THE RÔLE OF THE COMPONENTS OF COMPLEMENT IN SPECIFIC IMMUNE FIXATION*

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Although it is known that complement combines with most antigen-antibody compounds, the rôle of the components of complement in this union is not clearly understood.

It is generally stated that the mid-piece of complement is the combining component (1, 2). On the other hand, Brin states that the end-piece alone is fixed (3). Others assert that both the end-piece and mid-piece combine with antigen-antibody compounds (4–7). Deissler (8) contends that the fourth component of complement is utilized in complement fixation. Some workers claim to have confirmed this statement (9, 10), while others contradict it (11).

The present authors, in experiments preliminary to those reported in this paper, found indications that the fourth component and end-piece, as well as mid-piece in a smaller proportion, combine with antigen-antibody compounds.

There are several reasons for the various views of others. (1) With the exception of a few recent studies, investigations on the rôle of the complement components in fixation were performed before the discovery of the fourth component of complement. (2) Uniform methods for the preparation and identification of the individual complement components were not employed. (3) Much of the past work was qualitative in nature with little consideration of the physical and chemical conditions which affect the combination of complement with antigen-antibody aggregates. (4) The results obtained under one set of experimental conditions were frequently interpreted to fit all other conditions.

The need for more rigidly controlled studies on complement fixation is evident; and accordingly the present paper is concerned first with the conditions governing the fixation of the components of complement; and second with the identification of those components of complement which combine with specific antigen-antibody compounds.

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Résumé of the Nature of the Components of Complement

To assist in the interpretation of the data presented in this paper, a review of the existing knowledge of the constitution of complement is necessary. A more detailed discussion has been presented elsewhere (12).

Complement can be separated into two thermolabile components (destroyed by heating at 56° for 30 minutes) by treatment of serum with carbon dioxide-saturated water or dilute HCl, or by dialysis against distilled water (1). Accompanying each of these fractions are varying amounts of two relatively thermostabile components of complement, the third and fourth components. The fraction of fresh serum insoluble in a tenfold dilution of carbon dioxide-saturated water has been termed the "globulin fraction" or the "mid-piece," while the fraction soluble in the same medium has been termed the "albumin fraction" or "end-piece." The terms mid- and end-piece were formulated by the early investigators because they supposed that the carbon dioxide-insoluble fraction had first to combine with the sensitized red blood cells before the albumin fraction would act. It is also known that either cobra venom or yeast cells destroys a relatively heat-stable component (inactivated by heating at 66°C. for 30 minutes) which has been termed the third component (1). It is usually associated with the globulin fraction of serum, although, as will be shown below, it is also nearly always present in the end-piece. Pillemer and Ecker (13), investigating the nature of the yeast inactivation of the third component, isolated an insoluble fraction from yeast which adsorbs the third component specifically from fresh serum. Certain unpublished data are indicative of a lipid nature of this component (14). A fourth component of complement, inactivated by dilute ammonia, has also been discovered (1). It is usually associated with the end-piece, but, as will be shown below, under certain experimental conditions it also is often present in the mid-piece fraction of complement. Pillemer, Seifter, and Ecker (15) postulated that this component contains a reactive carbonyl group and is associated with a globulin fraction present in the so called albumin fraction.

Pillemer, Ecker, Oncley, and Cohn (16) have prepared in a highly pure state, and characterized, three of the components of complement. The mid-piece was found to be a euglobulin with an apparent isoelectric point of about 5.2. It has an electrophoretic mobility of 2.9×10^{-5} in a phosphate buffer of an ionic strength of 0.20 at pH 7.7; and a sedimentation constant of 6.4×10^{-13} in KCl of ionic strength of 0.20. It comprises only 0.60 per cent of the total serum protein; and each cubic centimeter of serum contains 0.060 mg. of mid-piece nitrogen. The end-piece and fourth component were found in the same serum fraction, which was characterized as a muco-euglobulin with an apparent isoelectric point of 6.3–6.4, and with an electrophoretic mobility of 4.2×10^{-5}. It contains 10.3 per cent carbohydrate; and it comprises less than 0.2 per cent of the total serum protein, thus accounting for 0.02 mg. of nitrogen per cc. of serum.

