RECENT ADVANCES IN THE CHEMISTRY OF COMPLEMENT[1]

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Complement is considered to be a chemical system composed of multiple components. Existing views on the chemical nature and the functions of each component of guinea pig complement are summarized.

Complement, also known as alexin, cytase, or addiment, is generally defined as the portion of fresh blood serum or plasma which is not increased on immunization and which, when added to certain sensitized cells, combines with these sensitized cells and, under appropriate conditions, results in their destruction or death.

Ever since its discovery, complement has continued to interest and puzzle investigators. Its theoretical importance was clearly shown by the basic experiments of Büchner, of Bordet, and of Ehrlich. Subsequent studies resulted in the practical use of complement in important diagnostic tests (complement fixation). It is especially significant in the part it plays in the actual destruction of pathogenic bacteria. Data accumulated over a number of years from several sources indicate that complement plays a major rôle in the destruction of certain microörganisms.

The bacteriologist and immunologist have made important strides in the elucidation of the biological significance of complement. Until recently, however, attempts to solve the riddle of the chemical nature and function of complement have proved difficult. The peculiar complexity and extreme perishability of complement have often discouraged the initiation or continuance of promising experiments.

Although chemists and physicists have been reluctant to enter into investigations on complement, the need of a closer coöperation between these workers and the immunologists is desirable, since the application of exact physical and chemical methods to this problem gives promise that it may be eventually solved.

It is the purpose of this review to consider complement as a chemical system composed of multiple components and to summarize, in the light of recent advances, existing views on the chemical nature and the function of each of the components of guinea pig complement.[3]

THE BIOLOGICAL SIGNIFICANCE OF COMPLEMENT

As early as 1792, John Hunter noted that blood resists putrefaction to a greater degree than other putrescible material. Later, several workers (8, 61, 98)

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independently showed that defibrinated blood, peptone blood, and blood serum exert measurable destructive influence upon bacteria. It was further shown (61) that this bactericidal power is weakened on standing and can be destroyed by heating to 60°C. Although several theories have been advanced as to the mechanism of the destruction of bacteria by blood serum, the humoral theory of immunity as conceived by Büchner offered a theoretical basis for future work. Büchner (8) looked upon blood as containing a constituent—which he named "alexin" (protective substance)—that is responsible for the bactericidal action of blood serum. He considered alexin to be comparable to an enzyme because of its heat lability and perishability. He showed that alexin operates optimally at body temperature and is capable of destroying bacteria without the co-operation of cellular elements. Meanwhile, Bordet's experiments (2) showed that fresh serum by itself has very slight or no bactericidal action. However, fresh serum combined with heated immune serum produced powerful and rapid bactericidal effects. Thus, the mechanism of the bactericidal action of blood became clearer. Immunization of an animal produces in its blood serum relatively heat-stable protective substances (antibodies), which are specific for the antigen used in immunization. Antibodies, alone, do not destroy bacteria, but depend on the auxiliary action of another substance present in normal fresh serum, corresponding to Büchner's alexin. This substance was found to be non-specific and not increased on immunization. The discovery that immune hemolysis is in every way analogous to immune bacteriolysis led to the common use of erythrocyte–hemolysin systems in most subsequent work. Owing to the work of Ehrlich and Morgenroth (27) further great strides were made in elucidation of the mechanism of immune hemolysis. Briefly, their experiments showed the following: Red blood cells undergo hemolysis when acted upon by two serum factors, the relatively thermostable antibody (hemolysin) and the thermolabile non-specific alexin or *complement*, as Ehrlich now more properly called this substance. The antibody unites with its homologous erythrocyte in the presence or absence of complement. Complement, however, does not combine with the red cell until antibody enters into union with the cell. When this takes place, complement combines, and under appropriate conditions lysis then occurs. Continued research has added little to these fundamental initial studies, except to show that the same mechanism holds true over a wide range of antigen–antibody systems. The essential facts remain that the union of complement through the medium of the specific antibody may lead to alterations of the antigen–antibody complex; that, secondly, in other cases, a similar combination with antigen–antibody complexes may take place without any visible change occurring, the fixation of complement being shown by the disappearance of complement from the medium when a suitable test is applied. *The basis of all complement-fixation tests rests on this last fact.* From the foregoing, it is apparent that the action of complement is dependent on the nature of the substrate with which it comes into contact. There appears to be no need at the present of postulating a multiplicity of complements. Instead, emphasis should be directed to the fact that variations in the activities of complement on different antigen–

