece)	Muco-euglobulin (end-piece and fourth component.)
Electrophoretic mobility. x 10^{5}, pH7.7; ionic strength 0.20.	2.9	4.2
% protein nitrogen.	16.3	14.2
% total serum protein.	0.72	0.17
% carbohydrate.	2.7	10.3
% phosphorus.	< 0.1	< 0.1
$[\alpha]_{25°C.}$	-28.7	-192.5
Apparent iso-electric point.	5.2-5.4	6.3-6.4
% Original complementary activity.	100.	85.
Heat stability.	Destroyed at 50 °C. for 30 minutes.	End-piece destroyed at 50° C. for 30 minutes; fouth component at 66 C. for 30 minutes.

evidence suggests that it acts as a carrier of this fourth component, which is relatively more heat stable and is held to be a carbohydrate complex of the end-piece.

Note on the heat lability of C'1 and C'2: Although it is generally agreed that a temperature of fifty-four to fifty-six degrees will inactivate C'1 and C'2 long before it will C'3 and C'4, several authors have claimed that in untreated serum C'1 is often more resistant to heat than C'2. This has been most extensively discussed by Parsons (1926), who concludes that these reports are erroneous or, at best, that different samples of serum vary greatly in this respect. Since then it has been generally assumed that heat inactivated whole serum has lost both C'1 and C'2. However, my own work on frog serum suggests that a re-investigation of this problem in guinea pig serum would be desirable, for it has shown that one component actually is more resistant than the other in whole serum and this resistance is lowered by complement "splitting" with carbon dioxide.

C'3 (formerly known as the third component of complement and discovered by von Dungern 1900): This component of complement is specifically inactivated by an insoluable carbohydrate present in yeast (Pillemer and Ecker, 1941), by cobra venom, and certain bacteria (Coca 1914). The usual method of inactivation now employed is to add the proper amount of zymin (see section on technique in the appendix) to the whole serum and then, after two and a half hours, to centrifuge this off, adjust the pH and dilute with saline to the proper concentration. There is some evidence that the yeast and cobra

venom do not act in exactly the same way for it is reported (Coca 1914) that occasionally fractions obtained by these two methods will reactivate each other when mixed. A detailed investigation of this point is yet to be made.

While the third component is often found to be largely precipitated along with C'1 during complement splitting, no precise predictions can be made on this point and, in any case where it is important, one must determine the relative amounts of C'3 associated with C'1 and C'2 experimentally.

The third component is relatively heat stable, losing only a little activity during the heat inactivation of the C'1 and C'2 fractions, or of whole serum, and in this respect it is similar to the fourth component. In spite of this relative heat stability, both Ecker and Osborn report that it is the first component to lose its activity in serum that is allowed to stand for a few days. However, as the other components also undergo various changes in such serum, this method of inactivation is not specific enough to be of any great value in the fractionation of complement.

There is general agreement that very little if any of this component is actually fixed or used up during a complementing reaction (Osborn 1937), suggesting in a general sense (Pillemer, Seifter, Chu and Ecker 1942) a catalytic role for this factor. However, more work will be necessary before such a characterization can be definitely ascribed to C'3. At present it has not been possible to obtain it in a pure enough state to study its physico-chemical properties.

It may be definitely stated, however, that the third

component is non-dialyzable against distilled water, that it is inactivated by heating at sixty-two degrees for thirty minutes, and that zymin inactivated serum showed an electrophoretic disturbance of its alpha-globulins, accompanied by a slight increase of the mobilities of the remaining serum proteins. Attempts are now in progress in Ecker's laboratory to obtain C'3 in pure state by absorption with purified yeast carbohydrate and subsequent elution.

Note on a "zone" phenomenon: It is of interest that the earlier discovery (Whitehead, Gordon and Wormall, 1925) that an excess of zymin during attempted inactivation leaves the serum as active as before has been confirmed in this laboratory not only for the guinea pig, but for the carp as well.

C'4 (formerly called fourth component and discovered by Gordon, ~~Robinson~~ Whitehead and Wormall in 1926): This most recently discovered component is peculiar in that it is specifically inactivated through the agency of dilute ammonia which, when added in the proper proportions to whole serum, brings about its effect in two hours or less at thirty-seven degrees. In relation to the affinity of C'3 for cobra venom, the demonstration that viper venom will specifically inactivate C'4 is of interest (see Takano 1936 for further references). Also of interest is the work of Tyler (1942) showing that the protein fertilizin, obtained from sea urchin eggs, will specifically inactivate C'4 and that serum containing C'4 will agglutinate these eggs. This work will be discussed in more detail in a later section.

Present evidence would seem to indicate that C'4

represents a carbohydrate complex carried by C'2, and that these two components are part of the same molecule. This latter conclusion can best be seen from the data in table 1. , while the former is reached by Pillemer, Seifter and Ecker (1941) on the basis of studies of the effect of amino compounds on the fourth component.

Like C'3, this last component is heat stable relative to C'1 and C'2, a temperature of sixty-five degrees for thirty minutes being required to inactivate it completely.

The Human

General comments: The presence of complement in man is well established, although surprisingly little work has been done upon this subject. One of the most well-known observations on this point is that fresh serum and cells of the appropriate blood group types often, but not always, show the phenomenon of isohemolysis, and the need for inactivating such fresh serum has been pointed out in the standard texts on blood grouping. Isohemolysis was first observed by Maraghano (1892), but more detailed information has been supplied by Williams (1920). He showed that isohemolysis never occurs in the absence of the proper isoagglutinins and that there seems to be no good correlation between the occurrence of hemolysis and the amount of agglutinin present, although, even in cases of no hemolysis, one can usually obtain it by increasing the amount of the specific serum employed. Further data on isohemolysis is to be found in the papers of Thomsen and Thisted (1928a and b), who confirmed the presence of human complement and its action upon human red cells sensitized with the appropriate isoagglutinin.

The phenomenon of isohemolysis was observed in Dr. Emerson's laboratory where several individual suspensions of type A cells were treated with several type O sera. The results varied from none to complete hemolysis and did not seem to be correlated with the agglutinating titer of the O sera, although this last point is not certain as only six combinations were made.

In spite of the great amount of work on the blood groups,

I was unable to find any more information on isohemolysis than that given above, other than the statement that it is often found that species will not lyse their own cells (Osborn 1937, Zinsser, Enders and Fothergill 1939), to which humans seem to be one of the exceptions.

The components: While it is probable that some of the earliest papers on complement splitting include experiments on human complement, it can be safely said that no one has completed a successful analysis of the components of human complement since the discovery of the fourth component in the guinea pig in 1926, the work of Hegedus and Greiner (1938) notwithstanding. Such work as was done before then is difficult to find and in general lacks details of value to this study. The only direct reference to an attempted demonstration of C'1 and C'2 in human complement that I can supply is to the work of Mackie (1920), who found that, after the fractionation of human serum with ammonium sulphate, the globulins contained all the activity. However, it has since been shown by Ecker and Pillemer (1942) that this method is not a reliable one for separating components in guinea pig serum.

That human serum can be inactivated by heating in the same way that guinea pig can has been shown by Thomsen and Thisted (1928a and b), Kolmer (1919) and many others. That it still retains heat stable parts is shown by Hyde's (1932) demonstration that heated, but not zymin treated, human serum will reactivate the serum of complement deficient guinea pigs, and by the work of Jonas (1913), both of whose findings unfortunately do not account for the possibility of fourth

component being involved.

It is worth pointing out that in 1928 Hyde obtained an active complement from a hemophiliac which supports the view of the relative independence of the clotting and complementing powers of the blood.

Human complement is generally believed to exist in rather low titer, but as most of the evidence for this has been obtained indirectly from observations on isohemolysis, more should be done with other systems of antigen and amboceptor before definite conclusions can be drawn. The recent work of Heidelberger and Mayer (1942) would indicate that in the proper system human complement may have a titer as high as that of guinea pigs. Of course, all comparisons of this sort are quite artificial, depending as they do upon the type of complementing activity being studied, as well as upon several other factors which will be discussed in detail in another section. In summarizing, one is forced to conclude from available material that, while heat labile and heat stable components comparable in degree of stability to those in the guinea pig are to be found in human complement, these have not been correlated to any certainty with their possible counterparts in the guinea pig, and that surprisingly little is known of this important property of human serum.

Other Mammals

General comments: Zinsser, Enders and Fothergill (1939) state that "there is, after all, much similarity between the alexins of different animals" and cite the work of Ritz and Sachs (1912) and of Marks (1910-11) on the cross-activation

(globulin + alexin fraction)

of $C'1_{\wedge}$ and $C'2_{\wedge}$ in various species of mammals. While this is probably true, the fact remains that no comparison apparently has been made that involves the identification of the four components possibly present in other mammals. The work that has been done has consisted almost entirely of the activation of the antibody, natural or induced, of one species with the complement of another and this in itself has shown that all the mammals studied had complementing powers in their sera which would work with some amboceptors and not with others. However, this work involving cross-activations will be considered under a separate section and it must be concluded here that no direct comparative data are available for mammals other than the guinea pig.

B. Aves

General Comments: Several kinds of birds have been shown to possess natural hemolysins for the red cells of other species, a case in point being the chicken which can, among others, lyse the red cells of the sheep and the rabbit. (See Hyde and Bailey, 1922.) This ability has been shown to be due to the action of agglutinative antibodies and a complement destroyed by heating (Hyde 1921). Aside from the inter-specific reactivations to be taken up later on, work on bird complement does not seem to have been extensive, although one should cite the work of Ehrlich and Morgenroth (1901) as showing the presence of complement in the goose and pigeon. It is interesting to note that these authors were able to produce an artificial anti-ox red cell serum in the goose, as Muir (1911-14) was also able to do in the duck.

The components: Hyde (1921) has shown that chicken serum can be inactivated by heating to or above fifty-three degrees for thirty minutes, but beyond this no one seems to have worked on the components as such, the little work that has been done being concerned with cross-activations between different species.

C. Reptilia

General comments: Much work has been done on the problem of snake poisons and in this connection the complement of the victim seems to play an important role. However, the situation is complicated by an effect of lecithin and a multiplicity of toxic factors in any one venom and for the present had best be considered as a problem beyond the scope of this paper, especially as Flexner and Noguchi (1902-3) have shown that venom itself lacks complement.

The presence of natural hemolysins in the sera of different reptiles was demonstrated as early as 1913 by Mazzetti, these being directed against mammalian and other types of erythrocytes. These hemolysins were thermolabile and were shown in one case at least to be due to amboceptor (antibody), and a heat labile complement. Of interest is the observation that hemolysis was brought about by these sera as readily at fifteen degrees as at thirty-seven.

More recently, Bond and Sherwood (1939) have reported that the normal serum of various species of snakes is frequently hemolytic for the red cells of man, the sheep, the rabbit and the guinea pig. In their experiments they used sheep cells sensitized with rabbit antibody, as well as unsensitized cells, and the sensitized cells were found to be hemolized to a higher degree than the unsensitized ones by everyone of thirty-eight different sera taken from eight genera of snakes. This not only showed the presence of an active complement, but also that it was capable of working with rabbit amboceptor (antibody). Further proof of the existence of complement

was given by adding non-hemolytic snake serum to hemolytic serum that had been inactivated by heating and thus obtaining a hemolytic mixture. This was also attempted using guinea pig complement, but without success. Other experiments showed that not only was the titer of snake complement acting against rabbit sensitized sheep cells comparable to that of guinea pig, but that snake complement was fixed about as well as guinea pig in the combination of rabbit anti-Salmonella serum with its antigen, and also by the Kolmer antigen syphilitic reagin complex.

Further parallels between snake and guinea pig complement are that both deteriorate at comparable rates upon standing, and that snake serum is inactivated in five minutes if heated to fifty-six degrees.

One should note that turtle and lizard complements apparently can not use rabbit antibodies (Amako 1912, Mazzetti 1913) although the frog can as will be seen later.

The components: Amako (1912) was able to split turtle complement into mid and end pieces by globulin precipitation and this is the only evidence known to me of C'1 and C'2 components possibly being present in reptilian serum.

The heat labile constituents of snake serum (species?) were shown by Mazzetti to be completely destroyed by heating to fifty degrees for thirty minutes, but to resist a forty-five degree temperature for the same length of time.

Bond and Sherwood found that the snake sera they used was inactivated in five minutes at fifty-six degrees. The possible presence of C'3 in snake serum is suggested by their

discovery that such serum loses its activity at about the same rate that guinea pig serum does when standing, this loss being generally attributed in the latter serum to the relatively rapid deterioration of this component.

D. Amphibia

General comments: As in other classes, the serum of members of this class was long known to contain hemolysins for many kinds of erythrocytes. One of the first forms to be investigated in this respect was the frog (Lazar 1904, Liefmann 1911, Frankel 1911) and for a while some argument existed as to whether the action of this serum was due to antibody and complement, or to some non-immunological factor. The work of Mazzetti (1913) decided the point in favor of the former idea. Among other findings of Mazzetti was that frog complement worked with rabbit antibodies against calf cells and as rapidly at fifteen as at thirty-seven degrees. This has been confirmed by Reiner and Strilich (1929), who give, in addition, extensive data showing that Wasserman tests on the same sera were closely parallel whether run at eighteen degrees with frog complement, or in the usual way with guinea pig complement.

In the light of these results, the early observation (Friedberger and Seelig 1908) that an antitoxin against the frog hemolysin could be made in rabbits is probably best explained as due to the fixation of frog complement rather than to a true toxin-antitoxin reaction.

As in other groups of animals there seems to be no obvious rules regarding complementing activity for Amako (1912) was not able to activate rabbit amboceptor (antibody) with toad complement.

The existence of natural hemolysins for fish, rabbit, sheep and even man (in contrast to the report of Allen and Mc Daniel 1937) in frog serum, as well as the action of frog

complement with rabbit antibodies and the temperature effect, has been confirmed in the present studies.

The Components in Species Other Than the Frog

The heat inactivation of toad serum has been studied by Mazzetti (1913) and found to take place at forty degrees in thirty minutes, but not at thirty-five degrees for the same length of time. Amako in 1912 reports the splitting of toad serum into mid (-C'2) and end (-C'1) pieces and that heated toad serum could be reactivated by turtle complement (and vice versa) showing that complement and amboceptor both occur in this species. Lazar (1904) found that frog serum was inactivated by heating to from forty-two degrees to forty-five degrees and that above this temperature the natural frog antibody was destroyed.

The Frog (Rana catesbiana)

General comments: The ability of this species to activate rabbit antibody, in addition to its large size, makes it a convenient amphibian for study and it was therefore selected for a relatively intensive comparison with the guinea pig. Unless otherwise credited, the work on this form was done for the present thesis and is here reported for the first time. All tests of complementary activity were carried out on sheep red cells sensitized with about two units of pooled rabbit amboceptor, the same pool of serum being used throughout. Frogs were bled from the heart and their serum separated and used either pooled or individually and within an hour of bleeding (see appendix for details of technique).

A comparison of the titers of frog and guinea pig complement, as well as of carp, has been made at three different temperatures (table 2.) and from it one can see the general nature of the differences that occur among the species in this respect. In the work on frog components, the temperature at which any experiment was run has been given on the data sheets. In order to avoid unnecessary repetition, a minimum of tables will be presented with the text, these being either summaries or selected examples of confirmed experiments. However, the reader is referred to the appendix where much of the original data is presented if he desires further detail.

The components: The terminology applied will correspond to that used for the guinea pig and is based upon similarities in the specific techniques of inactivation.

C'1 (also demonstrated by Liefmann, 1911, and by Frankel 1911): The treatment of normal frog serum with carbon dioxide split it into two fractions, as regards complementary activity, one of these being in the precipitate, the other in the supernatant. These fractions were inactive in themselves at dilutions of one to ten, but were hemolytic when recombined (table 3.). Like guinea pig C'1, the precipitated fraction was heat labile, being inactivated by heating at forty-seven degrees for twenty-five minutes, and lost its hemolytic properties within a few hours when suspended in saline. The precipitate in which it occurred was finer and more soluble in saline than that of the guinea pig, although apparently equivalent to it in amount.

Table 2.

A Comparison of the Activity of Carp, Frog and Guinea Pig Complements at Different Temperatures.

Carp.

Hemolytic System: One tenth cc. of a two-percent suspension of rabbit cells sensitized with the natural antibodies present in two tenths cc. of serial dilutions of the serum of one fish.

Temperature:	37°								16°								1°-2°							
Reciprocal of serum dilutions.	1	2	4	8	16	32	64	128	1	2	4	8	16	32	64	128	1	2	4	8	16	32	64	128
Time in minutes.																								
10	++++	++++	++++	++	o	o	o	o	++++	++++	++++	++	o	o	o	o	++	++	o	o	o	o	o	o
20			++++	++	o	o	o	o				++++	+	o	o	o	++++	++++	++++	o	o	o	o	o
30			++++	++	o	o	o	o					++	o	o	o				o	o	o	o	o
45				++	o	o	o	o					++	o	o	o				++	±	o	o	o
60				++	o	o	o	o					++	o	o	o				++++	++	o	o	o
90				++	+	o	o	o					++	o	o	o					++	o	o	o
240				++++	++	±	o	o					++	+	o	o					++++	+	o	o

Frog

Hemolytic System: One tenth cc. of a two-percent suspension of sheep cells sensitized with two units of rabbit antibody and complemented with pooled frog serum.