Pillemer and Ecker (17) on the basis of electrophoretic and functional studies, and Heidelberger (18, 22) have proposed a new nomenclature for the complement components. The symbols C′1, C′2, C′3, and C′4, which correspond to the mid-piece, end-piece, third component, and fourth component respectively in the older termi-

nology, were adopted. In the present paper the terms mid-piece and CO_2-insoluble fraction are used interchangeably, as are end-piece and CO_2-soluble fraction; and the symbols C'1 and C'2 represent the mid-piece and end-piece *components per se*. The terms third and fourth components are used interchangeably with the symbols C'3 and C'4 respectively.

Materials

Complement.—The blood of normal guinea pigs which had been maintained on a high vitamin C diet was placed in the ice box for about one hour. At the end of this time the serum was separated by centrifugation and immediately used in the experiments. In the protocols which follow, the serum, unless otherwise noted, was never allowed to stand more than one hour before use in fixation experiments.

Antigens.—Type III pneumococcus specific carbohydrate was used in the larger part of the experimental work. A stock solution of 0.1 per cent SIII[1] in 0.9 per cent saline was diluted with 0.9 per cent saline to the desired concentration.

Human serum, hemocyanin (from *Busycon canaliculata*), tobacco mosaic virus, and red blood cells of various species were also employed as antigens.[2] The amounts employed are indicated in the protocols.

Antisera.—A refined anti-Type III pneumococcus rabbit serum containing 4.57 mg. of antibody nitrogen per cc. was kindly furnished by the Lederle Laboratories, Inc. It was not anticomplementary in twice the quantities employed in the present experiments.

Antihuman rabbit serum, antihemocyanin rabbit serum, antitobacco mosaic virus (rabbit) serum, and rabbit antisera to red blood cells of sheep, dog, guinea pig, and man were also employed. Since the latter sera were found to be somewhat anticomplementary in the amounts necessary for optimum fixation, the procedure advocated by Sachs (19) was employed to remove the objectionable substances from them. This was successfully accomplished without much loss of the fixative powers of the antisera.

Chemicals.—All chemicals used were either best grade Eastman or Merck's blue label chemicals. Unless otherwise noted, all dilutions were made with 0.9 per cent NaCl. The insoluble carbohydrate from yeast used in the removal of C'3 was prepared by the method of Pillemer and Ecker (13).

Methods

General Method for Fixation.—To A cc. of antiserum, B cc. of undiluted fresh serum were added with thorough mixing. The mixture was then brought to the desired temperature, tonicity, and hydrogen ion concentration. C cc. of antigen, adjusted to the desired experimental conditions, were then added rapidly with constant stirring.

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[1] S with the appropriate numeral is used for the type-specific polysaccharide of the pneumococcus.

[2] We are indebted to Dr. M. Heidelberger for a generous supply of anti-Type III pneumococcus rabbit serum; to Dr. S. B. Hooker for hemocyanin and antihemocyanin rabbit serum; and to Dr. W. M. Stanley for crystalline tobacco mosaic virus.

The mixture was allowed to stand for the prescribed time at the required temperature. Following this incubation the mixture was centrifuged for 30 minutes at 2750 R.P.M.; and the clear supernatant was drawn off carefully with a pipette, adjusted to the required conditions, and finally made up with 0.9 per cent NaCl to a 10 per cent dilution of the original guinea pig serum. This solution was tested for hemolytic activity, and if found not to show more than 10 per cent of its original activity it was further tested for content of individual complement components.

Complement Titration.—A unit of complement, designated as producing 100 per cent hemolysis, was considered to be the smallest amount of a 10 per cent dilution of untreated serum which caused complete hemolysis of 1 cc. of 2.5 per cent suspension of sheep red blood corpuscles containing 5 units of antisheep cell rabbit serum per cc. An identical amount was employed in all reactivations and complement component determinations.