antibody systems may depend either upon differences in the combining affinities of the components of complement with various antigen–antibody aggregates, or upon the relative insensitiveness of certain antigen–antibody complexes to the action of the components of complement, or finally upon differences in the nature and quality of the substrate.

Among the many functions attributed to complement are: (*1*) the hemolysis of sensitized red blood cells; (*2*) the lysis of certain sensitized bacteria (65); (*3*) the capacity to kill sensitized bacteria in the absence of bacteriolysis (61); (*4*) the opsonization of bacteria in the absence of immune serum (51); (*5*) the activation of thermostable opsonins (93); (*6*) the alteration of the rate of aggregation of antigens by their homologous antiserums (13, 39); (*7*) the alteration of the state of aggregation of antigen–antibody systems (78, 81); (*8*) and finally the property of combining with most antigen–antibody systems even in the absence of any visible manifestation (complement fixation). There is also evidence to show that complement operates in the destruction of virus by immune serum (17). Recently, it has been shown that complement plays a part in tissue sensitization (50). It has also been suggested that toxin–antitoxin aggregates bind complement *in vivo* and that by this mechanism the toxin is neutralized (55). Complement has also been associated by some workers with the coagulative processes of blood (31, 92), but recent evidence appears to invalidate this view (23, 77). There seems to be some connection between the sedimentation rate of red blood cells and the complement activity of the serum (62). Finally, although never substantiated, complement has even been associated with the biochemistry of muscle contraction (47). The criteria employed in many of the above instances as to whether complement performs the above-mentioned functions were based on the fact that the function in question is destroyed by heating the serum to 56°C. for 30 min.; and it is apparent that several of the functions attributed to complement may be due to other thermolabile constituents of serum entirely independent of complement or its components. Work with pure components of complement should clarify the biological significance of complement. In any event, there can be little doubt that complement contributes greatly to both active and passive antibacterial immunity.

THE CHEMISTRY OF THE STRUCTURE OF COMPLEMENT

Investigations into the chemistry of complement have been confronted with peculiar difficulties arising partly from the fact that complement occurs as a relatively small part of a very heterogeneous mixture (serum), and partly because of the complexity and lability of complement itself. Workers failed to appreciate the fact that complement might comprise only a small part of the serum. Indeed, it has been stated (4) only recently that "complementary power comprises all of the activities of unheated serum." Furthermore, underestimation of the extreme lability of complement when subjected to prolonged standing, changes in pH, and high temperature, and of the composite nature of complement itself led to various views as to the nature of complement.

Büchner (8) suggested that complement is an enzyme or ferment. In further attempts more clearly to define this concept of Büchner's, others have suggested that complement is a lipase (94), a protease (14), a peptidase (62), an intracellular catalyst (45), or a combination of several of these. On the other hand, it has often been inferred that complement is a simple chemical, like oleic acid (99) or similar fatty acids. It has also been proposed that complement is a lipid or a combination of lipids, soaps, and proteins (38). However, there is no evidence that the nature of complement is similar to that of any of the recognized enzymes (7). Also, subsequent studies have refuted the lipin–soap–protein complex concept (7). More commonly, complement has been considered to be a colloidal attribute of fresh serum, i.e., the activity of complement being dependent on the physicochemical state of the serum.