Temperature:	37°								16°								1°-2°							
Reciprocal of serum dilutions.	1	2	4	8	16	32	64	128	1	2	4	8	16	32	64	128	1	2	4	8	16	32	64	128
Time in minutes.																								
10	++++	++++	++++	o	o	o	o	o	++++	++++	o	o	o	o	o	o	o	o	o	o	o	o	o	o
20			++++	++	o	o	o	o			++++	o	o	o	o	o	o	o	o	o	o	o	o	o
30			++++	++	o	o	o	o			++++	±	o	o	o	o	±	o	o	o	o	o	o	o
45				++	o	o	o	o				++	o	o	o	o	++	o	o	o	o	o	o	o
60				++	o	o	o	o				++	o	o	o	o	++++	+	o	o	o	o	o	o
90				++	o	o	o	o				++	++	++	o	o		++++	o	o	o	o	o	o
240				++	++	±	o	o				++++	++	++	++	o		++++	++	+	o	o	o	o

Table 2. (Continued.)

Guinea Pig.

Hemolytic System: The same as that used for the frog, except that the serum of only one individual was used.

Temperature:	37°								16°								1°-2°						
Reciprocal of serum dilution.	1	2	4	8	16	32	64	128	1	2	4	8	16	32	64	128	1	2	4	8	16	32	64
Time in minutes.																							
10	++++	++++	++++	++++	++++	++++	++++	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
20								++	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0
30								++	++++	++++	++++	±	0	0	0	0	0	0	0	0	0	0	0
45								++	++++	++++	++++	++	0	0	0	0	0	0	0	0	0	0	0
60								++	++++	++++	++++	++++	++	0	0	0	0	0	0	0	0	0	0
90								++				++++	++	++	0	0	0	0	0	0	0	0	0
240								++					++++	++++	++	±	0	0	0	0	0	0	0

Table 3.

A Comparison of the Components in the Serum of One Frog.

Test Antigen: one tenth cc. of a two-percent suspension of sheep cells sensitized with rabbit antibody.

Abreviations and their corresponding serum fractions:

M and E :: "globulin" and "albumin" fractions separated by the carbon dioxide method. (M contains C'1 ; E contains C'2, C'3 and C'4.) Diluted one to ten with saline.

M^h and E^h :; the above fractions heated at 47 for 25 minutes.

Z^1 and Z^2 :; two samples of whole serum treated with zymin for 60 and 30 minutes at room temperatureand diluted one to five.

H^1 and H^2 :: two samples of whole serum heated for 25 minutes at 47 and at 49-50 degrees and diluted one to five.

A :: serum inactivated with ammonia and diluted one to five.

Final volumes of all tubes made up to three tenths cc. with saline; all tests run at room temperature.

Amounts and kinds of fractions per tube.

Time in minutes.	.2cc M	.2cc E	.2cc Z^1	.2cc Z^2	.2cc H^1	.2cc H^2	.2cc A	.2cc M^h	.2cc E^h	.1+.1 M+E	.1+.1 M+A	.1+.1 M+Z^1
10	0	0	0	0	0	0	0	0	0	++++	0	0
20	0	0	0	0	0	0	0	0	0	++++	0	0
30	0	0	0	0	0	0	0	0	0	++++	0	0
45	0	0	0	0	0	0	0	0	0	++++	0	0
60	0	0	0	0	0	0	0	0	0	++++	0	0

	.1+.1 M+Z^2	.1+.1 M+H^1	.1+.1 M+H^2	.1+.1 E+A	.1+.1 E+Z^1	.1+.1 E+Z^2	.1+.1 E+H^1	.1+.1 H^1+A	.1+.1 H^2+A	.1+.1 H^1+Z^1	.1+.1 H^1+Z^2	.1+.1 A+Z^1
10	0	+	0	++++	0	++	0	++	++++	0	++	0
20	0	+++	0	++++	++	+++	0	+++	+++	++	++	+
30	0	+++	0	++++	+++	+++	0	+++	++++	++	+++	+++
45	0	++++	0	++++	++++	++++	0	++++	++++	+++	++++	++++
60	0	++++	0	++++	++++	++++	0	++++	++++	++++	++++	++++

	.1+.1 A+Z^2	.1+.1 M+E^h	.1+.1 M^h+E	.1+.1 M^h+Z^1	.1+.1 E^h+Z^1	.1+.1 M^h+A	.1+.1 E^h+A	.1+.1 E^h+H^1	.1+.1 M^h+H^1	.1+.1 M^h+Z^2	.1+.1 E^h+Z^2
10	0	0	0	0	0	0	0	0	0	0	±
20	±	++	0	0	0	0	++	0	0	0	++
30	++	+++	0	0	+	0	+++	0	0	0	++++
45	+++	+++	0	0	+++	0	+++	0	0	0	++++
60	+++	++++	0	0	+++	0	+++	0	0	0	++++

It was found possible to split frog complement using half the amount of distilled water ordinarily employed, this resulting in a one to five dilution rather than a one to ten. However, while the fractions seemed to behave as well at this dilution as at the higher one, it was subsequently shown that one could be less certain of obtaining a non-hemolytic supernatant at this greater concentration and therefore most of the experiments were carried out with mid and end piece diluted one to ten, as in the guinea pig.

C'2 (also demonstrated by Liefmann, 1911, and Frankel, 1911): The supernatant remaining from the carbon dioxide treatment, while relatively heat labile (forty-nine to fifty degrees) as in the guinea pig, was found to resist heating to a greater degree than C'1, particularly when heated in otherwise untreated serum (table 3.). This resistance was found to be somewhat variable so that, while the relative stability of C'2 can be assumed to be correct, the precise temperatures at which the inactivations occur vary among different sera. However, forty-seven degrees for twenty-five minutes seem to have been the closest to the critical point of differentiation between C'1 and C'2 in the tests made.

C'3: As in the guinea pig, serum heated sufficiently to inactivate C'1 and C'2 was found to contain still at least two constituents necessary to hemolytic activity. The first of these proved to resemble C'3 in that it was specifically inactivated by zymin. The specificity of this inactivation was found to be highest when zymin was used in the same proportions as in the guinea pig, but only allowed to act for one

hour and at room temperature. In at least one case, inactivation resulted after treating for half an hour. Similarly to the guinea pig, it was found difficult to reactivate the zymin fraction, sometimes with heated serum, sometimes with ammonia treated serum, sometimes with both. While this suggests some variation in the heat stability of C'3, and some non-specific effects of zymin and ammonia, no careful analysis was made along these lines for the specificity of the treatment was adequate for the purposes of comparison as can be seen in the tables in the appendix.

A further resemblance to C'3 of the guinea pig is suggested by one experiment indicating that frog C'3 is the first of the components to become inactive upon standing. This point, however, should be further investigated before it can be considered as fully established.

Where the C'3 activity of C'1 and C'2 serum fractions was tested, as in table 3., only the latter was found able to reactivate zymin inactivated serum. This is in contrast to the results usually observed for the guinea pig; however, there is not enough data to be sure that this is an invariable rule and, even if so, its significance from a comparative point of view remains to be determined.

C'4: Another relatively heat stable constituent of frog serum can be specifically inactivated by ammonia when the serum is treated in the same way as that of guinea pig and is therefore referred to the fourth component. The main difference between the two species in this respect is that, while one can inactivate frog serum by treating for ninety minutes,

one must treat guinea pig serum for thirty to firty-five minutes longer. The mixture of heated and ammonia inactivated serum is alike in both species in that it is usually the most active of the various recombinations. There is one experiment to indicate that C'4 is relatively stable in aging serum.

Like C'3, C'4 has always been found associated with C'2 when frog serum has been fractionated by the carbon dioxide method (table 3.).

Summary: As can be seen from the above, the complementary activity of frog serum can be shown to depend upon four components with properties sufficiently like those in the guinea pig to enable one to consider them under the same specific names. These components have been shown to reactivate each other in original dilutions as high as one in ten for C'1 and C'2, and as high as one in five for C'3, C'4 and heated inactivated serum. Their further similarity to guinea pig complement is shown by their combined ability to react with rabbit antibody in the same way.

E. Pisces

General Comments: Fishes are no exception in having sera very hemolytic for many kinds of erythrocytes. In fact, some of the earliest work on toxins was upon the hemotoxin of eel blood and protective antibodies against it (Korsel, 1898). Other workers subsequently have attempted to show that this hemolytic property of fish serum depends upon the presence of natural antibody and complement, the most recent paper being that of Nardi (1938). Nardi reviews the not too extensive earlier literature and says that two lines of thought are to be distinguished, one that the active principles in fish sera is due to simple hemotoxins and the other that it is due to complement and amboceptor. While he himself favors the latter view and attempts to prove it by cross-reactivations of heated eel (Anguilla vulgaris) and tench (Tinca vulgaris) sera, his results, while positive, are not adequately controlled and therefore are open to some question. However, it would seem probable that he did reactivate heated eel serum with non-hemolytic tench serum and thus demonstrate the presence of an amboceptor complement complex in fish.

These results are supported by the work in this laboratory, where it has been possible to induce artificial antibodies in carp against sheep erythrocytes and, using cells sensitized with these, to show the presence of hemolytic complement in this species. In addition, it has been possible to show hemolysis of the cells of different species that were agglutinated by the natural antibodies present in heated carp

sera by adding non-hemolytic dilutions of unheated carp sera. So far it has not been possible to activate rabbit antibody with carp complement, a result confirming that of Nardi.

Of interest, in contrast to human isohemolysis, is the fact that, while both natural and induced carp antibodies against carp and goldfish erythrocytes have been obtained, no hemolysis results even though carp complement is present. This conforms to the not inviolate, but often quoted rule, that one individual of a species will not lyse the red cells of another individual even though adequate amounts of complement and antibody are present (Osborn 1937). The reason for this is obscure although the work of Liefmann and Stutzer (1910) may be cited as having some bearing on the point. They report that normal fresh sheep serum has a heat labile constituent that protects sensitized sheep cells from guinea pig complement.

The Components of Fish Other Than Carp

The heat lability of fish serum has been known for some time, the critical temperature, for instance, of eel serum being given by Nardi as between forty and forty-two degrees. However, I know of only one previous attempt to fractionate eel complement, this being successfully carried out by Liefmann and Andrew (1911). These men demonstrated the presence of a globulin and albumin fraction in eel serum and concluded that a decomposable complement must be present.

The Carp (Cyprenius carpio)

General comments: The following determinations, unless otherwise credited, were carried out as part of this

thesis. The complement used was obtained by bleeding carp from the heart as explained in the appendix, and was used either pooled or individually. The tables presented in the text are selected as representative of the material they portray and are supported by the data given in the appendix.

While carp have a rather high titer complement, it is not possible to make as direct a comparison with the guinea pig as it was in the case of the frog. This is because two different sets of cells and antibodies are involved and it is well known that such combinations vary greatly in their hemolytic properties. However, it may be stated that carp have an abundant supply of complement, although possibly not as much as do the other two forms.

The experiments on the components of carp complement were conducted on rabbit red cells, and utilized the natural antibody present in the various samples being tested. It is important to note that none of the specific inactivations destroyed this natural antibody, a fact that was readily ascertained by observing whether or not such inactivated serum could still agglutinate rabbit cells. These antibodies were not precipitated by carbon dioxide, but the precipitate was not useful in the experiments as will be seen.

The components:

a. Heat labile. While it was found possible to distinguish heat labile and heat stabile fractions of carp complement, it was not possible to separate the serum into two parts with the use of carbon dioxide. This was only tried twice, but the results, as seen in table 4. , were clear cut and

The Components of Carp Serum.

Inactivations were made according to the methods given under technique.

The same abbreviations applied for fractions as were used for those of the frog(sheet).

Rabbit red cells sensitized wih the natural antibody present in the fractions were used as the test antigen.

Tests were made at room temperature, about twenty two degrees.

One tenth c.c..of a two percent suspension of cells used ih each tube, all final volumes three tenths of a c.c..

Serum fractions of Carp A.

Fraction.	Z	A	H	M	E	M+E	Z+M	Z+E	Z+H	Z+A	A+M	A+E	A+H	H+M	H+E	M^H+E
Amount.	.2	.2	.2	.2	.2	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1
Time in minutes. 10	0	0	0	0	++++	0	0	++++	0	+	0	++++	++++	0	++++	0
20	0	0	0	0	++++	0	0	++++	0	++++	0	++++	++++	0	++++	0
30	0	0	0	0	++++	0	0	++++	±	++++	0	++++	++++	0	++++	0
45	0	0	0	0	++++	±	0	++++	++	++++	0	++++	++++	0	++++	0
60	0	0	0	0	++++	+	0	++++	++++	++++	0	++++	++++	0	++++	0

Fraction.	E^H+M	A+M^H	A+E^H	Z+M^H	Z+E^H	M^H+H	E^H+H	E	E^H
Amount.	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1	.1
Time in minutes. 10	0	0	+	0	⊕	0	0	0	0
20	0	0	++	0	⊕	0	0	0	0
30	0	0	+++	0	⊕	0	±	0	0
45	0	0	+++	0	⊕	0	+	±	±
60	0	0	+++	0	⊕	0	++	+	±

Serum fractions of Carp B.

Fraction.	Z	A	H	M	E	M+E	Z+M	Z+E	Z+H	Z+A	A+M	A+E	A+H	H+M	H+E	M^H+E
Amount.	.2	.2	.2	.2	.2	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1
Time in minutes. 10	0	0	+	0	++++	0	0	++++	±	0	0	++++	++++	0	++++	0
20	0	0	+++	0	++++	±	0	++++	++++	0	0	++++	++++	+	++++	0
30	0	0	++++	0	++++	++	0	++++	++++	0	0	++++	++++	++++	++++	±
45	0	0	++++	0	++++	++++	0	++++	++++	0	0	++++	++++	++++	++++	++++
60	0	+	++++	0	++++	++++	0	++++	++++	+	0	++++	++++	++++	++++	++++

Fraction.	E^H+M	A+M^H	A+E^H	Z+M^H	Z+E^H	H+M^H	H+E^H	E	E^H
Amount.	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1	.1
Time in minutes. 10	0	0	++	0	0	0	+	0	0
20	0	0	++	0	0	0	++	0	0
30	0	0	+++	0	0	+	+++	⊕	0
45	0	0	+++	0	0	++++	++++	+	±
60	0	0	+++	0	0	++++	++++	++++	±

Titers; M and E 1/10, Z, A, and H 1/5.

showed that, while a globulin precipitate was obtained, all the hemolytic activity remained with the supernatant. It is my personal belief that proper, slight variations in technique, such as splitting by dialysis, will show that C'1 and C'2 are present and separable. In this I am supported by the work of Liefmann and Andrew (1911) who were able to split eel serum into these two fractions, and also by the fact that even the best of technique sometimes leaves enough C'1 in solution in guinea pig serum to result in a hemolytic supernatant. However, until this is done it is safer to think in terms of the heat labile fraction of carp complement and to realize that this may mean either C'1 or C'2, both, or neither, in terms of the guinea pig.

The best temperature at which to carry out the heat inactivation of carp serum is at fifty-three degrees for twenty-five minutes, fifty-six degrees being too drastic and lower temperatures or shorter times not always being sufficient. This temperature is about ten degrees higher than that reported by Nardi for eel serum, but as a similar range of variation exists between the toad and the frog, this difference would not seem to be of great significance.

The partial activity of the combination of heated albumin fraction and heated whole serum to be seen in table 4. is not explained, but may involve inhibition or other obscure effects. This has only been observed once.

b. <u>The zymin inactivated fraction</u>: Carp serum is specifically inactivated by zymin, differing from the frog

and guinea pig in that only one sixth as much zymin, applied for thirty minutes at room temperature, gives an optimum inactivation. More drastic treatments than this result in a large amount of non-specific inactivation, unless a great excess of zymin is used when no appreciable inactivation takes place. While heated normal carp serum was able to reactivate zymin treated serum, the same reactivation was not obtained with heated albumin fraction; however, too much significance should not be placed upon this latter observation for the same combination in frog serum, while active, is quite a bit weaker than that of heated and zymin treated whole serum. Further, it is to be seen that the carbon dioxide treatment lowers the heat resistance of the end-piece and may well do so for C'3, and also that the heat resistance of C'3 varies among the individual guinea pigs.

Although carp complement is similar to the other two species in the rate at which it deteriorates with age, no data has yet been secured as to the relative rates at which its various components become inactive during this process.

While the data show that carp complement possess a heat stable, zymin removable fraction, caution should be applied in considering it essentially like that of the frog or guinea pig until the nature of the heat labile fraction has been better analyzed. Therefore, while the term C'3 will be used as a matter of convenience, the amount of implied homology stops with the facts. A further necessity for this will be seen in consideration of the inter-specific reactivations between the

frog and the carp.

c. C'4. The technique of inactivating carp serum with ammonia is very like that employed against the guinea pig and the frog, in fact, the same proportion of ammonia to serum is used and at the same temperature, the only difference being that an hour's treatment is sufficient for inactivation. Serum so treated may be reactivated by heat or zymin inactivated serum, demonstrating that, like other forms, this component of carp complement is heat stable and distinct from C'3. Other similarities with the higher species are to be seen in that the reactivation is usually most readily obtained between the ammonia and heat inactivated fractions, and the C'4 activity is not precipitated upon carbon dioxide treatment.