Estimation of the Individual Component Activity in Fixed Complements.—A unit of the complement rendered inactive by fixation was added to an equal amount of each of the specifically inactivated complement components described below. The mixture was incubated at room temperature for 15 minutes, 1 cc. of sensitized sheep red cell suspension added, and the hemolytic titer read after 30 minutes of incubation at 37.5°C. The amount of hemolysis produced was estimated by comparison with a series of standard hemoglobin solutions, and expressed as percentage of complete hemolysis.

It is pointed out that the data presented in this paper do not indicate the absolute quantity of each component present, but only represent the residual activity present after fixation or specific inactivation.

For the further clarification of the data, the per cent hemolysis resulting from the inter-reactivations of the "specifically inactivated complements" (see below) is included in each protocol.

Success in the reactivations depends largely on preventing the loss of activity of the complement components which might occur during the course of an experiment. Therefore, all serum reagents should be kept at 0°C. (±1°) until they are to be used. Precautions of this kind are necessary for obtaining comparable, reproducible data. Furthermore, slight deviations from any technique described here may alter the end-result of an experiment.

In the interpretation of data on hemolysis no significance is attached to differences of 10 per cent hemolysis or less, because of the proscribed limit of accuracy of the method.

The Preparation of Complements Deprived of Various Components

These are hereafter designated "specifically inactivated complements."

1. Complement was deprived of active C'4 by appropriate treatment of serum with hydrazine or ammonia (15, 20).

2. Complement was deprived of C'3 by treatment of serum with zymin or the insoluble carbohydrate prepared from fresh yeast (13).

3. Complement was deprived of C'1 and C'2 activity by heating serum at 56°C. for 30 minutes.

4. The CO_2-soluble and CO_2-insoluble fractions were prepared by saturation of diluted serum with CO_2 as described elsewhere (12). While this method results in the sharp separation of C'1 and C'2 from one another, marked variation occurs in the distribution of C'3 and C'4 in the fractions. The CO_2-soluble fraction or end-piece, contains all of the active C'2, usually from 70 to 100 per cent of the C'4, and from 20 to 40 per cent of the C'3. The CO_2-insoluble fraction, or mid-piece, contains all of the C'1, usually about 60 to 80 per cent of C'3, and from 0 to 30 per cent of the C'4. In this laboratory it is believed that the residual C'4 manifest in the mid-piece is accompanied by a small amount of C'2, since it is the opinion here that C'2 and C'4 are associated in the same guinea pig serum protein constituent.

EXPERIMENTAL

Factors Which Influence the Fixation of the Components of Complement.—In these experiments complement fixation was carried out with anti-Type III pneumococcus rabbit serum compounds.

1. The Effect of Aging of Complement.—

A pool of fresh guinea pig serum was divided into two portions, one of which was allowed to incubate for one hour with the specific antipneumococcus complexes, at the end of which time the fixability of the complement components was determined. The second portion of serum was allowed to stand for 19 hours in the ice box (5°C.) and was tested in a like manner. The serum lost 50 per cent of its complementary activity during the 19 hours in the cold. Complete hemolysis was brought about by 0.07 cc. of 10 per cent dilution of fresh serum, while 0.14 cc. of 10 per cent dilution of aged serum was required.

The results of this experiment, performed in quintuplicate, are tabulated in Table I. An inspection of the data shows that when serum stood for 19 hours and lost 50 per cent of its complementary activity the following changes in the fixability of the complement components occurred: (*a*) C'4 became more resistant to fixation; (*b*) C'3 underwent no change in fixability; (*c*) there was less reactivation upon the addition of the CO_2-insoluble fraction, and increased reactivation upon the addition of the CO_2-soluble fraction in the case of the aged serum.

Most investigators have allowed complement to age or "cure" in an ice chest overnight before using it in fixation experiments (18, 21); however, it is apparent from the data presented here that this procedure may lead to serious error in the determination of the fixability of the various components of complement. In this laboratory, therefore, it is the practice to allow serum complement to stand not more than one hour before use in fixation experiments.

2. The Effect of Time of Incubation.—An experiment was performed to determine the optimum time necessary for the fixation of the various components of complement.