THE COMPONENTS OF COMPLEMENT

It is well established that guinea pig complement is composed of four functionally distinct components whose interactions result in what is commonly called complementary activity. Treatment of serum with carbon dioxide water (52) or dilute hydrochloric acid (82) or by dialysis against distilled water (9) has been shown to separate complement into two relatively thermolabile fractions. The globulin fraction or the fraction insoluble in dilute acids or in salt-free media has been designated the midpiece, and the so-called albumin fraction or the fraction soluble under the above-mentioned conditions has been termed the endpiece of complement. The activities of both endpiece and midpiece are abolished in a few minutes at 56°C. In addition, it has been shown that cobra venom (79), yeast cells (97), or an insoluble carbohydrate (67) isolated from fresh yeast inactivates or removes a relatively heat-stable fraction of complement, the third component. It has also been shown that dilute ammonia (32) and other amino compounds (68) capable of reacting with carbonyl groups destroy another thermostable component of complement, the fourth component. These two latter fractions may be distinguished from the thermolabile components by their resistance to destruction by heat up to 60°C. Claims have been made for the existence of other components, but evidence of their actual existence is lacking.

The two thermolabile components of complement owe their terminology to their supposed action rather than to their nature. The thermostable components were named according to the order of their discovery. However, functional studies raise question as to the desirability of employing the above terminology (39, 73). In addition, electrophoretic diagrams (71) (figure 1) of midpiece and endpiece prepared by the carbon dioxide method, as well as of complement deprived of its third and fourth components, indicate that the so-called globulin fraction or midpiece contains at least four distinct proteins, two of which have mobilities faster than any of those found in whole serum; while the endpiece or the so-called albumin fraction also contains at least four distinct boundaries, one of which corresponds to the gamma globulin of the normal serum. No significant difference was detected electrophoretically between normal serum

and serum deprived of the fourth component of complement. Serum lacking in third component shows a disturbance of the alpha globulins. On the basis of the above findings, it has been suggested (71, 73) that the four components of complement be designated by the following symbols: C′1, midpiece; C′2, endpiece; C′3, third component; and C′4, fourth component. In the following portion of the present review the terms "midpiece" and "carbon dioxide-insoluble fraction" are used interchangeably, as are the terms "endpiece" and "carbon

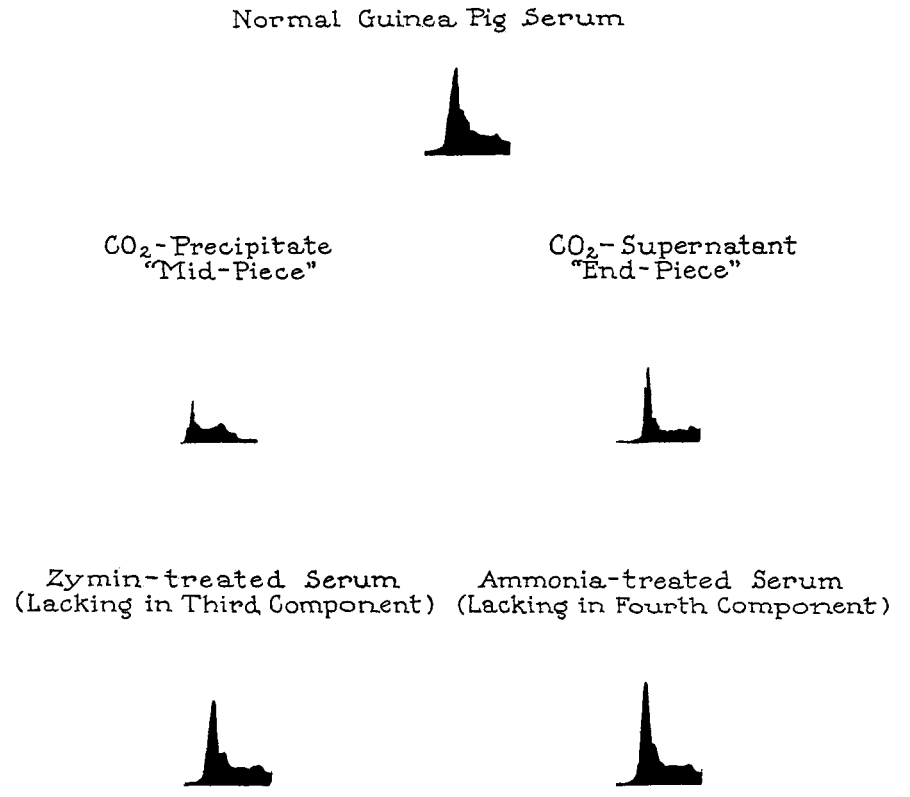

FIG. 1. Electrophoretic schlieren patterns of normal guinea pig serum and guinea pig serum treated with various reagents which separate or destroy the components of complement. Descending boundaries of the proteins in phosphate buffer of pH 7.7 and ionic strength 0.2. Scanning exposures made after electrolysis for 2½ hr. Pillemer and Ecker (71).