These facts show that it is reasonable to consider the ammonia inactivated component of carp serum as comparable to the C'4 of the frog and guinea pig. It must still be remembered, however, that the validity of this consideration is subject to further work on the heat labile fraction.

Summary: While it is probable from earlier work that two heat labile components exist in carp serum, it has only been possible to demonstrate that carp complement has one heat labile part; however, two heat stable components have also been shown. Of these, one closely resembles the C'4 component in other forms, the other the C'3 component; the latter conclusion, however, being less secure than the former.

The Complement of Elasmobranchs and Cyclostomes

While nothing definite can be stated on this subject, a

statement was made in Karsner and Ecker (1921) to the effect that rays and lampreys have a toxic principle in their blood similar to that found in eel serum. A similar reference has been made to shark serum by Ruediger and Davis (1907) who report a thermolabile opsonin in dog-fish serum. These two papers are of passing interest as the fishes mentioned are the lowest forms of vertebrate life known to me about which a probable reference to the presence of complement has been made.

Complement in the Invertebrates

No one has definitely demonstrated the presence of hemolytic complement in the invertebrates, but several observations have been made that are of interest in this connection. The most suggestive of these have been reviewed by Huff (1940) and consist of the statement of Cantacuzene that the serum of the hermit crab (Eupagurus prideauxii), which is naturally hemolytic for unsensitized sheep cells in low dilutions, will hemolyze such cells in much higher dilutions if they are sensitized with anti-sheep serum (presumably from rabbits). Another statement by the same author is that the arachnolysin obtained from the eggs of Eperia diadematа when inactivated by heating to sixty-two degrees, can be reactivated by adding very small amounts of non-lytic eggs from the same or closely related species.

Tyler (1942a) has recently shown that the sperm of the key-hole limpet (Megathura) and of the abalone (Haliotis) are associated with proteinaceous lysins specific for the membranes of their own eggs. These lysins deteriorate with age and can

be inactivated by heating to temperatures as low as forty-four degrees, which process still leaves anti-fertilizin intact. As anti-fertilizin is itself a highly specific protein found in sperm that, together with the fertilizin of eggs, shows many of the properties common to antigen-antibody complexes, the analogy between the labile lysin and complement is strong, as Tyler has pointed out.

Evidence of a more indirect sort for the presence of complement in the invertebrates is the universal occurrence of phagocytosis in all forms of animals. It is well known that in the vertebrates this phenomenon is often affected by the presence of complement, and mammalian leuckocytes have even been shown (Maltaner 1935) to contain at least the C'4 component. With this in mind the paper of Ruediger and Davis (1907) is significant for they report that phagocytosis in representative forms of the great groups of animals, down to and including the echinoderms, seems to depend largely upon the presence of opsonins in their sera. The paper includes such facts as the following: sea urchins will sensitize staphlococci for phagocytosis by human sera, while lobsters, spider crabs, flounders and other forms will not, and that the opsonin in Limulus is thermolabile. As this paper was written long ago, one should not rely upon its evidence too heavily, It is, however, suggestive and particularly so since Tyler (unpublished) has shown the presence of many specific agglutinins in lobster serum.

In concluding this section, it should be pointed out that in general anticomplementary effects have received little

attention, particularly as regards their specificity, although much might be learned from them. Such effects as the removal of third component by certain bacteria (Coca 1914), as well as by cobra venom and yeast cells, the action of zymin upon mid and end piece, and the anticomplementary modification that mid-piece and whole serum undergo upon standing (Osborn 1937) are just a few that should be investigated and correlated.

Especially pertinent to the problem of complement in the invertebrates is Tyler's (1942b) discovery of the anti-complementary action of fertilizin. Fertilizin is a protein that occurs at the surface of sea urchin and other eggs of many marine invertebrates and is peculiar in that it combines with a related substance in the sperms of these species.

This combination is species-specific and, depending upon the experimental conditions, can result in sperm or egg agglutination. In the attempted fixation of guinea pig complement by this combination, Tyler found that fertilizin specifically inactivates C'4 of guinea pig serum, that anti-fertilizin added to serum so treated causes reactivation by "releasing" C'4, and that the fourth component will agglutinate sea urchin eggs. While it is not clear what general significance can be attached to all these findings, it is apparent that more work on the invertebrates is highly desirable for determining further the occurrence and properties of complement.

A final paper may be cited as related to this section, and that is by Adams (1931) on an anticomplementary substance that was extracted in saline from the salivary glands and mid-

intestine of the tsetse fly (Glossina palpalis). This substance was found to inactivate the hemolytic complement of mammalian, avian and reptilian sera in vitro, and was assumed to be the cause of the rapid in vivo inactivation of such sera in flies. Correlated with this was the fact that while gut forms of several strains of Trypanosoma gambiense and T. rhodesiense were soon killed by sera containing hemolytic complement, the salivary forms were not, this immunity being an obvious necessity if the flagellate is to enter and live in the blood of its vertebrate host. The relation of this immunity to the anticomplementary factor in the fly is not known, but it can be seen that the factor prevents sterilization of the gut of the fly by vertebrate blood during feeding, which makes one wonder if other biting flies do not need to possess such factors in order to preserve their intestinal flora and fauna. Similarly one would like to know the relationship between complement and Trypanosoma hippicum which is transmitted through the bite of the vampire bat (Allen 1940).

III. Inter-specific Combinations of the Components of Complement

Introduction

The part of the thesis immediately preceeding this contained a comparative survey of the present knowledge of the occurrence of complement and its composition in different species. It is apparent that, aside from the guinea pig, frog and carp, little work has been done upon the intra-specific inactivation of the various components of complement, and this includes even those of man. However, relatively more comparative work has been published upon the cross-reactivations among different species and it is upon this type of work that the various conclusions as to the presence, absence, amount and specificity of the components in different species have been based almost exclusively. This part of the thesis proposes to review this work and to present original data on the same subject.

Inter-specific Reactions Between Whole Complement and the Antigen-Antibody Complex

The most frequent tests of cross-activity among species have been the addition of the normal serum of one species to the hemolytic, but heat-inactivated serum of another. Almost invariably the heat inactivation has been carried out at fifty-six degrees, which leaves the results subject to the unknown influence of heat stable components in the inactive serum as well as to the effects of heating upon the natural antibody where this was involved. A good review of much of this work has been made by Zinsser, Enders and Fothergill (1939) and one can see from it that no phylogentic deductions are possible,

there being no apparent regularity about the results. This is probably best illustrated by the report of Ehrlich and Morgenroth (1901) that, while hen and goose complement would activate rabbit antibody, pigeon would not, although it would work with goose amboceptor (antibody). This suggests some phylogentic correlation, but only until one tries to explain the additional facts that rat, rabbit, and guinea pig complement also worked with goose antibody. (Also that frog complement works with rabbit amboceptor (antibody), while horse will not.)

A further complication is introduced into studies of this type by the fact that different kinds of antigens vary in the degree to which they react with various combinations of complement and amboceptor. This means that in comparative studies one must be very careful to interpret the results in terms of the combinations involved, particular in the case of negative ones for a combination may work under one set of conditions, but not another. The need for this care will be discussed in detail in the section on the specificity of complement.

Experiments similar to those above have been carried out among cold-clooded vertebrates with like results. The most extensive of these were done by Mazzetti (1913) on reptiles and amphibians, and by Nardi (1938) on fish. Both these authors furnish, in addition to their own work, reviews of preceeding studies. Neither of them, nor their predecessors was able to activate cold-blooded antibody with mammalian complement (guinea pig, rabbit and calf) and this has been the case with all later work on this point. Even Bond and Sherwood (1939) and Reiner and Strilich (1929) were not able to achieve this

activation in spite of the fact that they were able to activate rabbit antibody using snake and frog complements respectively.

In this laboratory it has not yet been possible to activate carp anti-red cell amboceptor with mammalian complement or to activate rabbit antibody with carp complement. The latter antibody, however, has been successfully combined with frog complement as will be seen.

The Exchange of Components Between Species

Introduction: Osborn states, in reviewing the data on the exchange of components between species, that no cases have been reported where the globulin fraction lacks C'2 of one species has failed to activate the albumin fraction lacks C'1 of another, and cites the work of Mackie and Finkelstein (1931) and of Marks (1910-12) as examples. This, however, is contradicted by the statement of Ritz and Sachs (1912, 1917) that the serum of some animals, as the mouse and horse, may possess a mid-piece lacks C'2 for certain sensitized cells, but not for others.

Jonas (1913) and Misawa (1934) have studied the thermostable components in different sera, and Hyde (1923) the reactivation of complement deficient guinea pig serum with that of various animals including the frog, but only Hagedius and Griener (1938) seem to have made an attempt to compare all the four components of complement in different species. As their paper has recently been quoted by some authorities (Heidelberger and Mayer 1942; Ecker, Pillemer and Kuehn 1942) it is necessary to point out here that it can not be considered accurate for

several reasons. The first of these is that they did not allow for the fact that their tests were made exclusively on sheep cells sensitized with rabbit anti-body, with the result that sheep serum, in common with cow, horse and ground squirrel, appears to lack C'2 and C'4 completely, an observation that could as well be explained on the basis of differences in specificity. As will be discussed in a later section, normal horse serum is hemolytic when used against unsensitized guinea pig cells or against ox cells sensitized with cat antibody and this, as well as the knowledge of the species specificity of complement, suggests caution in concluding that horse, or any other animal, lacks such components as end-piece (C'2) and the fourth (C'4). In addition, the fact that the determinations were made by adding the whole serum of the test animals to various fractions of guinea pig serum prevents an accurate interpretation of which components actually are involved. However, the paper does show that some sort of activations can be achieved by mixing the sera of two species, and that variations exist between species as to degree of activity under the conditions of the experiment.

This paper is the only one known to me in which all four components were considered for comparison, all others failing to account for this many and thus making it difficult to judge just what the activity observed, if any, was due to. As will be seen in the next section, the interpretation of the results obtained by cross-activation is not easy even when sufficient controls are run, this being due, in large part, to the type of technique necessarily employed.

The Cross-Activation of Frog and Guinea Pig Serum

General Comments: The fortunate circumstance that both frog and guinea pig have complements that will work with rabbit antibody and sheep cells to about the same degree, makes them of special use in studying inter-specific cross-reactivation for this obviously eliminates several unknown factors from an already complicated situation. The fact that frog and guinea pig sera would reactivate each other in some combinations has not been observed by other authors, and was determined only after it was found that the so-called standard one in ten dilutions of guinea pig serum were in general too high to permit cross-activity (a possibility already suggested by Reiner and Strilich in 1929). This situation was overcome by using dilutions of from one in two to one in five, excepting in the case of C'1 and C'2 fractions which usually could not be obtained in concentrations lower than one in ten.

The results of this analysis of cross-activity are presented in detail in the appendix, and have been summarized for discussion in table 5. in the text. All work was done with frog serum taken on the day of any particular experiment, and with guinea pig serum taken the evening before and kept in an ice box during the night. All fractions were made on the day of the experiment. While Pillemer, Seifter and Ecker (1941) have objected to the keeping of serum overnight before using it on the grounds that it reduces the amount of fixation, no differences were observed in preliminary experiments with fresh serum in the case of the guinea pig and it is not likely that such aging influenced the results of this work.

Table 5.

A Summary of the Cross-Activity between Frog and Guinea Pig Serum Fractions.

The following table represents a summary of the various combinations made between frog and guinea pig serum fractions and is based largely upon the data given in the appendix, sheets a to h. All combinations are represented with the frog serum fractions above those of the guinea pig, the presence or absence of a component being indicated by an x or o sign. (Parenthesis about a sign indicate that the presence of a component was not actually tested for.)

The probability that a given result is accurate as far as determined is noted in the appropriate column and is based upon clearly defined differences. These notations of course are subject to further determinations.

Abbreviations stand for the following;

M and E : "globulin"and "albuminin" fractions separated by the carbon dioxide technique.

Z : zymin inactivated serum.

A : ammonia inactivated serum

H : heat inactivated serum.

F : Serum from the frog.

G : serum from the guinea pig.

Doubly inactivated fractions are represented by two of the above letters as, for example, AZ ; mixtures of two fractions from the same species are indicated by two letters enclosed in parenthesis.

Table 5 .(Cont.)

Active Combinations.

Components.				Fractions.		Sheets.	Probability of Observation.
C'1	C'2	C'3	C'4	F/G			
x	x	x	o	A	Z		Good.
x	x	o	x				
x	x	o	x	Z	A		Good.
x	x	x	o				
x	x	o	x	Z	E		Questionable.
o	x	x	x				
x	x	o	x	Z	(EH)		Good.
o	x	x	x				
x	x	o	x	Z	H		Good.
o	o	x	x				
o	x	(x)	(x)	E	M		Good.
x	o	(x)	o				
o	(x)	x	o	AH	Z		Good.
x	x	o	x				
o	(x)	o	x	ZH	A		Good.
x	x	x	o				
o	(?)	x	x	H	A		Good.
x	x	x	o				
o	(?)	x	x	H	Z		Good.
x	x	o	x				
o	(?)	x	x	H	ZA		Good.
x	x	o	o				
x	x	o	x	Z	HA		Reaction quite weak.
o	o	x	o				

Inactive Combinations, lacking C'4.

Components.				Fractions.		Sheets.	Probability of Observation.
C'1	C'2	C'3	C'4	F/G			
x	x	x	o	A	A		Good, see ammonia ion effect in appendix.
x	x	x	0				
x	x	x	o	A	M		Good.
x	o	(x)	0				
x	x	x	o	A	AH		Good.
o	o	x	o				
x	x	x	o	A	ZA		Good.
x	x	o	o				
o	(x)	x	o	AH	A		Good.
x	x	x	o				
x	o	(o)	(o)	M	A		Good.
x	x	x	o				

Table 5.(Cont.)

Inactive Combinations, lacking C'3.

Components.				Fractions.	Sheets.	Probability of Observation.
C'1	C'2	C'3	C'4	F/G		
x	x	o	x	Z Z		Good.
x	x	o	x			
x	x	o	x	Z ZH		Good.
o	o	o	x			
x	x	o	x	Z ZA		Good.
x	x	o	o			
x	o	(o)	o	M Z		Good.
x	x	o	x			
o	x	(x)	x	E Z		Good.
x	x	o	x			
x	x	o	o	ZA Z		Good.
x	x	o	x			

Inactive Combinations, lacking C'2.

Components.				Fractions.	Sheets.	Probability of Observation.
C'1	C'2	C'3	C'4	F/G		
x	o	(o)	o	M M		Good.
x	o	(x)	o			
o	(o)	x	x	H H		Good, F heated to fifty degrees.
o	o	x	x			

Inactive Combinations, lacking C'1.

Components.				Fractions.	Sheets.	Probability of Observation.
C'1	C'2	C'3	C'4	F/G		
o	(x)	x	x	H H		Good.
o	o	x	x			
o	(x)	x	x	H ZH		Good.
o	o	o	x			
o	(x)	o	x	ZH H		Good.
o	o	x	x			
o	(x)	x	x	H AH		Good.
o	o	x	o			
o	(x)	x	o	AH H		Good.
o	o	x	x			
o	x	x	x	(EH)(EH)		Good.
o	x	x	x			
o	x	x	x	(EH) E		Good.
0	x	(o)	x			

Table 5.(Cont.)

Complete but Inactive Combinations.

Components.				Fractions.	Sheet.	Probability of Observation.
C'1	C'2	C'3	C'4	F/G		
x	x	x	o	A E		Good.
o	x	(x)	x			
x	x	x	o	A ZH		Good.
o	o	o	x			
x	x	x	o	A H		Good.
o	0	x	x			
x	x	x	o	A (EH)		Good.
o	x	x	x			
o	x	x	x	E (MH)		Good,an inhibition as
x	o	x	x			E/M is active.
x	o	(x)	o	M E		Good.
o	x	x	(x)			
x	o	(x)	o	M (EH)		Good.
o	x	x	x			
o	x	(x)	x	E A		Good,an inhibition as
x	x	x	o			E/M is active.
x	x	o	o	ZA H		Good.
o	o	x	x			
o	x	(x)	x	E Z		Good,not due to lack of
x	x	o	x			C'3 for (EH)/Z does not go.
o	x	x	x	(EH) Z		Good,possible inhibition
x	x	o	x			as E/M is active.

Combinations Demonstrating Inhibition.

These experiments show that inhibition occurs in some combinations,for while the first two are active,the rest are inactive,i.e. only as active as frog (MH) alone.

Components.				Fractions.	Sheet.	Probability of Observation.
C'1	C'2	C'3	C'4	F/G		
x	x	x	x	(MH) A		Good.
x	x	x	o			
x	x	x	x	(MH) H		Good.
o	o	x	x	(MH)		
x	x	x	x	(MH) Z		Good.
x	x	o	x			
x	x	x	x	(MH) M		Good.
x	o	x	o			
x	x	x	x	(MH) E		Good.
o	x	o	x			
x	x	x	x	(MH)(EH)		Good.
o	x	x	x			

As one can see from the original data, it was necessary to run a great many controls because of the somewhat variable nature of the specific inactivations. All conclusions that are drawn are based upon careful comparisons with these controls, and were considered significant only when the data showed marked and striking differences. Such cases that seemed doubtful will be found indicated as such where they are discussed. The experiments were often carried on simultaneously at thirty-seven degrees and at room temperature, but the differences found were not such as to confuse the results given below.