The reagents were mixed as described in the section of this paper dealing with the general method of fixation, and were allowed to stand at 25°C. for different periods of time. At the end of the allotted time the residual complement component activities were determined. In order to avoid occlusions and uneven distribution of reagents, separate tubes were employed for each incubation.

TABLE I

The Effect of Aging on the Fixation of Complement

1:8 pneumococcic antiserum	Guinea pig serum	1:40,000 SIII	Hemolysis produced by supernatant after fixation		Hemolysis produced after addition of specifically inactivated complements									
					Serum treated with hydrazine		Serum treated with insoluble carbohydrate		Serum heated at 56°C. for 30 mins.		CO_2-insoluble fraction ("mid-piece")		CO_2-soluble fraction ("end-piece")	
			I*	II‡	I	II	I	II	I	II	I	II	I	II
cc.	*cc.*	*cc.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0.2	1	0.2	0	0	0	25	50	55	10	5	65	40	20	35
0.2	1	0.2	0	0	0	25	60	60	Tr.	5	65	40	15	30
0.2	1	0.2	0	0	0	25	50	60	10	Tr.	65	40	15	25
0.2	1	0.2	0	0	0	25	50	60	10	Tr.	65	40	15	25
0.2	1	0.2	0	0	0	30	60	50	15	Tr.	65	45	15	30

Specifically inactivated complements	Hemolysis											
	I	II										
	per cent	*per cent*										
Serum treated with hydrazine	0	0	—	—	80	60	80	90	40	60	90	90
Serum treated with insoluble carbohydrate	0	0	80	60	—	—	60	35	60	60	40	25
Serum heated at 56°C. for 30 mins.	0	0	80	90	60	35	—	—	0	0	0	0
"End-piece"	0	0	90	90	40	25	0	0	85	90	—	—
"Mid-piece"	0	0	60	40	60	50	0	0	—	—	85	90

* I, fresh sera.

‡ II, sera after standing 19 hours at 5°C.

The results of this experiment show that C'4 was fixed in the first 5 minutes of incubation and remained fixed for the duration of the 80 minute incubation. Maximum fixation of mid-piece occurred after 40 minutes of incubation; while little or no variation in the fixation of end-piece occurred that could be attributed to the effect of time of incubation. On the basis of these data an incubation time of 60 minutes was chosen as the optimum time to be used in the remaining fixation experiments.

3. The Effect of Temperature.—Although earlier workers (23, 24) determined the optimum temperature for the fixation of complement (considered as a single entity), no knowledge exists concerning the influence of temperature on the fixation of the individual components of complement. The results of an experiment performed to determine the effect of temperature on the fixation of the individual components reveal that maximum fixation of all the com-

TABLE II

The Effect of Varying Hydrogen Ion Concentration on the Fixation of Complement

pH*	Relative quantity and appearance of precipitate	Hemolysis produced by supernatant after fixation	Hemolysis produced after addition of specifically inactivated complements				
			Serum treated with hydrazine	Serum treated with zymin	Serum heated at 56°C. for 30 mins.	CO_2-soluble fraction ("end-piece")	CO_2-insoluble fraction ("mid-piece")
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
5.3	++++ plaque	0	15	15	20	50	0
6.1	+++ plaque	0	15	20	20	45	0
7.0	++ granular	0	0	60	0	35	0
7.1	++ granular	0	0	40	0	15	0
8.1	++ granular	0	0	50	0	25	0
8.8	++ plaque	0	0	50	0	25	0

Specifically inactivated complements	Hemolysis					
	per cent					
Serum treated with hydrazine..	0	—	70	90	90	0
Serum treated with zymin.....	0	70	—	80	25	60
Serum heated at 56°C. for 30 mins.....	0	90	80	—	0	0
"End-piece".....	0	90	25	0	—	85
"Mid-piece".....	0	0	60	0	85	—

* 1 cc. of guinea pig serum was fixed by the aggregate from 0.2 cc. of 1:4 antipneumococcus rabbit serum and 0.2 cc. of 1:10,000 SIII. All reagents were made up to volume with phosphate buffers of ionic strength 0.15. The pH indicated here is the pH of the supernatants after fixation.

ponents occurred at 22°C. Although slight differences in the fixability of the components were evident at 0° and 22°, marked differences occurred as between these temperatures and 37°C. At the latter temperature C'4 was more resistant to fixation. On the basis of these data further fixation experiments were conducted at temperatures between 20 and 25°C.