dioxide-soluble fraction," and the symbols C′1 and C′2 represent the midpiece and endpiece *components per se*. The third and fourth components are used interchangeably with the symbols C′3 and C′4, respectively.

The recent work of Hegedüs and Greiner (35) should be mentioned. These authors found that the differences in the hemolysis of sensitized sheep red cells by the complements from various species of animals are due to variations in the content or activities of the different complement components in each serum.

These differences were found to be constant and characteristic of each species. They claim that the complement titer of serum is determined by the component present in the lowest titer. These components are midpiece for the guinea pig, endpiece for the human and rabbit, and C'4 for the hog. Guinea pig complement contains a fuller quota of all components than other serums, which accounts for its powerful complementary action against sensitized sheep cells. Hegedüs and Greiner further showed that cow, horse, sheep, and squirrel serums do not hemolyze sensitized sheep cells because of the absence of endpiece activity in all of these. It is evident from the results of Hegedüs and Greiner that great caution must be observed in interpreting data derived from studies on the complement activities of one species to fit the activities of the complements of all other species. However, confirmation of the Hegedüs and Greiner findings with the use of *purified* components of complement is desirable. In any case, the value of their results lies in the exposition that complement activity is determined by the activity of the weakest component present and that one component of complement cannot replace another.

It is apparent that complement function is associated with the protein consituents of blood serum, and is distributed over several protein fractions, which interact with one another and not merely in the manner of summation. Most attempts to separate complement from whole serum have yielded results too indefinite to allow the characterization either of complement or of any of its components as functionally distinct entities. Indeed, Browning (7) in summarizing the literature on complement up to 1931 stated that, "at present it is impossible to determine whether complement is of compound nature, as is believed to be the case for enzymes, or whether it may represent a physicochemical state of a mixture of proteins, lipins and other constituents of the serum." However, some advance in the purification and characterization of the components has been made, and for greater ease in presentation and for the sake of clarity, the chemistry of the existing knowledge of each component of complement will be reviewed and discussed.

THE CHEMISTRY OF MIDPIECE

The globulins, which separate out when a complement-containing guinea pig serum is treated with either carbonic acid or weak hydrochloric acid or by dialysis against distilled water, contain all of C'1, a major portion of C'3, and a small part of C'4 of complement. As prepared by carbonic acid precipitation, this fraction, judged electrophoretically (71), consists of at least four serum proteins, one of which has an electrophoretic mobility of 2.9×10^{-5} in phosphate buffer of pH 7.7 and an ionic strength of 0.20. Eagle (19a) has recently investigated the midpiece of complement in the ultracentrifuge and found that this fraction of complement sediments faster than the other components of complement. The isoelectric point of midpiece has been reported variously between pH 5 and 6. It is non-dialyzable through cellophane against distilled water, hypertonic salt solution, or hypotonic salt solution (25). A dilute solution of midpiece in 0.9 per cent sodium chloride loses its activity in a few hours at