Interpretation and Discussion

Although it was relatively easy to show that some combinations of fractionated guinea pig and frog complement were hemolytic, while others were not, the technical difficulties in obtaining some combinations were such that it is difficult to say, in several cases, whether the absence of activity is due to differences in specificity, to the interference of accessory components, to high dilution, or to other causes. It is also hard to determine which components are actually at work in the active combinations. However, some conclusions can be made and these will now be discussed, the reactions upon which they are based being summarized in table 5. . While some of these reactions were carried out several times, others were, for lack of time, performed only once. The estimates of their probability are based less upon this consideration than upon that of the controls for a given experiment, and are, of course, subject to further experimentation.

The first conclusion to be reached is that all four components must be present at least once before a reaction will occur. This is almost certainly so for C'1, C'3, and C'4, and quite probably so for C'2 (which, unfortunately, has not been tested in as many combinations as the other components because of the relatively late discovery of the difference in heat stability between frog C'1 and C'2). This conclusion may be taken as further evidence that a given specific treatment inactivates equivalent components in the serum of each animal. (In this connection, the apparent activity of a mixture of the two ammonia fractions has been found to be due to the hemolytic effect of the ammonia ion, rather than to complementary activity. This is discussed in the appendix.)

Next, it can be seen that even though a mixed serum may have at least a single dose of each component in its make-up, it is not always active. The determination, however, of which inter-specific combinations of components are active is not a simple matter for in most cases it has not been easy, because of technical difficulties, to manipulate the components readily, especially where one would like to introduce a single component of one species into the system of the other. This should eventually be possible, but until then all that one can safely do is to indicate which combinations have been active and which have not. The advisability of such caution is supported by the results to be seen in the last part of table 5. where the inhibition of active units by the presence of extra components is clearly indicated.

One may, however, come to some tentative conclusions

that at this point can only be accepted as little more than suggestions. In the first place, it looks as if C'3 will prove to be the least specific of the various components in either species for it seems as though it does not matter which contributes the C'3 in combinations otherwise active. Then, it will be noted that guinea pig C'1 is present in eight out of the twelve active combinations, but in only four out of the eleven inactive ones. It should also be noted that while frog C'2 and guinea pig C'1 (E/M) are active in a dilution of one in ten, the reciprocal mixture (M/E) is not active even at a dilution of one to five. These observations suggest that only guinea pig C'1 and frog C'2 are active in cross-combinations involving the heat labile fractions. This activity may be inhibited, under some conditions, however, as can be seen in such inactive combinations as E/(MH), E/Z, E/A, and (EH)/M.

Finally, it looks as if it may eventually prove that C'4 is not interchangable in these two species, but that each requires at least its own C'2 as a "carrier" before it can be potentially active. This last conclusion is the least secure of any, largely because of the scarcity of data definitely bearing on the point.

While it is to be hoped that with better techniques one can eventually make more definite assertions as to the role of the various components in cross-activations, still it does seem clear from the present work that the complements of such widely divergent forms as the frog and guinea pig are very similar in function and in constitution and are therefore quite stable from an evolutionary point of view. This concept of

stability is borne out in the two comparisons to follow. In spite of this stability, however, at least one striking adaptation has occurred and this is in the relative speed of hemolytic action at different temperatures, the optimum temperature being higher in the guinea pig than the frog, and higher in the frog than in the carp. It is to be hoped that the technique of cross-activation will eventually be advanced to the point where the specific components involved in this adaptation can be found, for at present it is not possible to determine this fact.

The Cross-Activation of Carp and Guinea Pig Sera

While the experiments are not nearly as complete for this series of activations as for the preceeding, they are of interest in that several positive results were obtained which further indicates the essential homology of complement throughout the vertebrates. The fish and guinea pig are not known to use the same antibody, so all combinations were run in duplicate, one with unsensitized rabbit red cells which are known to be hemolized by normal fish serum, and one with sheep red cells sensitized with rabbit amboceptor as test antigen for the guinea pig. As C'1 and C'2 have not been demonstrated in the carp as yet, and as these experiments were primarily designed to show merely that cross-activity does occur, the fractions used are best considered as to how they were inactivated rather than as to what components they contain. The results are summarized in table 6., which is largely explained by its legend.

Of particular note is the difference between the action

Table 6.

A Summary of the Cross-Activity between Carp and Guinea Pig Sera.

The following table summarizes the active and inactive combinations of various carp and guinea pig serum fractions as determined from the data given in sheets λ to ȷ in the appendix.

Abbreviations are the same as those used for the different fractions in the table summarizing the cross- activity between frog and guinea pig.

Active combinations are indicated with an x, inactive with an o.

Activity with Rabbit Red Cells Sensitized with Natural Carp Antibody.

Guinea Pig.

Carp.	H	Z	A	Diluted whole normal serum. 1/2	1/4	1/6
H	o	o	o	x	x	o
Z	o	o	o	x	x	x
A	o	o	o	x	x	x

Activity with Sheep Red Cells Sensitized with Rabbit Antibody.

Carp.

Guinea Pig.	H	Z	A	Diluted 1/2	normal 1/3	serum. 1/6
H	o	o	o	o	o	o
Z	x	o?	x	x	x	o
A	x	x	o	x	x	o

of whole guinea pig serum and its fractions when mixed with the fish fractions and rabbit cells. Three explanations are possible; one, that whole guinea pig serum can use fish antibody; two, that combinations of components are to be found in this mixture that do not occur in those previously made; and three, that the fractionation techniques injure the linkages in guinea pig complement that permit activity with carp components. The only evidence on this point is that the one to six dilution of normal guinea pig serum did not activate heated fish serum, although it still reacted with the other two fractions. This suggests that the guinea pig complement is not working with fish antibody, but instead that the activity is due to some sort of interaction between components.

As in the frog-guinea pig tests, all reactivations were successful only in dilutions lower than one to ten, those at one and two and one and three being usually employed.

The Cross-Activation of Carp and Frog Sera

In concluding the cross-activation work for the purposes of this thesis, two experiments were run between frog and carp complements. Here the relation of complement to amboceptor is even more confusing, for while only the frog can use rabbit antibody, both carp and frog have rather a large amount of natural antibody against rabbit erythrocytes in their sera. Therefore, while again it is possible to demonstrate cross-activations, the relationships are too involved to permit an analysis of what is taking place.

The experiments are presented in tables 7, 8 and 9 in both a summarized and an original form. The most interesting

Table 7.

A Summary of the Cross-Activity between Carp and Frog Sera.

The following table summarizes the active and inactive combinations of various carp and frog serum fractions as determined from the data for the two experiments given in sheets 8. to 9. ~~in the appendix.~~

Abbreviations are the same as those used for the different fractions in the table summarizing the cross-activity between frog and guinea pig.

Active combinations are indicated with an x, inactive with an o.

Activity with Rabbit Red Cells Sensitized with the Natural Antibody Present in the Sera.

		Frog. H	Frog. Z	Frog. A
Carp.	H	o	x	o
	Z	x	x	o
	A	x/o	x	o

Activity with Sheep Red Cells Sensitized with Rabbit Antibody.

		Frog. H	Frog. Z	Frog. A
Carp.	H	o	x	o
	Z	o	x	o
	A	o?	x	o

Cross-activity between Carp and Frog Sera.

Abbreviations the same as those used for the cross-activation experiments between the frog and the guinea pig, C standing for carp fractions, F for frog fractions.

One tenth c.c. of a two percent suspension of rabbit cells, sensitized with the natural antibodies present in the fractions, used as test antigen throughout.

All fractions diluted one to three before testing.

Cross-activity observed at room temperature(twenty two degrees).

Species.	C-	C-	C-	C+	C+	C+	F-	F-	F-	F+	F+	F+
Fraction.	Z	A	H	Z+A	Z+H	H+A	Z	A	H	Z+A	Z+H	H+A
Amount.	.2	.2	.2	.1+.1	.1+.1	.1+.1	.2	.2	.2	.1+.1	.1+.1	.1+.1
Time in 10 minutes. 10	0	0	0	±	±	0	0	0	0	0	0	0
20	0	0	0	±	±	+	0	0	0	0	0	0
30	0	0	0	±	±	+++	0	0	0	0	0	0
45	0	0	0	+	+	+++	0	0	0	0	0	±
60	0	0	0	+	+	+++	0	0	0	0	0	+
100	0	0	0	+	+	+++	0	0	0	0	0	+

Species.	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	Highest titer of natural antibody.	F Normal		C Normal	
Fraction.	Z+A		A+Z		Z+H		H+Z		A+H		H+A		Z+Z		A+A		H+H			1/16	1/32	1/16	1/32
Amount.	.1+.1		.1+.1		.1+.1		.1+.1		.1+.1		.1+.1		.1+.1		.1+.1		.1+.1						
Time in 10 minutes. 10	0		0		0		0		0		0		0		0		0			0	0	0	0
20	+++		0		++		0		0		0		0		0		0			0	0	0	0
30	+++		0		+++		±		0		0		0		0		0			0	0	0	0
45	+++		0		+++		+		0		0		+		0		0			±	0	0	0
60	+++		0		+++		++		0		0		+++		0		0			+	0	++	0
100	+++		0		+++		++		0		0		+++		0		0			++	0	+++	0

Cross-activity observed at eleven degrees.

Species.	C-	C-	C-	C+	C+	C+	F-	F-	F-	F+	F+	F+
Fraction.	Z	A	H	Z+A	Z+H	H+A	Z	A	H	Z+A	Z+H	H+A
Amount.	.2	.2	.2	.1+.1	.1+.1	.1+.1	.2	.2	.2	.1+.1	.1+.1	.1+.1
Time in 10 minutes. 10	0	0	0	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0	0	0	0	±
45	0	0	0	0	0	++	0	0	0	0	0	++
60	0	0	0	±	0	+++	0	0	0	0	0	++
100	0	0	0	++	0	+++	0	0	0	++	0	+++

Species.	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	Highest titer of natural antibody.	F Normal		C Normal	
Fraction.	Z+A		A+Z		Z+H		H+Z		A+H		H+A		Z+Z		A+A		H+H			1/16	1/32	1/8	1/16
Amount.	.1+.1		.1+.1		.1+.1		.1+.1		.1+.1		.1+.1		.1+.1		.1+.1		.1+.1						
Time in 10 minutes. 10	0		0		0		0		0		0		0		0		0			0	0	0	0
20	0		0		0		0		0		0		0		0		0			0	0	0	0
30	0		0		0		0		0		0		0		0		0			0	0	0	0
45	++		0		++		0		0		0		0		0		0			0	0	0	0
60	+++		0		+++		0		0		0		+		0		0			+	0	++	0
100	+++		0		+++		0		0		0		++		0		0			++	0	++	0

+ , − :Indicate presence or absence of hemolysis after 24 hours.

Sheet 9.(Feb.16,1945.)

Cross-activation with Carp and Frog Sera.

Conditions the same as for sheet 8, excepting that sheep cells sensitized with rabbit antibody were used as a test antigen as well as rabbit cells.

Experiments were conducted at room temperature(twenty- two degrees.)

Rabbit cells(unsensitized).

Species.		C	C	F	F	C	C	C	C	C	C	F
Fraction.		Normal	Normal	Normal	Normal	Z	A	H	Z+A	Z+H	A+H	Z
Amount.		1/8	1/16	1/16	1/32	.2	.2	.2	.1+.1	.1+.1	.1+.1	.2
Time in 10	Titer of natural antibody.	0	0	++++	0	0	0	0	+++	++	++	0
minutes. 20		0	0	++++	+±	0	0	0	++++	++	++++	0
30		0	0	+++	++	0	0	0	++++	+++±	+++	0
45		±	0	+++	++	0	0	0	++++	+++±	+++	0
60		++	0	+++	++	0	0	0	++++	+++±	+++	0
100		++	0	+++	++	0	0	0	++++	+++±	+++	0

Species.	F	F	F	F	F	F	C	F	C	F	C	F	C	F	C	F	C
Fraction.	A	H	Z+A	Z+H	H+A	Z+A	A+Z	Z+H	H+Z	A+H	A+A	H+A	Z+Z				
Amount.	.2	.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1				
Time in 10	0	0	0	0	±	0	0	0	+++±	0	++++	0					
minutes. 20	0	0	0	0	+	+	0	+±	++++	0	++++	0					
30	0	0	0	+	+++±	++	0	++	++++	0	+++	0					
45	0	0	0	+	+++±	+++	0	++	++++	0	+++	0					
60	0	0	0	++	++++	+++±	0	+++±	++++	0	++++	±					
100	0	0	0	×	×	+++±	0	+++±	++++	0	+++	++					

Sheep cells(sensitized).

Species.	F	C	F	C		F	C	F	F	F	F	F	F
Fraction.	A+A	H+H				Normal	Normal	Z	A	H	H	Z+A	Z+H	H+A
Amount.	.1+.1	.1+.1				1/32+	1/2	.2	.2	.2	.1	.1+.1	.1+.1	.1+.1
Time in 10	0	0				++++	0	0	0	0	0	0	0	++±
Minutes. 20	0	++++				+++	0	0	0	+	+±	++	+++	++++
30	0	++++				+++	0	0	0	++	++	+++	+++	++++
45	0	++++				+++	0	0	0	+++	++++	++++	++++	++++
60	0	++++				+++	0	0	0	+++	++++	++++	+++	++++
100	0	+++				+++	0	+	0	+++	++++	++++	++++	++++

Titer of complement with sensitized sheep cells.

Species.	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	G
Fraction.	Z+A	A+Z	Z+H	H+Z	A+H	H+A	A+A	Z+Z	H+H							
Amount.	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1							
Time in 10	0	0	0	0	0	0	0	0	0							
minutes. 20	0	0	0	0	0	0	0	0	0							
30	+	0	0	0	0	0	0	0	0							
45	++	0	+++	0	0	+	0	++	+							
60	++	0	+++	0	0	+	0	++	++							
100	+++	0	+++	0	0	++	0	++	+++							

feature observed is that the two zymin fractions show a fair amount of complementary activity when mixed and used with either unsensitized rabbit cells, or sheep cells sensitized with rabbit antibody. This seems to be a valid activation (see appendix for a discussion of the invalid ammonia ion effect) and may be due to a different qualitative effect of zymin upon the components of carp serum, or to differences in the inter-relationships of residual C'3 and other components in the mixed sera of the two species. Unfortunately it is not possible to distinguish between these points as yet.

Discussion and Summary

The work on the inter-specific reactivations shows that, while these occur, they take place only at dilutions that are considerably lower than the intra-specific ones, and one may ask why this should be. The situation is analagous to that observed in comparing the reactions of homologous and related but heterologous antigens with a given antiserum. Here one also observes that the former reaction always can be obtained at much higher dilutions than the latter. Landsteiner (1936) explains this on the basis of chemical similarity, the more similar the haptenes of the antigens being compared, the greater the reaction between heterologous antigen and the antibody. This explanation may also be applied to complement, but not until more is known concerning the kind of linkages involved, and the other related aspects of the problem have been further studied. The fact that C'4 of guinea pig serum will agglutinate sea urchin eggs (Tyler, 1942) might be used as a starting

point for this kind of work, particularly as something is known of the type of compounds able to specifically inactivate this component (Pillemer, Seifter, and Ecker, 1941).

In summarizing this part of the thesis, it may be said that the preceeding sections have shown that, contrary to former work, some of the components of complement can be successfully interchanged among the carp, frog, and guinea pig. The results of such an exchange were extensively studied in the case of the frog and guinea pig, and it was concluded that, here at least, one member of each component had to be present before an action might occur. However, as all combinations in which this was true were not active, a further attempt at analysis was made. While some tentative conclusions were reached, it became apparent that the techniques of inactivation were not refined enough to carry the matter further at this time. This circumstance made it unfeasible to attempt a similar analysis of the more complicated combinations between carp and frog and carp and guinea pig, which, however, do show a fair degree of activity. The general conclusion to be reached is that complement is very similar throughout the vertebrates and is, therefore, a relatively stable character from an evolutionary point of view. Such differences that have occurred do not seem to parallel phylogentic relationships, as will be seen in the section on specificity, with the possible exception of that of optimum activity at different temperatures.

The Complement-Deficient Guinea Pig

This strain of guinea pig, discovered by Downing

(Moore 1919), was distinguished by having a serum with only one percent of the hemolytic activity of normal guinea pig serum (Hyde 1932). This loss of activity has been shown by Rich and Downing (1923) to be due to the activity of a recessive mutation, inherited in simple Mendelian fashion, the homozygotes of which were clearly marked as complement-deficient. Unfortunately, the strain became extinct before the discovery of the fourth component so that the work done on it could not account for this factor. Enough was done, however, to permit some speculations as to the effects of the mutation upon the components then known, and the general conclusion reached at first was that the deficient serum was lacking or inactive in third component (e.g. Hyde 1923). However, Parsons (1926), after an intensive study, concluded that the component inactivated by mutation was not identical with that removable by yeast as it was "impossible to activate them in an identical manner with the fractions obtained by splitting guinea pig serum with carbon dioxide or ammonia sulphate".