4. The Effect of Hydrogen Ion Concentration.—The results of an experiment to determine the effect of hydrogen ion concentration on the fixation of the individual complement components are shown in Table II.

In this experiment all reagents were either diluted or made up to volume with phosphate buffers of various hydrogen ion concentrations and of ionic strength 0.15. The complement was incubated with the antigen-antibody complexes for one hour at a temperature of 23°C. Before determination of their complement component activities the supernatants were adjusted to a neutral pH with 0.1 N HCl or 0.1 N NaOH.

Table II shows the following: (*a*) at pH's below 7.0, less fixation of C'4 and C'1 occurred than at pH 7.0, but there was an enhancement of the fixation of C'3; (*b*) at pH's above 7.0 up to 8.8 there occurred complete fixation of C'4 together with an increased fixation of C'1; and (*c*) optimum fixation of the components of complement occurred at neutral pH's.

In this experiment the CO_2-insoluble fraction contained no C'4; and no reactivation resulted upon the addition of CO_2-insoluble fraction to complement rendered inactive by fixation. This demonstrates that C'2 was bound along with C'4. If, however, C'4 is present in the CO_2-insoluble fraction, reactivation by the latter of fixed sera in which no C'4 and C'2 can be demonstrated, is due to the presence of small amounts of C'2 in the CO_2-insoluble fraction.

Another point of interest evident from the experiment is that complementary activity was removed by specific aggregates over an extremely wide pH range, although there was marked variation in the fixation of the individual components at different pH's. On the basis of these data all other fixation experiments were conducted at pH's between 7.0–7.2.

5. The Effect of Tonicity.—Although the effect of varying salt concentration on the fixation of complement is well known (21), nothing is known of the effect of variations of tonicity on the fixation of the individual complement components. The results of an experiment performed to determine this effect revealed the following: (*a*) complement in slightly hypertonic NaCl solutions was fixed incompletely; and (*b*) complement in hypotonic NaCl solutions was fixed completely with little or no variation in the fixation of the individual components.

$CaCl_2$ is known to interfere with complementary activity (1), and is further known to interfere with certain adsorption reactions (25). An experiment performed to show the effect of $CaCl_2$ on the fixation of the components revealed the following points: (*a*) in amounts over 0.2 per cent, $CaCl_2$ interfered with the fixation of C'4 and C'1; and (b) in all amounts used $CaCl_2$ did not influence the fixation of C'3.

6. The Effect of Propionaldehyde.—Pillemer, Seifter, and Ecker (15) have shown that propionaldehyde protects the fourth component from inactivation by hydrazine. Since the direct and immediate inactivation of complement by specific aggregates appears to involve C'4, experiments were performed to determine if propionaldehyde would interfere with the fixation of the components. The results of such an experiment are given in Table III. It is seen

that propionaldehyde in amounts far greater than that needed to oppose the anti-C′4 effect of hydrazine failed to inhibit the fixation of complement. However, slightly less C′4 was fixed in the presence of propionaldehyde, while there was no interference with the fixation of the other components.