Specifically, she showed that yeast inactivated serum is best reactivated by mid-piece (lacks C'2), though end-piece (lacks C'1) also worked to a lesser extent, and that heating to fifty-five degrees for thirty minutes left these fractions still capable of reactivation to a fair, though impaired degree. The mutant serum, however, was best activated by end-piece (lacks C'1), and only a little by mid-piece (lacks C'2), this power being completely lost by both when heated. This last point is in contrast to Hyde's (1923) observations that heated whole guinea pig serum will reactivate the deficient serum, but to a less degree than intact serum. No one seems

to have tried adding heated serum, to yeast absorbed mutant serum, although it was shown that a mixture of yeast absorbed normal serum and of deficient serum was not active.

Whitehead, Gordon and Wormall (1927), in connection with their discovery of fourth component, suggest that this may be the one inactivated by the mutation and offer explanations that cover the discrepancies noted. Unfortunately, the race was then extinct, and while the fourth component may have been involved, no tests could be made and the authors' explanations are just complicated enough to require an experimental check. The safest conclusion to be made from the various experiments is that the mutant probably affected a heat stable constituent, but in a way not identical with those of the artificial specific inactivations.

The other studies that were made upon this strain include work on anaphylaxis and susceptibility to various diseases; these are, however, a little beyond the scope of this thesis and the reader is referred to Hyde (1923, 1932) and Parson (1926) for further sources of information.

The Specificity of Complement

The complementing action of serum has often been referred to as non-specific as compared to the antigen-antibody reaction, in that the same complement can react with a variety of antigen and antibody complexes. Nevertheless, complement may be said to have several other orders of specificity, not quite comparable with that above although all related, of course. The first of these is that of the several components in any one system, each of which has its own characteristics. The second of these includes the differences that occur among species in these characteristics, expressed at present in differences in optimum of inactivation and so on. The third of these is that observed in the different degrees to which a given complement participates in various reactions.

Complement, in addition to its hemolytic activity, is known to act in the lysis of certain sensitized bacteria, the non-lytic destruction of others, the phagocytosis of almost all that are so removed from the animal body, and in several other related activities (see Ecker and Pillemer, 1942, for a complete list). These activities are probably the expression of a single basic reaction, which however remains to be proven. In spite of former arguments, it is now generally accepted that any one animal possesses only one kind of complement which acts as occasion demands, in all the different types of activities given above. There is some question as to whether or not all components act to equal degrees in each kind of reaction, but although it would seem from the material given below that they probably do not, this does not invalidate the evidence that

those which do act are all part of the same complementing system. In other words, while any one animal has only one complement, this complement exhibits specificity in that it participates differently in different reactions. As this final aspect of the specificity of complement is very confusing and little understood in spite of the amount of work done on it, what is known will now be discussed in terms of whole complement, antigen and antibody without attempting to consider the role of single components, although these may ultimately be found involved in the differences observed.

The easiest way to assimilate the various data is to consider the possible combinations in terms of the effects of the sources of the three entities involved (complement, antibody, and antigen), and this has been done in the accompanying table. They show that all three are capable of influencing the success of a given complementing reaction, whether it is fixation, hemolysis or a bactericidal effect. That is, ~~the~~ same combinations of antibody and complement from the same source will or will not react depending upon the antigen added; similarly, the same antibody and antigen will react with one individual's complement and not others; and finally, the same complement and antigen will react with one individual's ~~kind of~~ specific antibody and not others. This leads one to conclude that the result of an untried mixture can not in general be safely predicted on the basis of species or other known relationships, although one might suspect that complement and antibody from the same species should work more frequently than other combinations. However, the data available show

Table 10.

The following is a list of examples showing that the activity of a complementing reaction is influenced by the kinds of complement, antigen and antibody involved, and that variations in any one of these may bring about different results even though the other two are held constant.

Active combinations are indicated by an x, inactive by an o, excepting that in cases where differences in degree of activity occur the stronger reaction is shown by a large X in contrast to the weaker.

Numbers in last column indicate references at end of table.

Reactions in which the Antigen is Varied.

Complement.	Antibody.	Antigen.	Activity.	Criteria.	Ref.
guinea pig	horse	pneumococcus capsule.	o	fixation	1
"	"	pneumococcus protein.	x	"	"
"	"	pneumococcus C. fraction.	o	"	"
"	"	H. influenzae capsule.	o	"	2
"	"	H. influenzae protein	x	"	"
"	"	B. pestis	x	"	3
"	"	Various toxins.	o	"	4
guinea pig	rabbit	pneumococcus capsule	x	fixation	1
"	"	pneumococcus protein	x	"	"
"	"	pneumococcus C fraction	o	"	"
"	"	H. influenzae capsule	x	"	2
"	"	H. influenzae protein.	x	"	"
"	"	S. hemolyticus C fraction	o	"	1
"	"	S. hemolyticus M protein	x	"	"
guinea pig	rabbit	guinea pig red cells	o	hemolysis	4
"	"	sheep red cells	x	"	"

Table 10. (Cont.)

Reactions in which the Antibody is Varied.

Complement.	Antibody.	Antigen.	Activity.	Criteria.	Ref.
guinea pig	horse	pneumococcus capsule.	o	fixation	5
"	human	"	o	"	"
"	mouse	"	o	"	"
"	cat	"	o	"	"
"	dog	"	o	"	"
"	goat	"	o	"	"
"	rabbit	"	x	"	"
"	guinea pig.	"	x	"	"
"	rat	"	x	"	"
"	sheep	"	x	"	"
guinea pig	guinea pig	human red cells.	x	hemolysis	6
"	rabbit	"	x	"	"
"	dog	"	X	"	"
"	cat	"	X	"	"
"	chicken	"	o	"	"
rabbit	guinea pig	"	X	"	"
"	rabbit	"	X	"	"
"	dog	"	x	"	"
"	cat	"	x	"	"
"	chicken	"	o	"	"
horse	rabbit	ox red cells.	o	"	7
"	cat	"	x	"	"
"	goat	"	o	"	"
"	duck	"	o	"	"

Reactions in which the Complement is Varied.

Complement.	Antibody.	Antigen.	Activity.	Criteria.	Ref.
horse	rabbit	sheep red cells.	o	hemolysis	8
guinea pig	"	"	x	"	4
frog	"	"	x	"	9
carp	"	"	o	"	10
snake	"	"	x	"	11
sheep	"	"	o	"	12
human	human	pneumococcus capsule.	x	fixation	13
guinea pig	"	"	o	"	5
goat	goose	ox red cells.	o	hemolysis	14
rat	"	"	x	"	"
human	horse	pneumococcus capsule.	o	fixation	13
guinea pig	"	"	o	"	"

Table 10.(Cont.)

Reactions in which the Complement is Varied(Cont.).

Complement.	Antibody.	Antigen.	Activity.	Criteria.	Ref.
guinea pig	natural rabbit.	Brucella suis.	o	bactericide	15
ox	"	"	x	"	
sheep	"	"	x	"	"
guinea pig	guinea pig	Hemophilus influenzae.	x	"	16
rabbit	"	"	x	"	"
human	"	"	x	"	"
guinea pig	rabbit	"	o	"	"
rabbit	"	"	x	"	"
human	"	"	x	"	"

Reactions where Heterologous Combinations of Complement and Antibody are more active with a given Antigen than are Homologous Ones.

Complement.	Antibody.	Antigen.	Activity.	Criteria.	Ref.
horse	horse	Hemophilus influenzae.	O	bactericide	16
guinea pig	"	"	o	"	"
human	"	"	x	"	"
rabbit	"	"	o	"	"
ox	ox	ox red cells.	o?	hemolysis	7
rabbit	"	"	x?	"	"
guinea pig	rabbit 30-units.	sheep red cells.	XX	"	17
rabbit	"	"	x	"	"
human	"	"	X	"	"

References.(See bibliography for complete reference).

1. Goodner and Horsfall,1936.
2. Pittman and Goodner,1935.
3. Kolmer and Tuft,1941.
4. Osborn,1937.
5. Horsfall and Goodner,1936.
6. Noguchi and Bronfenbrenner, 1911.
7. Muir,1911-14.
8.-16. Dingle,Fothergill, and Chandler,1938.
9. Reiner and Strilich,1929.
10. Cushing,unpublished.
11. Bond and Sherwood,1939.
12. Ecker,Weisberger,and Pillemer,1942.
13. Heidelberger and Mayer, 1942.
14. Ehrlich and Morgenroth, 1901.
15. Shrigley and Irwin,1937.
17. DaCosta,Cruz,and Penna, 1930.

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that even this point is not secure, notably in the human species. Here it is known that isohemolysis does not occur consistently and that its occurrence is apparently not related to the amount of antibody present. Of great interest in this connection is the report of Heidelberger and Mayer (1942) that human complement is not always fixed by the complex of human antibody and pneumococcus capsular antigen, for this raises the possibility that the same factor determines fixation, both here and in isohemolysis. Also of striking interest is the fact that horse antibody will not work with its own complement in the killing of H. influenzae, while it will with human complenent (Dingle, Fothergill and Chandler, 1938).

It will be further noted that, although given sources of complement and antibody may effect one activity together, such as hemolysis, they will not necessarily effect another, as the killing of bacteria.

All the evidence, then, shows that we are not yet aware of the properties that would make possible accurate predictions of how a given combination of complement, antibody and antigen will react. The obvious place to look for evidence on this is in the work done on the conditions influencing fixation. In this connection, Horsfall and Goodner (1936) have shown that different species, as far as studied, may be grouped in two classes as to whether or not their antibodies will fix guinea pig complement when used with the capsular antigen of pneumococcus, the active ones being the rabbit, guinea pig, sheep, and rat, and the inactive ones being the horse, human, mouse, cat, dog and goat. They also state that these groups are also

separable as to whether or not lecithin or cephalin is the lipoid necessary for the agglutinating action of their antibodies. However, a glance at the tables shows that a rearrangement on the basis of other antigens or complements no longer permits separation into these groups and suggests that further work will be necessary, which of course does not invalidate that already accomplished by these authors.

Other studies of fixation have been reviewed by Boyd (1943), who says that the formation of a precipitate as such is not necessary for fixation, nor is its presence a guarantee that it will occur. In spite of this, however, fixation in active combinations would seem to depend in part upon a certain optimum size of the antigen-antibody complex, which of course is also one of the factors influencing the amount of precipitate that will occur. It is apparent that more factors than the size of the complex are involved, but these are as yet unknown although it has been suggested that the physical state at the surface of the molecule is important, and also that complement and the antigen-antibody complex combine in a purely chemical way. The problem is a difficult one to attack because so many variables must be considered; however, as the work of Pressman, Campbell and Pauling (1942) shows that one can use relatively simple antigens in the fixation of guinea pig complement, a comparative study, making use of this fact, might be of some interest. The value of such work would be increased where the antibodies of two animals were compared whose molecular sizes, shapes and other properties have been studied, as in the case of the horse and rabbit. While it would also seem

logical to use homologous complements at first, as these should be best adapted to react with their own antibodies against the widest variety of antigens, the choice of constituents would have to be determined by results; the ultimate point being, to compare the actions of at least two sources of complement and antibody.

In studies of complement fixation, the role of individual components has been neglected and this is an important omission when comparing different active reactions for it is clear that those which merely removed C'3, for instance, could hardly be considered comparable to those fixing C'1, C'2, and C'4, even though both resulted in inactivation. This brings up the fact that we need to know more of the nature of the basic actions of complement in order that we may have a definite foundation on which to form a definition of fixation and subsequent activity that will be useful in comparative studies.

Enough has been said to show that at present we do not know the laws governing the reaction between complement and the antigen-antibody complex. However, these do transcend phylogentic relationships to a degree where it is not safe to predict even the outcome of reactions involving homologous complement and antibody. This lack of knowledge has led to various difficulties, examples of which are to be seen in some of the attempts to correlate hemolytic and phagocytic complements (e.g. Ecker, Pillemer, and Kuehn, 1942), to compare the complements of various species (e.g. Hegedus and Greiner, 1938), and in the studies on natural and immune antibodies (e.g. Kolmer, 1919). All three of these cases, for instance,

did not take into account the fact that the complement some species, such as the sheep and guinea pig, will not hemolize their own cells, although quite capable of hemolizing others. Several additional instances could be cited where the failure to account for the possibility of an effect from varying the relationship among complement, antigen and antibody has led to untenable conclusions, but the above should suffice to show the complications involved. Of interest in this respect is the statement in Osborn (1937) that toxin-antitoxin complexes will not fix complement for it seems probable that a source of antibody and complement other than the horse and guinea pig could be used to good advantage, and similar application might also be made in other cases where fixation tests would be desirable, though not yet demonstrable. Of further interest is the possibility that many of the apparent differences between the opsonic and hemolytic complementing properties of a serum are due to lack of consideration of the specificities involved, rather than to actual qualitative differences in the serum complement as such.

One final point may be discussed in concluding this section and that is the relationship of antigen, antibody and complement in phagocytosis. While antibody and complement alone are often ineffective in destroying bacteria, their action as a catalyzer of phagocytosis is much more effective and general in occurrence. This is important in that phagocytosis is held to be the most efficient natural agent of those that are active in the successful destruction of pathogens (Boyd 1943, Kolmer and Tuft, 1941, etc.). Many other factors are involved, of course, but this does not invalidate the

premise that it should be a great advantage in combating a disease to be able to utilize one's phagocytes as efficiently as possible, and on this premise it can be suggested that some of the failures in serum therapy have been due to the use of antibodies that will not work efficiently in the human system involved. This is an idea going back to Ehrlich, but, in spite of the fact that it has been restated by some later authors, it seems to have received hardly any attention. Specific cases where serum has been tried and found to fail for unknown causes, as in that of plague, should be re-investigated along the line suggested above. This applies even to cases where human convalescent serum has been used, for it is by no means certain that the action of complement is not inhibited in some individuals or that a foreign antibody might not be more destructive than the homologous one and so on.

Such work would of course be greatly facilitated if some generalities could be developed as to the critical points involved in a successful phagocytic reaction. It is conceivable, for instance, that altering the size of the antibody artificially might be a useful way to adapt it toward a more efficient activity in some cases, this of course being but one of many possibilities that await a fuller knowledge of the factors governing a complementing reaction.

In closing, it does not seem out of place to re-emphasize the fact that complement is an important factor in the chain of reactions between living cells and their action on the various antigens in the body, and that the understanding

of the nature and activity of complement is a necessary step before full advantage can be taken of the antigen-antibody reaction as such, itself often merely a relatively impassive starting point in the complete reaction system.

Appendix I: Technique

The Guinea Pig

Bleeding: A colony of about a dozen guinea pigs was kept as a source of complement during the experiments. It was found that these animals could be bled rather easily by one person in the following way. The animal to be bled was etherized until fully unconscious and then placed on its back in a hammock made from a towel and so designed that the animal's front legs could be passed through and held by small slits on either side. The towel was suspended only a few inches above the top of a rabbit dissecting board in such a way that the animal's back rested on the board, while the sides of the towel prevented it from rolling. It was further secured with a cord passed around its lower legs which were enfolded in the towel. The animal was then bled from the heart in the usual way, using a twenty-four gauge needle on a five ml. syringe. Care must be taken not to give too much ether, for, while this is not fatal, it causes convulsive movements that prevent the safe removal of blood. These will stop after a short time if the ether can is removed and work can be resumed. While not definitely established, it does seem that convulsions occur less readily if the ether is given rather slowly.

Treatment of Whole Blood: The whole blood was transferred into twelve cc. centrifuge tubes and these placed in an ice box until ready to use. In most of the work the blood was taken in the late afternoon of the day preceeding an experiment for this gave sufficient time in which to carry out

the extensive operations necessary next day. On the morning of such an experiment the blood was centrifuged and serum used immediately, either separately or pooled as the case might be.

C'1 and C'2: These components were separated by the modified carbon dioxide treatment described by Ecker and Pillemer, 1942, and partially quoted here. Distilled water is saturated at room temperature with pure carbon dioxide gas which is allowed to bubble through the water for thirty minutes under slight pressure. One cc. of serum is added to nine cc. of this water, and carbon dioxide is again passed through the mixture for twenty minutes. Foaming produced by the bubbling is reduced by the addition of capryl alcohol with a wooden applicator. Care should be taken to avoid an excess of alcohol because of its hemolytic quality. The globulin (C'1) precipitated after twenty minutes is settled by rapid centrifuging and the supernatant (C'2) is removed, freed of its carbon dioxide in vacuo, and made isotonic with sodium chloride. Meanwhile, the precipitate is washed several times in distilled water, redissolved in ten cc. of saline and freed of its carbon dioxide. The pH of each fraction is brought to neutrality and the fractions are then ready for use. They should be made last in an experiment as C'1 loses its activity in a few hours. One can use lesser amounts of serum, such as a half cc., provided the amount of distilled water is proportionally reduced. It is also possible to make these fractions in a one in five dilution, but the separation is not always as consistent.

C'3: This component was inactivated by the use of zymin powder, prepared from yeast according to the method described

by Ecker, Jones and Kuehn, 1941. The usual technique was to add .12 gms. of this powder to .7 cc. of normal serum and incubate, with occasional stirring, at thirty-seven degrees for two and a half to three hours. The mixture was then rapidly centrifuged for a short time and the supernatant serum, now about .5cc. in volume, was pipetted off and made up to the required dilution with .9 percent saline, after first being adjusted to approximate neutrality with .1 normal sodium hydroxide. The hydrogen ion concentration was determined with Braun's Universal pH Indicator Paper and adjusted for each individual sample used.

As the zymin powder seems to lose some of its inactivating power after three or four months, it is advisable to prepare fresh samples from time to time.

C'4: In order to inactivate this component, .12 cc. of N/65 of ammonium hydroxide was added to .5 cc. of normal serum which was then incubated for one and one half to two hours at thirty-seven degrees in a stoppered tube. After this the hydrogen ion concentration was adjusted to that of normal serum with one tenth normal hydrochloric acid and the sample brought to the proper dilution with .9 percent saline (see Osborn, 1937, for further information if desired).

Heat inactivation: Samples of the serum to be inactivated by heat were first diluted to the required concentration and then placed for thirty minutes in a water-bath at fifty-five degrees. This treatment is generally believed to inactivate both C'1 and C'2; however, some authors (see Parsons, 1926, for a review) have held that C'1 is more stable in whole serum

than is C'2, while a few others believe the opposite to be true. Until this matter is further clarified, it would be well to withhold too definite an opinion.

The Frog (Rana catesbiana)

Care: The frogs used were bullfrogs six to eight inches long, exclusive of legs, and were kept during the experiments in a shallow water tank. It was necessary to feed them about once every two weeks with a mixture of ground meat and vegetables (Daily Made Pet Food) which had to be pushed down their throats as they would not feed voluntarily. Much trouble was caused by a disease that was probably the result of the activity of Proteus hydrophilus, the organism causing "red-leg". Toward the end of the experiments a paper on this organism with advice on the preparation of a vaccine against it was discovered (Kulp, 1942). While Dr. Kulp kindly sent me a strain of P. hydrophilus to make a vaccine from, it is too soon to state what effect it has had.

Bleeding: It was found that frogs could be bled from the heart, and as much as six cc. were obtained from some individuals without ill effect. The best procedure was to etherize the animal and lay it on its back where it would remain perfectly immobile for some time, then enter the heart with a very sharp twenty-four gague needle attached to a five cc. syringe. One can determine the location of the heart with one's fingers where it lies under the pectoral girdle, but even so considerable practice is necessary before one can acquire a fairly reliable technique. However, when this is done, it will

be found that sufficient blood can be obtained, and that the same frogs can be used many times. Sterile precautions are necessary in order to avoid introducing Proteus hydrophilus or other organisms into the frog.

Treatment of whole blood: Once obtained, the whole blood is put in centrifuge tubes and allowed to clot in the ice box for about an hour, present results indicating that it should be taken and used on the same day. It is advisable to break up the clot before centrifuging for otherwise a cell-free, but clotted plasma is often obtained. The serum is pipetted off and may be used individually or pooled as the supply warrants. Frog serum was fractionated by the following techniques, modified from those used on the guinea pig.

C'1 and C'2: The same treatment as applied to the guinea pig was found suitable for the frog, and the resulting fractions appeared to be the same excepting that the frog globulin fraction was precipitated in finer particles and was more readily soluable in saline than that of the guinea pig.

C'3: Frog serum could be inactivated by the use of zymin powder; however, it was found that the optimum requirements of specific inactivation differed from those of the guinea pig in that, while the same proportions of zymin and serum were used, best results were obtained when the serum was treated at room temperature and only for an hour. Once treated the serum was prepared for further study just as that of the guinea pig.

C'4: This component was inactivated by the same treatment as that applied to the guinea pig, the only difference being that the serum was removed from the thirty-seven degree

water bath after an hour and a quarter, a shorter length of treatment than that given the guinea pig.

Heat inactivation: The optimum for heat inactivation was found to be close to forty-seven degrees for twenty-five minutes. However, it was also found rather late in the experiments that C'1 of frog serum was more labile than C'2, and that sometimes enough C'2 would remain in heat inactivated whole frog serum to reactivate the globulin fraction. The limits of the variability of this difference have not been fully investigated, although it seems that forty-nine to fifty degrees may be found to destroy both heat labile components and still leave C'3 and C'4.

The Carp (Cyprenius carpio)

Care: Carp were easily kept in cement tanks and required relatively little care, being fed on a mixture of ground meat and vegetables (Daily Made Pet Food) once a week or so. One source of trouble was a fungus that attacked and killed several fish; however, by raising the salt content of the water rather slowly (about a week) with sodium chloride, it was possible to adapt the fish to a higher concentration of salt than the fungus could stand. (The proper dosage can be obtained from any book on gold-fish raising.) In spite of this, fish would die from time to time of other diseases, but the incidence was very low and could be neglected. As the water had to be kept well salted, it was not possible to have it circulate, compressed air being bubbled through the tanks instead. The water was changed about every two months, which seemed adequate to insure the health of the fish.

One more important precaution that had to be observed was to keep the tanks covered with chicken wire in order to prevent the fish from jumping out, which they often tried to do.

Bleeding: In bleeding carp, individuals averaging about one kg. in weight were conveniently used, although larger or smaller fish were also easily handled. The fish to be bled was netted and held ventral side up by one person, while the other made a heart puncture, entering just posterior to the pectoral girdle and on the mid-ventral line with a sharp twenty-two gauge needle on a five cc. syringe. Some practice was necessary, but after this it was possible to take as much as six cc. from a sigle fish without ill effects to it. The highest frequency with which fish were successfully bled can be seen in the paper on temperature effect.

Subsequently, it was found that the fish could be readily held in the wooden frame shown in the accompanying drawing, and therefore bled easily by one person.

No particular sterile precautions were found necessary to protect the fish from internal infections.

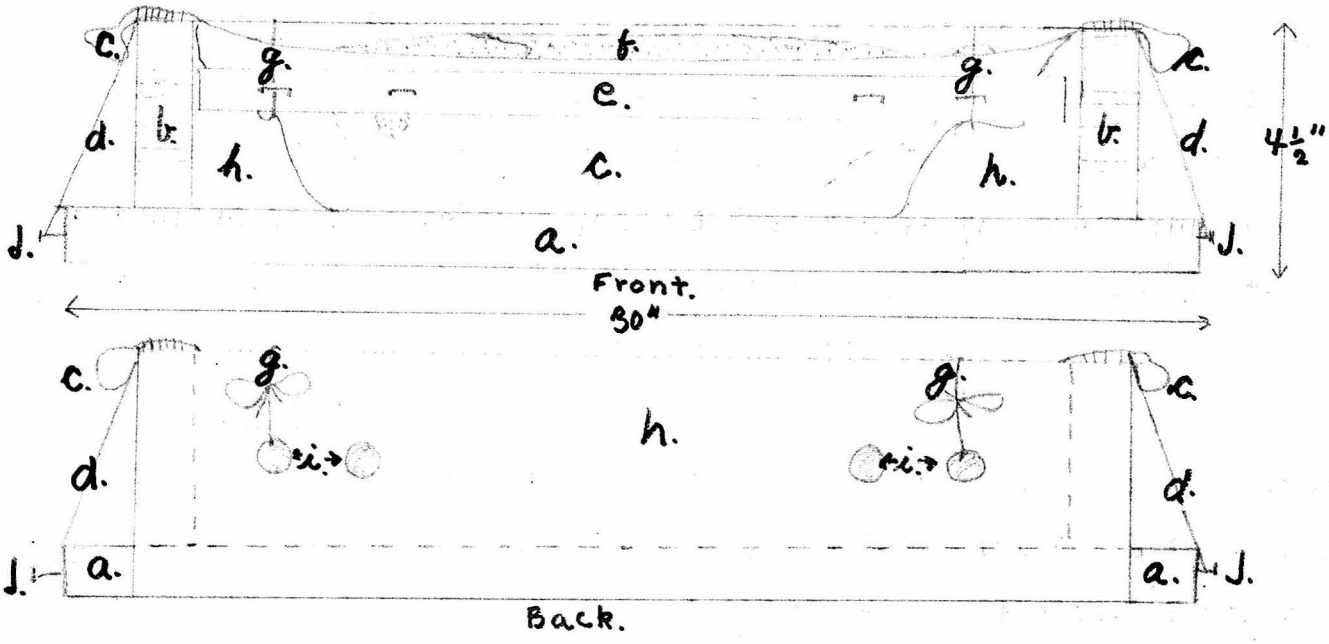
Carp serum was fractionated by the following techniques, modified from those used on the guinea pig.

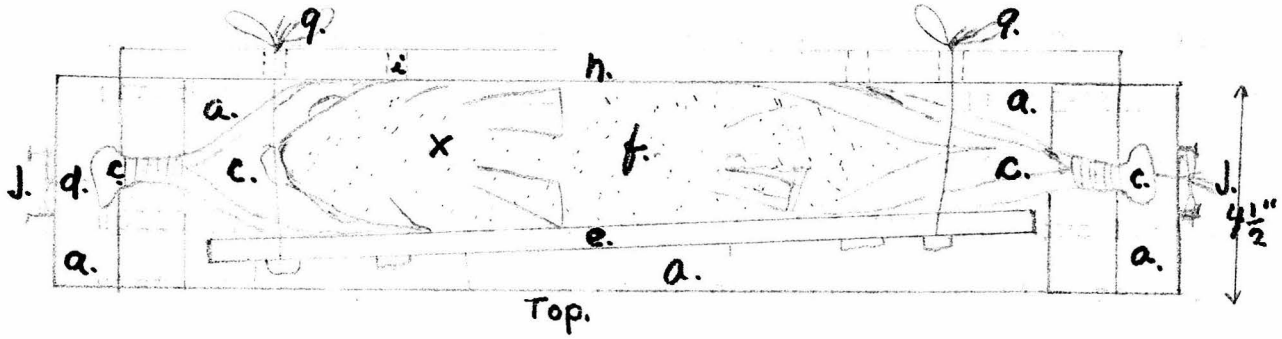
C'1 and C'2: It was not possible to split carp serum using the same carbon dioxide technique that was successful for the guinea pig and frog. A globulin precipitate was obtained by such treatment, but all the hemolytic activity of the serum remained in the supernatant. However, this does not necessarily mean that a slightly modified technique would

Table II.

A Wooden Frame for Holding Fish.

The following diagrams show the apparatus devised for holding fish while bleeding from the heart.





a; base.

b: side.

c: towel hammock.

d: string.

e: loose slat.

f: fish.

g: string.

h: backboard.

i: hole in backboard.

j: nails for securing hammock.

x: approximate location for heart puncture.

not split carp serum, as Liefman and Andrew (1911) have done in the case of the eel. Such variations in technique have not yet been tried.

C'3: Fish serum is very easily inactivated specifically with zymin by adding only .02 gms. to six cc. of serum and incubating for thirty minutes at room temperature. Serum so treated is then centrifuged and prepared as for the other two species. Care should be taken not to exceed the above treatment for non-specific inactivations will result.

C'4: Ammonia treated fish serum is specifically inactivated when the same proportions of serum and ammonium hydroxide are used as in the guinea pig, and this mixture is incubated in a closed tube for an hour at thirty-seven degrees. Further treatment follows the same course as for the two other species.

Heat inactivation: The optimum inactivation of fish serum by heating is brought about by keeping it for twenty-five minutes at fifty-three degrees. As this fraction can not be split, it can only be said that C'3 and C'4 are more heat resistant than at least one other constituent of fish serum.

Notes on Experimental Technique

General: The manner in which individual experiments were set up is best seen in the data sheets in the following section of the appendix. However, some points of general application may be given here. Five cc. test tubes were used in all experiments and, in all cases of reactivation, the sera involved were thoroughly mixed before the cells were added. All sheep cells were thoroughly washed and then mixed with about

two units of a pooled source of rabbit antibody that had a titer of 1/3000. The proportions of the two were so controlled that the final product was a two percent suspension of sensitized sheep cells which was then added to the sera to be tested in units of one tenth of a cc. per tube. All the rabbit cells used were thoroughly washed, diluted to two percent and added unsensitized to serum mixtures containing natural antibody against them. The differences in the way these two kinds of cells were sensitized may prove of interest in further work, as Hyde (1921) has shown that the order in which the various reagents are mixed in a tube can influence the result in some cases.

All estimations of hemolysis were made on the basis of four plus for complete lysis through three, two, and one plus to zero for no lysis at all, a method as satisfactory as any provided the proper precautions are used and that one draws conclusions from marked differences only.

Saline: While there is a difference in the salinity of carp, frog and guinea pig blood, it was found that nine-tenths percent saline was the most practical to use in the experiments. This is most nearly isotonic with the erythrocytes used as test antigen and preliminary results indicate that the use of this saline did not give results that differed from those where concentrations of salt more nearly isotonic with cold-blooded serum were employed.

However, it is important to note that while in general it was found that sodium chloride or filtered sea water, properly diluted, could be used interchangeably as a source of

saline, one exception was discovered.

This was that some element in sea water saline prevented the recombination of C'1 and C'2 in the guinea pig. What this element was has not been determined, but that it may have been the calcium ion is suggested by work reported in Osborn (1937). It is advisable, therefore, because of the possible complicating effects of the various ions in sea water, to use sodium chloride as the source of salt in complement splitting studies.

The hemolytic effect of the ammonia ion: It was found that .2 cc. of a one to two dilution of guinea pig or frog ammonia treated serum often had enough of this ion to completely hemolize the sheep cells being used as test antigen, while .1 cc. of the same serum did not have enough ion to produce any hemolysis. Some variations were noticed, but the effect was constant enough to establish the above concentrations as the critical point, the variations probably being due to incomplete inactivations or individual differences in cells and serum.

As is apparent from the data, this effect made it appear at first that frog and guinea pig sera which lacked fourth component were active when mixed. However, experiments with unsensitized cells and proper controls showed conclusively that this hemolysis was due to the action of the ammonia ion.

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Original Data on Cross- Activations, Sheets a to h.

The Cross- Activity of Frog and Guinea Pig Serum.

The following conditions obtain for all data included in the numbers given above:

Test Antigen: one tenth cc. of a two percent suspension of sheep erythrocytes sensitized with two units of rabbit anti-sheep serum as described under technique.

Aberviations: these pertain to sera that have been inactivated according to the methods described in the section on technique and are as follows:

M and E : soluable and insoluble fractions obtained by treating serum with carbon dioxide.

Z: serum that has been treated with zymin.

H ; serum that has been inactivated by heat.

A : serum that has been inactivated with ammonia.

g : shows that the fraction is from the guinea pig.

f : shows that the fraction is from the frog.

Double inactivations have letters for each treatment.

Mixtures of two homologous fractions are in parentesis, as follows : (M+E)

Degree of Hemolysis: is graded from 0 (none) to four plus (complete).

Volume of Tubes: all volumes equalized with saline to the amount indicated after the heading "total volume" on each sheet.

Final dilution of each fraction given on the separate sheets.

(Additional information will be found in section on technique.)

Sheet a.(Nov.26,1942.)

Conditions the same as for preceeding sheets.

Titer of frog serum —1/ 128 : of guinea pig 1/256.

Fractions and combinatons.

Species	F	F	F	F	F	F	F	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
Fraction.	Z	A	H	Z+A	Z+H	A+H		Z	A	H	Z+A	Z+H	A+H	Z+A	A+Z	Z+H	H+Z	A+H	H+A	Z+Z	A+A	H+H		
Amount of serum.cc.	.1	.1	.1	.1+.1	.1+.1	.1+.1		.1	.1	.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1		
Time in minutes.																(exchmage exhumed)								
10	0	0	0	±	+	+		0	0	0	|||	|||	|||	++	0	0	|||	0	0	|||	0	|||	0	0	|||	0
20	0	0	0	+	++	++		0	0	0	|||	|||	|||	|||	0	0	++	|||	0	0	|||	0	|||	0	0	|||	0
30	0	0	0	++	|||	++	0	0	0	|||	|||	|||	|||	0	0	|||	|||	0	0	|||	0	|||	0	0	|||	0
45	0	0	0	|||	|||	++	0	0	0	|||	|||	|||	|||	0	0	|||	|||	0	0	|||	0	|||	0	0	|||	0
60	0	±	0	|||	|||	++	+	0	0	|||	|||	|||	|||	++	++	|||	|||	0	+	|||	0	|||	0	0	|||	0
360	+	±	0	|||	|||	++	|||	0	0	|||	|||	|||	|||	|||	|||	|||	0	|||	|||	0

Sheet b.(Feb.25, 1943.)

Conditions the same as above, excepting as noted.

Sera of one frog and two guinea pigs used.

Fractions and combinations,one to two dilution.

Species.	F	F	F	F	F	F	F	F	F	G	G	G	G	G	G	G	G	G	F	G	F	G	F	G	F	G	F	G
Fraction.	Z	Z	A	A	H	H	Z+A	Z+H	A+H	Z	Z	A	A	H	H	Z+A	Z+H	A+H	Z+A	A+Z	Z+H	H+Z	A+H					
Amount.cc.	.2	.1	.2	.1	.2	.1	.1+.1	.1+.1	.1+.1	.2	.1	.2	.1	.2	.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1					
Time in minutes.																												
10	0	0	++	0	0	0	++	+	+	0	0	+	0	0	0	±	0	|||	0	0	0	0	0				
20	++	+	++	0	0	0	|||	|||	|||	0	0	+	0	0	0	++	0	|||	|||	0	|||	0	0				
30	|||	|||	|||	0	0	0	|||	|||	|||	0	0	+	+	0	0	|||	0	|||	|||	+	|||	0	0				
45	|||	|||	|||	0	0	0	|||	|||	|||	0	0	+	+	0	0	|||	++	|||	|||	+	|||	0	0				
60	|||	|||	|||	0	0	0	|||	|||	|||	0	0	+	+	0	0	|||	++	|||	|||	++	|||	+	0				
120	|||	|||	|||	0	0	0	|||	|||	|||	0	0	+	+	0	0	|||	++	|||	|||	++	|||	++	0				

Fractions and combinations,one to three dilution.