7. The Effect of Dilution of Complement.—Table IV summarizes the results of an experiment undertaken to determine the fixability of the components of complement which had been diluted previous to fixation. The table shows

TABLE III

The Effect of Propionaldehyde on the Fixation of Complement

1:8 pneumococcic antiserum	Guinea pig serum	0.16 molar propionaldehyde	0.9 per cent saline	1:40,000 SIII	Hemolysis produced by supernatant	Hemolysis produced by supernatants after addition of specifically inactivated complements: Serum treated with hydrazine	Serum treated with insoluble carbohydrate	Serum heated at 56°C. for 30 mins.	CO_2-soluble fraction ("end-piece")	CO_2-insoluble fraction ("mid-piece")
cc.	*cc.*	*cc.*	*cc.*	*cc.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0.2	1	1	0	0.2	0	10	50	10	15	55
0.2	1	0.5	0.5	0.2	0	10	50	10	10	60
0.2	1	0.25	0.75	0.2	0	5	60	10	15	70
0.2	1	0.125	0.87	0.2	0	10	60	Tr.	10	65
0.2	1	0	1	0.2	0	0	60	Tr.	10	65

Specifically inactivated complements	Hemolysis					
	per cent					
Serum treated with hydrazine.....	0	—	80	80	90	40
Serum treated with insoluble carbohydrate.....	0	80	—	60	40	60
Serum heated at 56°C. for 30 mins.....	0	80	60	—	0	0
"End-piece".....	0	90	40	0	—	85
"Mid-piece".....	0	60	60	0	85	—

that: (*a*) the fixation of C′4 was inhibited only in very large dilution; and (*b*) there was an increase of fixation of the mid-piece and end-piece on dilution.

8. The Effect of Varying Amounts of Complement.—The results of an experiment performed to determine the variations which occur in the fixation of the complement components when increasing amounts of complement are added to a standard amount of antigen-antibody aggregate, show that increasing amounts of complement resulted in a slight increase in the fixation of C′4; a marked decrease in the fixation of C′1; and little or no variation in the fixation of C′3.

9. The Effect of Varying Amounts of Antigen and Antibody.—The results of

an experiment dealing with the effects of varying amounts of antigen and antibody on complement fixation, show the following: (*a*) in extreme excess of antigen or antibody the fixation of complement or its individual components was markedly inhibited; (*b*) when very dilute solutions of antigen and antibody were used C′4 did not fix completely; (*c*) a slight excess of antibody resulted in maximum fixation of the combining components of complement. Therefore, in the experiments reported here, antibody was employed in a slight excess.

TABLE IV

The Effect of Dilution on the Fixation of Complement

1:8 pneumococcic antiserum	Guinea pig serum	1:40,000 SIII	0.9 per cent sodium chloride solution	Relative quantity and appearance of the precipitate	Hemolysis produced by supernatant after fixation	Hemolysis produced after addition of specifically inactivated complements				
						Serum treated with hydrazine	Serum treated with insoluble carbohydrate	Serum heated at 56°C. for 30 mins.	CO_2-soluble fraction ("end-piece")	CO_2-insoluble fraction ("mid-piece")
cc.	*cc.*	*cc.*	*cc.*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0.2	1	0.2	0	++ gran.	0	0	60	0	50	70
0.2	1	0.2	1	++ gran.	0	0	50	0	30	50
0.2	1	0.2	2	+ gran.	0	0	45	0	20	30
0.2	1	0.2	3	+ gran.	0	0	45	0	20	30
0.2	1	0.2	4	±	0	0	50	0	20	Tr.
0.2	1	0.2	5	0	0	20	60	0	20	Tr.

Specifically inactivated complements	Hemolysis					
	per cent					
Serum treated with hydrazine	0	—	60	70	95	50
Serum treated with insoluble carbohydrate	0	60	—	60	35	90
Serum heated at 56°C. for 30 mins.	0	70	60	—	0	0
"End-piece"	0	90	35	0	—	95
"Mid-piece"	0	50	90	0	95	—

The Fixation of Normal Guinea Pig Complement and Specifically Inactivated Guinea Pig Complement.—In order to determine whether the removal of one or more components of complement interferes with the fixation of the remaining components, an experiment such as the one summarized in Table V was carried out. In this experiment normal complement and specifically inactivated complements were added to the specific aggregates of Type III pneumococcus specific substance. The table reveals the following points: (1) Almost all of C′4, 25 per cent of C′3, 75 per cent of C′1, and nearly all of C′2 were removed or inactivated during fixation of normal complement. This is at variance with

the assumption of most workers that the mid-piece is the single combining component, as all of the components were fixed in varying amounts under the conditions of the experiment. (2) Inactivation of C'4 by hydrazine or the