Species.	F	G	F	G	F	G	F	G		F	F	F	G	G	G	F	G	F	G	F	G	F	G	F	G	F	G	F	G
Fraction.	H+A		Z+Z		A+A		H+H		1/3	Z+A	Z+H	A+H	Z+A	Z+H	A+H	Z+A	A+Z	Z+H	H+Z	A+H	H+A	Z+Z	A+A	H+H						
Amount of serum.cc.	.1+.1		.1+.1		.1+.1		.1+.1		dilutions	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1						
Time in minutes.									start here →																				
10	0		0		|||	0			0	0	0	±	0	|||	0	0	0	0	0	0	0	±	0				
20	++		0						0			+	+	0	++	0					++	0	0	0	0	0	±	0				
30	++		+		++		0			|||	|||	0	|||	0	|||	|||	0	0	0	±	±	0				
45	++		++		++		0			||||	|||	+	|||	++	|||	|||	0	|||	0	0	±	++	±	0				
60	++		|||	++		0			|||	|||	++	|||	++	|||	|||	⊕	|||	0	0	±	|||	+	0				
120	|||	|||	++		0			×	×	×	×	×	×	|||	+	++	±	0	++	|||	+	0				

Sheet c. (Nov.24,1942)

(Temperature 37 degrees) All fractions diluted one to two.

All tubes volumes total three tenths cc. DHIjil

(Pooled serum) Titration of whole serum

	Frog								Guinea Pig							
Reciprocal of serum dilutions.	2	4	8	16	32	64	128	256	2	4	8	16	32	64	128	
Time in minutes. 10	++++	++++	±	0	0	0	0	0	++++	++++	++++	++++	++++	++++	++++	
20	++++	++++	+++	0	0	0	0	0								
30	++++	++++	++++	++++	0	0	0	0								
45	++++	++++	++++	++++	0	0	0	0								
60	++++	++++	++++	++++	±	0	0	0								
240	++++	++++	++++	++++	+++	++	++	0								

Fractions and Combinations.(One tenth cc. serum per fraction per tube)

Fractions.	F Z	F A	F H	F Z+A	F Z+H	F A+H	G Z	G A	G H	G Z+A	G Z+H	G A+H	G Z+A	G A+Z	G Z+H	G H+Z	G A+H	G H+A
Time in minutes. 10	0	0	++++	++	++++	++++	0	0	0	++++	++++	++++	+	0	++	0	++++	0
20	0	0	++++	++	++++	++++	0	0	0	++++	++++	++++	+++	0	++++	0	++++	0
30	0	0	++++	++++	++++	++++	0	0	0	++++	++++	++++	++++	++++	++++	+	++++	0
45	0	0	++++	++++	++++	++++	0	0	0	++++	++++	++++	++++	++++	++++	++	++++	0
60	0	0	++++	++++	++++	++++	0	0	±	++++	++++	++++	++++	++++	++++	++	++++	0
240	0	0	++++	++++	++++	++++	+	0	++	++++	++++	++++	++++	++++	++++	++	++++	0

Sheet d.(Nov.27,1942)

The same conditions as above.

Titration of whole serum.

	Frog								Guinea Pig							
Reciprocal of serum dilutions.	2	4	8	16	32	64	128	256	2	4	8	16	32	64	128	256
Time in minutes. 10	++++	++++	++++	++++	0	0	0	0	++++	++++	++++	++++	++++	++++	+++	0
20	++++	++++	++++	++++	0	0	0	0							++++	++
30	++++	++++	++++	++++	++	0	0	0								+++
45	++++	++++	++++	++++	+++	0	0	0								+++
60	++++	++++	++++	++++	++++	0	0	0								++++
240	++++	++++	++++	++++	++++	+++	0	0								++++

Fractions and Combinations.

Fractions.	F Z	F A	F H	F Z+A	F Z+H	F A+H	G Z	G A	G H	G Z+A	G Z+H	G A+H	G Z+A	G A+Z	G Z+H	G H+Z	G A+H	G H+A	G Z+Z	G A+A	G H+H
Time in minutes. 10	0	±	0	±	0	±	0	±	0	++++	++++	++++	++++	0	++++	0	++++	0	0	+++	0
20	0	±	0	±	0	+++	+	+	0				++++	±	++++	0	++++	0	+	++++	0
30	0	±	0	±	0	+++	+++	++	0				++++	±	++++	0	++++	0	++	++++	0
45	0	±	0	±	0	+++	+++	+++	0				+++	±	++++	0	++++	0	+++	++++	0
60	0	+	0	±	0	+++	++++	+++	0				++++	±	++++	0	++++	0	++++	++++	0
240	0	+	0	±	0	++++	++++	++++	0				++++	+	++++	0	++++	0	++++	++++	0

Sheet e.(DEC.10,1942)

Conditions the same as for preeeeding sheets excepting that here further inactivations(indicated by double letters) were carried out on the single,inactivated fractions.

Pooled sera from each species used and all fractions diluted one to two.

Fractions and combinations.

Species.	F	F	F	F	F	F	F	F	F	F	F	F	G
Fraetions.	Z	A	H	ZH	ZA	HA	Z+A	Z+H	A+H	ZH+A	ZA+H	HA+Z	Z
Amount of serum used.	.1cc	.1cc	.1	.1	.1	.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1
Time in minutes. 10	O	⊕	O	X	O	X	O	O	O	O	O	O	O
20	O	⊕	O	X	O	X	++	+	++	O	O	O	O
30	O	⊕	O	X	O	X	+++	+	++	O	O	O	+
45	O	⊕	O	X	O	X	+++	+++	+++	O	O	O	++
60	O	⊕	O	X	O	X	++++	+++	++++	O	O	O	+++
240	O	⊕	O	X	O	X	++++	+++	++++	±	O	+	++++

Species.	G	G	G	G	G	G	G	G	G	G	G	G F	G F
Fractions.	A	H	ZH	ZA	HA	Z+A	Z+H	A+H	ZH+A	ZA+H	HA+Z	Z+A	A+Z
Amount of serum used.	.1	.1	.1	.1	.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1
Time in minutes. 10	+	O	X	++	X	+++	+++	+++	+++	+++	+++	++	±
20	++	O	X	+++	X	+++	+++	+++	+++	+++	+++	+++	±
30	++	O	X	+++	X	+++	+++	+++	+++	+++	+++	+++	±
45	+++	O	X	+++	X	+++	+++	+++	+++	+++	+++	+++	±
60	+++	O	X	+++	X	++++	+++	+++	++++	+++	+++	+++	±
240	+++	O	X	+++	X	+++	+++	+++	+++	+++	+++	+++	±

Species.	G F	G F	G F	G F	G F	G F	G F	G F	G F	G F	G F	G F	G F
Fractions.	Z+H	H+Z	A+H	H+A	Z+Z	A+A	H+H	Z+HA	HA+Z	A+HA	HA+A	H+HA	HA+H
Amount of serum used.	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.H.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1
Time in minutes. 10	+++	O	++	O	O	+++	O	++	O	++++	O	O	O
20	++++	O	+++	O	O	+++	O	++++	O	++++	O	O	O
30	+++	O	+++	O	O	++++	O	+++	O	++++	O	O	O
45	+++	O	+++	O	O	+++	O	++++	O	++++	O	O	O
60	+++	O	++++	O	+	+++	O	+++	O	++++	O	O	O
240	+++	++	++++	O	++	+++	O	+++	++	+++	O	O	O

Species.	G F	G F	G F	G F	G F	G F	G F	G F	G F	G F	G F	G F
Fractions.	Z+ZH	ZH+Z	A+ZH	ZH+A	H+ZH	ZH+H	Z+ZA	ZA+Z	A+ZA	ZA+A	H+ZA	ZA+H
Amount of serum used.	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.H.1	.1+.1	.1+.1	.1+.1	.1+.1
Time in minutes. 10	+	O	+++	O	O	O	O	O	O	+++	O	++
20	++	O	+++	O	O	O	+++	O	++	++++	O	++++
30	+++	O	+++	O	O	O	+++	O	+++	++++	O	+++
45	+++	O	+++	O	O	O	+++	O	+++	++++	O	+++
60	++	O	+++	O	O	O	+++	O	+++	++++	O	+++
240	++++	O	++++	O	O	O	+++	++	++++	++++	O	++++

Sheet f.(Jan.4, 1943.)

Cross-activations between C'1 and C'2.

Dilution of fractions: frog and guinea pig: M 1/10,E 1/10.

Activity at thirty seven degrees.

Species.	F	F	F	F	G	G	G	G	G F	G F
Fraction.	normal 1/10	M	E	M+E	normal 1/10	M	E	M+E	M+E	E+M
Amount.	.2	.2	.2	.1+.1	.2	.2	.2	.1+.1	.1+.1	.1+.1
Time in 10	++++	0	0	++++	++++	0	0	++++	0	0
minutes. 20	++++	0	0	++++	++++	0	0	++++	++	0
30	+++	0	+	++++	++++	0	0	+++	+++	0
45	+++	0	+++	++++	+++	0	0	+++	+++	0
60	+++	0	+++	++++	+++	0	0	+++	+++	0

Activity at room temperature(twenty two degrees).

Species.	F	F	F	F	G	G	G	G	G F	G F
Fraction.	normal 1/10	M	E	M+E	normal 1/10	M	E	M+E	M+E	E+M
Amount.	.2	.2	.2	.1+.1	.2	.2	.2	.1+.1	.1+.1	.1+.1
Time in 10	++++	0	0	+++	++	0	0	±	0	0
Minutes. 20	+++	0	0	++++	++++	0	0	++	+	0
30	+++	0	0	++++	+++	0	0	++	++	0
45	+++	0	+	++++	++++	0	0	+++	++++	0
60	+++	0	++	++++	+++	0	0	++++	++++	0

Dilution of fractions; frog and guinea pig; M 1/5 ,E 1/5.

Activity at thirty seven degrees.

Species.	F	F	F	F	G	G	G	G	G F	G F
Fraction.	normal 1/5	M	E	M+E	normal 1/5	M	E	M+E	M+E	E+M
Amount.	.2	.2	.2	.1+.1	.2	.2	.2	.1+.1	.1+.1	.1+.1
Time in 10	x	0	+++	++++	x	0	0	++++	+	0
minutes. 20	x	0	++++	++++	x	0	0	++++	++	0
30	x	0	++++	++++	x	0	0	++++	+++	0
45	x	0	+++	++++	x	0	0	++++	+++	0
60	x	x	x	x	x	x	x	x	x	x

Activity at room temperature(twenty two degrees).

Species.	F	F	F	F	G	G	G	G	G F	G F
Fraction.	normal 1/5	M	E	M+E	normal 1/5	M	E	M+E	M+E	E+M
Amount.	.2	.2	.2	.1+.1	.2	.2	.2	.1+.1	.1+.1	.1+.1
Time in 10	x	0	+	++++	x	0	0	0	0	0
minutes. 20	x	0	++++	++++	x	0	0	0	++	0
30	x	0	++++	++++	x	0	0	0	++++	0
45	x	0	++++	++++	x	0	0	+	++++	0
60	x	x	x	x	x	x	x	x	x	x

Cross-activations involving C'1 andC'2.

Dilution of fractions: Frog ;M 1/10 ,E 1/10, Z 1/5,A 1/5, H1/5.
Guinea pig; M 1/10, E 1/10, Z 1/10,
A 1/10, H 1/10.

Some combinations of more than two fractions were made,the constitution of these is shownas in the following example: where frog M and E are shown as though added to guinea pig E : $(^{F}_{ME}) + ^{G}_{E}$
Total volume all tubes equals four tenths c.c.

Activations observed at thirty seven degrees.

Species.	F	F	F	F	F	F	F	F	F	F	F	F
Fractions.	A	Z	H	M	E	M+E	Z+E	Z+M	Z+A	Z+H	A+E	A+M
Amount.	.2	.2	.2	.2	.2	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1
Time in 10	0	0	0	0	0	0	0	0	0	0	0	0
miutes. 20	0	0	0	0	0	0	0	0	0	0	0	0
30	0	0	0	0	0	+	0	0	0	0	++	0
45	0	0	0	0	0	+++	0	0	0	0	+++	0
60	0	0	0	0	0	+++	0	0	0	0	++++	0
240	0	+++	0	0	0	+++	±	0	+	+	++++	±

Species.	F	F	F	G	G	G	G	G	G	G	G	G
Fractions.	A+H	H+M	H+E	A	Z	Z	H	M	E	M+E	Z+E	Z+M
Amount.	.1+.1	.1+.1	.1+.1	.2	.2	.1	.2	.2	.2	.1+.1	.1+.1	.1+.1
Time in 10	0	0	0	0	+	+	0	0	0	++	0	0
minutes. 20	0	0	0	±	+++	++	0	0	0	+++	±	+
30	0	±	0	++	+++	++	0	0	0	+++	+	++
45	++	++	0	+++	+++	++	0	0	0	++++	++	+++
60	+++	+++	0	+++	+++	+++	0	0	0	++++	++	++++
240	++++	+++	0	++++	++++	++++	0	0	0	++++	++++	++++

Species.	G	G	G	G	G	F G	F G	F G	F G	F G	F G	F G
Fractions.	Z+A	Z+H	A+E	A+M	A+H	A+(MH)	(MH)+A	(MH)+Z	(MH)+H	(MH)+M	(MH)+E	(MH)(EH)
Amounts.	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+(.1+.1)	.1+.1+.1	.1+.1+.1	.1+.1+.1	.1+.1+.1	.1+.1+.1	.1+.1+.1
Time in 10	+++	0	++++	0	++++	0	0	0	0	0	0	0
minutes. 20	+++	+++	++++	0	++++	0	++	++	++	0	0	0
30	+++	++++	++++	0	+++	0	+++	+++	+++	±	0	X
45	++++	+++	+++	0	+++	0	+++	+++	+++	+++	±	X
60	++++	++++	++++	0	++++	0	++++	++++	++++	++++	++	+++
240	++++	++++	++++	0	++++	0	++++	++++	++++	++++	++++	++++

Species.	F G	F G	F G	F G	F G	F G	F G	F G	F G	F G
Fractions.	(EH)(EH)	(EH)+A	(EH)+Z	(EH)+M	(EH)+E	A+(EH)	Z+(MH)	E+(MH)	Z+A	Z+H
Amounts.	.1+.1+.1	.1+.1+.1	.1+.1+.1	.1+.1+.1	.1+.1+.1	.1+(.1+.1)	.1+.1+.1	.1+.1+.1	.1+.1	.1+.1
Time in 10	0	0	0	0	0	0	0	0	0	0
minutes. 20	0	0	+	0	0	0	0	0	0	0
30	0	0	++	0	0	0	0	0	0	0
45	0	+	++	0	0	0	0	0	0	0
60	0	+	+++	0	0	0	0	0	±	±
240	0	++	++++	0	0	0	+	0	++++	++++

Cross-activations involving C'1 and C'2.

Activations observed at room temperature(twenty two degrees.)

Species.	F	F	F	F	F	F	F	F	F	F	F	F
Fractions.	A	Z	H	M	E	M+E	Z+E	Z+M	Z+A	Z+H	A+E	A+M
Amount.	.2	.2	.2	.2	.2	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1
Time in 10	0	0	0	0	0	0	0	0	0	0	0	0
minutes. 20	0	0	0	0	0	0	0	0	0	0	0	0
30	0	0	0	0	0	++	0	0	±	++	+++	0
45	0	0	0	0	0	+++	±	0	+++	++	+++	0
60	0	0	0	0	0	++++	++	0	++++	+++	+++	0
240	0	++	0	0	0	++++	++++	0	++++	++++	++++	H

Species.	F	F	F	G	G	G	G	G	G	G	G	G
Fractions.	A+H	H+M	H+E	A	Z	Z	H	M	E	M+E	Z+E	Z+M
Amount.	.1+.1	.1+.1	.1+.1	.2	.2	.1	.2	.2	.2	.1+.1	.1+.1	.1+.1
Time in 10	0	0	0	0	0	0	0	0	0	0	0	0
Minutes. 20	0	0	0	0	±	0	0	0	0	0	0	0
30	++	++	0	0	±	0	0	0	0	0	0	0
45	+++	+++	0	0	++	0	0	0	0	0	0	±
60	+++	+++	0	0	++	+	0	0	0	0	0	+
240	+++	+++	0	+	+++	+++	0	0	0	+	+++	+++

Species.	G	G	G	G	G	F	G	F	G	F	G	F	G	F	G	F	G
Fractions.	Z+A	Z+H	A+E	A+M	A+H	A+(MH)	(MH)+A	(MH)+Z	(MH)+H	(MH)+M	(MH)+E	(MH)+(EH)					
Amount.	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1					
Time in 10	0	0	0	0	0	0	0	0	0	0	0	0					
minutes. 20	0	0	0	0	0	0	++	0	+++	0	0	0					
30	+	0	0	0	0	0	++++	0	++++	±	0	x					
45	+++	0	0	0	0	0	+++	+	++++	+++	+++	+++					
60	+++	0	0	0	±	0	+++	++	+++	+++	+++	+++					
240	+++	x	+++	0	+	0	+++	+++	+++	+++	+++	+++					

Species.	F	G	F	G	F	G	F	G	F	G	F	G	F	G	F	G	F	G		
Fractions.	(EH)+(EH)		(EH)+A		(EH)+Z		(EH)+M		(EH)+E		A+(EH)		Z+(MH)		E+(MH)		Z+A		Z+H	
Amount.	.1+.1		.1+.1		.1+.1		.1+.1		.1+.1		.1+.1		.1+.1		.1+.1		.1+.1		.1+.1	
Time in 10	0		0		0		0		0		0		0		0		0		0	
minutes. 20	0		0		0		0		0		0		0		0		0		0	
30	0		0		0		0		0		0		0		0		0		0	
45	0		0		0		0		0		0		0		0		0		0	
60	0		0		0		0		0		0		0		0		0		0	
240	0		0		0		0		0		0		+++		0		+++		+++	

Sheet i.(Feb.20,1943).

Cross-activity between Carp and Guinea Pig Sera.

Abbreviations the same as for sheet a-h, C standing for carp fractions, G for guinea pig.

One tenth c.c. of a two percent suspension either of unsensitized rabbit cells or of sheep cells sensitized with rabbit antibody used for test antigen.

All fractions diluted one to three before testing.

Experiment made at thirty seven degrees.

Rabbit cells(unsensitized).

Species.	Titer of natural antibody.	G	G	C	C	C	C	C	C	C	G	G	G	C	G
Fraction.		Normal	Normal	Z	A	H	Z+A	Z+H	A+H	Z+A		A+Z		Z+H	
Amount.		1/3	1/6	.2	.2	.2	.1+.1	.1+.1	.1+.1	.1+.1		.1+.1		.1+.1	
Time in 10		++++	0	0	0	0	++++	0	++++	0		0		0	
minutes. 20		++++	0	0	0	0	++++	0	++++	0		0		0	
30		++++	0	0	0	0	++++	0	++++	0		0		0	
45		++++	0	0	0	0	++++	0	++++	0		0		0	
60		++++	0	0	0	0	++++	0	++++	0		0		0	

Species.	C	G	C	G	C	G	C	G	G	G	C	G	Whole guinea pig serum(1/6), added to carp Z, A, H.	C	C	C		
Fraction.	H+Z		A+H		H+A		Z+Z		A+A		H+H			Z	A	H		
Amount.	.1+.1		.1+.1		.1+.1		.1+.1		.1+.1		.1+.1			.1+.1	.1+.1	.1+.1		
Time in 10	0		0		0		0		0		0			++++	++++	0		
minutes. 20	0		0		0		0		0		0			++++	++++	0		
30	0		0		0		0		0		0			++++	++++	0		
45	0		0		0		0		0		0			++++	++++	0		
60	0		0		0		0		0		0			++++	++++	0		

Sheep cells (sensitized).

Species.	Titer of natural antibody.	C	C	G	G	G	G	G	G	G	G	C	G
Fractin.		Normal	Normal	Z	Z	A	A	H	Z+A	Z+H	H+A	Z+A	
Amount.		1/3	1/6	.2	.1	.2	.1	.2	.1+.1	.1+.1	.1+.1	.1+.1	
Time in 10		++++	0	+	++	+++	±	0	++++	++++	++++	+	
minutes. 20		++++	0	+++	+++	+++	±	0	++++	++++	++++	+	
30		++++	0	++++	++++	+++	±	0	++++	++++	++++	+	
45		++++	0	++++	++++	+++	±	0	++++	++++	++++	+	
60		++++	0	++++	++++	×	×	0	++++	++++	++++	+	

Species.	G	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	Whole carp serum (1/6), added to g.pig Z, A, H.	G	G	G
Fraction.	A+Z		Z+H		H+Z		A+H		H+A		Z+Z		A+A		H+H			Z	A	H
Amount.	.1+.1		.1+.1		.1+.1		.1+.1		.1+.1		.1+.1		.1+.1		.1+.1			.1+.1	.1+.1	.1+.1
Time in 10	++++		0		++++		0		+		++		+++		0			0	0	0
minutes. 20	++++		0		++++		0		++		+++		+++		0			0	0	0
30	++++		0		++++		0		++		+++		+++		0			0	0	0
45	++++		0		++++		0		++		+++		+++		0			0	0	0
60	++++		0		++++		0		×		+++		×		0			0	0	0

Sheet j .(Oct.10,1942)

Cross-activity between Carp and Guinea Pig Sera.

Conditions the same as for sheet i, excepting that all fractions were diluted one to two before using.

Rabbit cells(unsensitized)

Species.	G	G	C	C	C	C	C	C	C G	C G	C G
Fraction.	½ Normal	¼	Z	A	H	Z+A	Z+H	A+H	Z+A	A+Z	Z+H
Amount.			.1	.1	.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1
Time in 15	±	0	0	0	0	++++	0	++++	0	0	0
minutes.30	±	0	0	0	0	++++	±	++++	0	0	0
45	±	0	0	0	0	++++	±	++++	0	0	0

Titer of natural antibody

Species.	C G	C G	C G	C	C	C
Fraction.	H+Z	A+H	H+A	Z	A	H
Amount.	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1
Time in 15	0	0	0	++++	++++	++++
minutes.30	0	0	0	++++	++++	++++
45	0	0	0	++++	++++	++++

Whole guinea(¼) pig serum added to carp A.Z.H

Note: ¼ quinea pig was not tested for natural antibody.

Sheep cells(sensitized)

Species.	C	C	G	G	G	G	G	G	C G	C G	C G
Fraction.	normal	normal	Z	A	H	Z+A	Z+H	A+H	Z+A	A+Z	Z+H
Amount.	½	⅓	.1	.1	.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1
Time in 15	±	0	0	+	0	++++	++++	++++	++++	0	0
minutes.30	++	0	+	++	0	++++	++++	++++	++++	++	0
45	++	0	+	++	0	++++	++++	++++	++++	++	0

Titer of natural anti-body.

Species.	C G	C G	G G	G	G	G
Fraction	H+Z	A+H	H+A	Z	A	H
Amount.	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1
Time in 15	0	0	++	+	++	0
minutes.30	+	0	++	++	+++	0
45	+++	0	+++	++	++++	0

Whole carp serum added to g. pig. ZAH.

Sheet 4.(Oct.2o,1942).

Cross-activity between Carp and Guinea Pig Sera.

Conditions the same as for sheet , excepting that all fractions were diluted one to two before using.

Rabbit cells(unsensitized)

Species.		G	G	C	C	C	C	C	C	C G	C G	C G
Fraction.		normal	normal	Z	A	H	Z+A	Z+H	A+H	Z+A	A+Z	Z+H
Amount.		1/2	1/4	.1	.1	.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1
Time in 15	Titer of natural antibody	0	0	0	0	0	++++	++++	++++	0	0	0
minutes.30		0	0	0	0	0	++++	++++	++++	0	0	0
45		±	0	0	0	0	++++	++++	++++	0	0	0

Species.	C G	C G	C G	whole guinea pig(1/4) added to carp.	C	C	C
Fractions.	H+2	A+H	H+A		Z	A	H
Amount.	.1+.1	.1+.1	.1+.1		.1+.1	.1+.1	.1+.1
Time in 15	0	0	0		++++	+++	++
minutes.30	0	0	0		x	x	x
45	0	0	0		++++	++++	++++

Sheep cells(sensitized)

Species.		C	C	G	G	G	G	G	G	C G	C G	C G
Fraction.		normal	normal	Z	A	H	Z+A	Z+H	A+H	Z+A	A+Z	Z+H
Amount.		1/1	1/2	.1	.1	.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1	.1+.1
Time in 15	Titer of natural antibody	±	0	+	0	0	++++	++++	++++	+++	++++	0
minutes.30		++	+	+++	+++	0	++++	++++	++++	++++	++++	0
45		++	+	++++	+++	0	++++	++++	++++	++++	++++	0

Species.	C G	G G	C G	whole carp(1/4) added to guinea pig	G	G	G
Fraction.	H+2	A+H	H+A		Z	A	H
Amount.	.1+.1	.1+.1	.1+.1		.1+.1	.1+.1	.1+.1
Time in 15	++++	0	++++		++++	+++	0
minutes.30	++++	0	++++		++++	++++	0
45	++++	0	++++		++++	++++	0

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AN EFFECT OF TEMPERATURE UPON ANTIBODY-PRODUCTION IN FISH

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A recent paper by Ellingson and Clark (1) on the effects of artificially induced fevers upon the production of antibodies in rabbits refers to a temperature experiment by Allen and McDaniel (2) on frogs, showing that lysins against human red cells could be developed at room temperature, but not at a temperature of 8 to 10 C. These results suggest, in common with those of Wollman (3) and Widal and Sicard (4) on frogs, and of Nybelin (5) and Pliszka (6) on fish, that antibodies are not formed by animals living at 10 C. That this is not necessarily the case, however, has been demonstrated recently by W. W. Smith (7) whose work indicates that carp and trout held at this temperature are capable of forming agglutinins against *Bacterium salmonicida*, provided the antigen is given in sufficient quantity, and the animals are kept for a long enough time. As all these experiments indicate an effect of temperature upon the rate of antibody-formation and as only one previous paper is known to the author that deals specifically with this point, the following experiment, carried out in relation to other serological studies now in progress, may be of interest in that it shows such an effect. The previous paper is that by Widal and Sicard (4) which suggests that antibodies against typhoid bacteria were formed more rapidly by frogs living in higher than in lower temperatures.

For the present experiment, two kinds of closely related fish were used; carp (*Cyprenius carpio*) from one of the fresh water lakes of California purchased from a dealer, and "feral" gold fish (*Carassius auratus* var. fan tail) obtained through the courtesy of Mr. D. A. Clanton, Superintendent Southern California Hatcheries of the State Division of Fish and Game, from Crystal Lake, Los Angeles, Cal. The author would like to express his indebtedness to the California Division of Fish and Game, and especially to Mr. Clanton for help in getting these fish and advice on handling them. The fish were kept in two constant-temperature tanks, one held at approximately 15 degrees and the other at 28 degrees; four carp and four gold fish, averaging respectively about one kg and 460 g in weight and individually distinguishable, being put in each tank. After an acclimatization period of a few days each fish was bled and then, five days later, injected intraabdominally with five ml of sea urchin (*Strongylocentrotus purpuratus*) sperm suspension (one part of "dry" sperm to two parts of sea water, see Tyler (8) on the use of this sperm for antigenic purposes). The fish were bled three days after the injection and then at intervals of every four days (unless otherwise indicated on the graphs) until the experiments were terminated almost a month later. The blood samples were obtained from the heart by

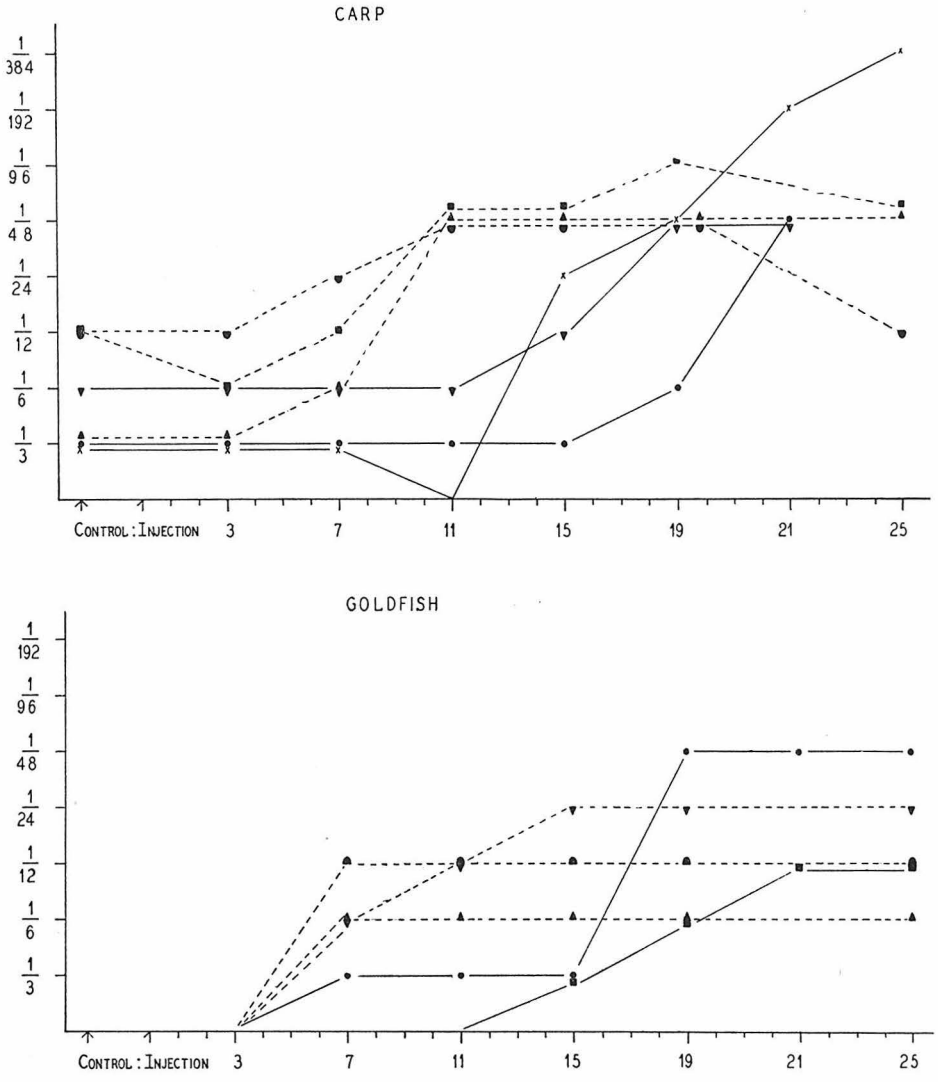
means of a 22-gauge hypodermic needle attached to a 5 ml syringe, 3 to 4 ml being taken at a time. This bleeding was done from the ventral side, one person holding the fish and another operating the syringe. (Subsequently it has been found possible to hold the fish in a wooden frame with equally successful results.) The operation did not appear to bother the fish in any way except in rare instances when they would lie on their sides or swim abnormally for a short time after being released. One possible exception was the death of two of the carp in the cold tank, a day or so after their seventh bleeding; however, it is more probable that this was due to disease as a few other fish were later lost in this way.

The whole blood obtained was centrifuged shortly after being taken, the separated serum being pipetted off and stored in an ice-box until wanted for testing. The tests were run after the bleedings were all completed, 4 drops of fresh "dry" sperm diluted in 10 ml of sea water being used as the test antigen. All sera of a given fish were tested at the same time with the same fresh motile antigen preparation in order to insure uniformity of relative reactivities within the group.

The results of the experiments are presented in the accompanying graphs from which it can be seen that in the fish kept in the warm tank a rise in antibody-titer occurred within eleven days, while in those kept in the colder water such a rise was not observed until fifteen days after injection. (The five fish not recorded showed no change in antibody-titer during the experiment.) In addition to the induced sperm agglutinins, two other types of agglutinins were found. These were natural agglutinins against sperm of relatively low titer, occurring only in the carp and an unknown factor, probably associated with the antigen that caused a rather weak clumping of a few of the sperm in most of the preparations irrespective of the presence of the other two antibodies. These three types of agglutinins were not sharply separable from each other, which necessitated a comparison of relative, rather than absolute titer differences, adequate serum and antigen controls, of course, being included in such comparisons. However, in spite of this, it can be seen that sufficiently marked differences exist among the sera of various fish to show a definite temperature effect.

The way in which temperature acts upon antibody-production cannot, of course, be determined from the above data. However, it should be noted that the fish in these experiments were injected intraabdominally as were the frogs studied by Allen and McDaniel, the carp of Pliszka, and the fish of W. W. Smith. With this the case, it is possible that the observed action of temperature upon the formation of antibodies could in large part be due to an effect upon the relative rate of the entrance of the antigen into the circulation, rather than to a direct effect upon the mechanism of antibody-production itself. Further information will be necessary to clarify this point.

In concluding, the author would like to thank Dr. Sterling Emerson for his advice and generous help throughout the experiment.



Graphs showing the change in titer of sperm agglutinins in goldfish and carp sera following a single injection with *Strongylocentrotus purpuratus sperm*. Broken lines indicate fish kept at 28 C; solid lines those kept at 14 C. Of the original sixteen fish injected, only those that showed any change in titer are recorded. Numbers along the abscissa indicate number of days after injection on which successive bleedings were made, those along the ordinate successive serum dilutions.

SUMMARY

A study was made upon the rate of production of agglutinins against sea urchin sperm in carp and gold fish held at two different temperatures (15 and 28 degrees). The fish, four of each kind at each temperature, were bled every

few days from the heart and agglutinative tests made on their sera. These showed that the antibody-titer of the fish kept in the warm tank rose at least four days sooner than that of those kept in the cold tank. This indicates, in agreement with other work, that temperature does influence the rate of antibody-production in cold-blooded vertebrates.

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