TABLE V

The Fixation of Normal and Specifically Inactivated Guinea Pig Complements

Pneumococcic antiserum 1:8	1:40,000 SIII	1 cc. of various serums or serum fractions	Hemolysis after fixation	Hemolysis produced after addition of specifically inactivated complements					
				Serum treated with hydrazine	Serum treated with insoluble carbohydrate	Serum heated at 56°C. for 30 mins.	Serum treated with hydrazine and insoluble carbohydrate	"End-piece"	"Mid-piece"
cc.	*cc.*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0.2	0.2	Guinea pig serum	0	5	65	10	10	20	Tr.
0.2	0.2	Serum treated with hydrazine	0	0	80	0	0	Tr.	0
0.2	0.2	Serum treated with insoluble carbohydrate	0	10	Tr.	0	0	0	0
0.2	0.2	Serum treated with hydrazine and insoluble carbohydrate	0	0	10	0	0	0	0
0.2	0.2	Serum heated at 56°C. for 30 mins.	0	95	90	0	75	0	0
0.2	0.2	End-piece	0	95	40	0	40	0	45
0.2	0.2	Mid-piece	0	0	40	0	0	0	0

Specifically inactivated complements	Hemolysis						
	per cent						
Serum treated with hydrazine.....	0	0	65	90	0	95	0
Serum treated with insoluble carbohydrate.....	0	65	0	90	10	45	60
Serum treated with insoluble carbohydrate and hydrazine.....	0	0	10	60	0	25	0
Serum heated at 56°C. 30 mins.....	0	90	90	0	60	0	0
"End-piece".....	0	90	45	0	25	0	90
"Mid-piece".....	0	0	60	0	0	90	0

removal of C'3 by the insoluble carbohydrate from yeast did not markedly influence the fixation of the other components. (3) The inactivation of both C'1 and C'2 by heat inhibited the fixation of C'4 and C'3. (4) When end-piece, which contained C'2, C'4, and a small amount of C'3 was added to the specific aggregates, no fixation of C'3 and C'4, and partial inactivation of C'2 resulted. Although, C'1 is fixed in the absence of C'4, functionally in so far

as hemolysis is concerned, it is inactive. In studies on the mechanism of immune hemolysis (26), it will be shown that unless C'4 is either fixed previously to or simultaneously with C'1, no hemolytic activity occurs. (5) All of C'1 but only a small amount of C'3 were removed in the fixation of mid-piece which contained all of C'1 and the major portion of C'3.

The results of many experiments similar to the one above show that the direct and immediate inactivation of complement which occurs in fixation is due to the fixation of almost all of C'4 and C'2 and of a part of C'1. C'3 does not appear to play a major part in such fixation reactions. However, it appears that the inactivation of the thermostable components (C'4 and C'3) does not interfere with the fixation of the thermolabile components (C'1 and C'2). Further, in the absence of mid-piece, C'2 and C'4 are not adsorbed or inactivated by fixation; while in the absence of C'2 and C'4, C'1 is still adsorbed by specific aggregates. The full significance of these results will be elaborated in a subsequent paper dealing with immune hemolysis since they are best interpreted when a visible reaction, such as hemolysis, occurs.

The Fixation of the Components by Complement by Various Antigen-Antibody Systems.—In order to determine whether the nature of the antigen influences the fixability of the components of complement, a series of experiments was conducted in which antigens of different characteristics and molecular sizes were used.

The components of complement were fixed quite similarly by human serum-antihuman rabbit serum, tobacco mosaic virus-antitobacco mosaic virus rabbit serum, and by hemocyanin-antihemocyanin rabbit serum aggregates. The fixation of the components by these systems was qualitatively similar to fixation by SIII-antipneumococcus Type III rabbit serum (Table V).

Since sensitized red blood cells of different species are commonly employed in the assay of complementary activity, an experiment was performed in which complement was allowed to fix to the specific aggregates of sheep, dog, human, and guinea pig erythrocytes at 1° C. The results obtained were similar to those described for other aggregates, except for a few minor deviations. Whereas the fixation of C'4–C'2 in these cases was almost identical with their fixation by other systems, C'3 appeared not to be adsorbed at all. Marked variations occurred in the amounts of C'1 which combined with the different red blood cell aggregates, as expressed in the following order: guinea pig > sheep > human > dog.

DISCUSSION AND SUMMARY

From the experiments reported here it is evident that the amount of each complement component which combines with specific immune aggregates depends upon a number of factors, including the age of the complement; the concentrations of antigen, antibody, and complement; hydrogen ion and electrolyte concentrations; and time and temperature of incubation for fixation.

The experiments also reveal the following:

1. C′4 must be considered a combining component of complement because it is invariably inactivated or adsorbed by specific aggregates.

2. C′3, although necessary for the final action of complement, *e.g.*, hemolysis or bactericidal action, is fixed only partially or not at all by antigen-antibody compounds.

3. C′2 is fixed together with C′4, as is evident from those experiments in which reactivations were conducted with a CO_2-insoluble fraction which contained no C′4 activity.

4. Mid-piece, which contains C′1, generally stated to be the single combining component of complement, is adsorbed by immune aggregates in varying amounts depending on the experimental conditions employed.

5. No inactivation or adsorption of C′4 and of C′3 occurs upon the addition to specific aggregates of serum which has been heated at 56°C. for 30 to 50 minutes. This indicates that certain thermolabile constituents of serum are necessary for the fixation of C′4. Inactivation of C′4 by hydrazine or the removal of C′3 by the insoluble carbohydrate from yeast does not markedly influence the fixation of the other components.

6. The assumption that the mid-piece is the single combining component of complement is further questioned for the following reasons.

First, C′4 in fresh, untreated serum combines with specific aggregates, but C′4 in heat-inactivated serum does not. It is, therefore, apparent that a part of the combining complement must be attributed to C′4 and its carrier C′2. Second, it will be shown in a subsequent paper that C′1, heated at 56°C. for 45 minutes, combines with specific immune complexes, and in doing so may in fact inhibit the further combination of the components of untreated complement. This effect, first noted by Ehrlich and Sachs (27), is termed a "complementoid" action. Thirdly, it will also be shown on the basis of quantitative nitrogen data (28), that the combining nitrogen, in those instances in which large amounts of serum are added to a constant amount of aggregate, should for the most part be attributed to C′4 and its carrier C′2.

7. The nature and the molecular size of the antigen does not influence the qualitative picture of fixation of the complement components.

8. While it is tempting to speculate on the mechanism of complement fixation, it probably is advisable that such speculation be postponed until experiments similar to those reported here are performed with purified components. However, certain differences between the fixation of the complement components to specific aggregates and the adsorption of these components to non-specific agents, are apparent. For example, in specific immune fixation C′4, C′2, and varying amounts of C′1 are fixed, while in non-specific adsorption both to inorganic adsorbents (29) and to untreated bacteria (30, 31), no fixation of C′4–C′2 occurs while all of the other components of complement are adsorbed. Furthermore, C′3 is bound only partially or not at all to specific

aggregates, while it is completely adsorbed or inactivated by non-specific adsorbents. It appears, therefore, that the highly reactive component, C'4, is directly and immediately involved in specific immune fixation.

9. A very small amount of specific immune aggregate combines with a large amount of complement; whereas a large amount of non-specific agent adsorbs only a small amount of complement. This difference is undoubtedly due to the marked chemical affinity of C'4–C'2 and C'1 for the surfaces presented by immune aggregates.

10. It can be now stated that when complement exerts its activity in hemolytic, bacteriolytic, or bactericidal reactions, C'4–C'2 and varying amounts of C'1 must first combine (fix) with the antigen-antibody compound in question; and that any secondary manifestation is dependent both on the adjunctive action of the unbound C'3 (26) and on the nature of the substrate employed.

The chemical and immunological implications of these results will be further elaborated in subsequent papers.

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