room temperature or at ice-box temperature (5°C.), in 30 min. at 54°C., and almost instantaneously at temperatures above 60°C. (25). It has been reported (57) that after midpiece combines with antigen–antibody complexes, its further activity is then unaffected by heating at 56°C. for 30 min. While midpiece is extremely unstable in 0.9 per cent sodium chloride, it can be kept for days suspended in distilled water or dissolved in hypertonic salt solution at a pH between 5.8 and 6.2 (25). It is partially inactivated at a pH of 8.8 or 4.2, and completely inactivated at pH 9.5 or over, or pH 4.0 or under, being more stable in acid than in alkaline solution (70). Midpiece is inactivated by shaking, by ultraviolet light (33, 84), and by organic solvents (66), e.g., ether, alcohols, benzene, chloroform, and acetone. Its activity is inhibited or destroyed by colloids possessing strong acidic groups (23), such as cellulose sulfuric acid, crystalline heparin, hyaluronic acid, and chondroitinsulfuric acid, and also by protamine sulfate and diethylamine. Midpiece is susceptible to adsorption by inorganic adsorbents such as lead phosphate and titanium dioxide (24), by sensitized and unsensitized cells (63), and even by filtration through a Berkefeld filter (87). Summarizing, midpiece has the character of a globulin, and is extremely sensitive to physical or chemical conditions different from those encountered in its natural environment.

Attempts have been made to purify midpiece. Browning and Mackie (6) and Parsons (64) were unable definitely to establish midpiece in any distinct serum protein fraction. Tokunaga (90) even casts doubt on the individuality of midpiece. However, all of the above-named authors agreed on the globulin nature of midpiece. Ferranti (30) later characterized midpiece as a subfraction of the acid globulins and as having a low isoelectric point. Doladilhe (16) also established midpiece as being an acid globulin, insoluble in distilled water or carbonic acid at low salt concentrations, but soluble in carbonic acid in the presence of 0.9 per cent sodium chloride. However, none of the above preparations was fully characterized and therefore their exact identities are unknown.

THE PURIFICATION AND CHARACTERIZATION OF GUINEA PIG C′1

The separation of C′1 from guinea pig serum has been achieved (72) by adherence to exact physicochemical manipulations with rigidly controlled conditions of temperature, hydrogen-ion concentration, and ionic strength. The procedure in brief consisted of the precipitation of the active principle by 1.4 *M* ammonium sulfate at low temperatures, followed by isoelectric precipitations and extractions. Final purification was aided by the observation that, whereas the active principle C′1 was quantitatively precipitated at an ionic strength of 0.02 at pH 5.2, a fair proportion of other euglobulins as well as pseudoglobulins remained in solution, thus leading to a high degree of purification of C′1. Fully 95 per cent of the euglobulin separated by the above procedures showed an electrophoretic mobility of 2.9×10^{-5} when measured in phosphate buffers of ionic strength of 0.2 at pH 7.7, and all of the molecules sedimented with the same velocity in the ultracentrifuge, calculated to be 6.4×10^{-13} Svedberg units, when measured in 0.2 *M* potassium chloride. The final yield of this material from

1070 ml. of serum was 400 mg. or 0.6 per cent of the total serum proteins. Stability studies on the purified C′1 revealed that while it was unstable at neutral pH, its activity remained intact at pH of 5.4 to 6.0. No C′1 activity was observed at protein concentrations exceeding 0.02 per cent, while full activity was observed at protein concentrations between 0.002 and 0.02 per cent. The presence of excess lipoidal material also interferes with the activity of C′1, which may account for the failure of workers to obtain midpiece activity in the proteins precipitated at one-third saturation ammonium sulfate. The properties of C′1 are summarized in table 1.

TABLE 1*

Properties of C′1

PROPERTIES	C′1
Mobility in phosphate buffer, at pH 7.7, 0.2 ionic strength	2.9×10^{-5}
Sedimentation constant, $s^{1\%}_{20^\circ,w}$	6.4×10^{-13}
Protein nitrogen, per cent	16.3
Carbohydrate, per cent	2.7
Phosphorus, per cent	0.1
Optical rotation, $[\alpha]_D^{25^\circ}$	−28.7
Apparent isoelectric point	5.2–5.4
Fraction of total serum protein, per cent	0.6
Heat stability of complement, activity (destroyed in 30 min. at tabulated temperature), °C	50

* Reference 72.

THE CHEMISTRY OF ENDPIECE

The fractions of guinea pig serum which remain in solution when the serum is treated with carbonic acid or weak hydrochloric acid or by dialysis against distilled water, contain all of C′2, a major portion of C′4, and a small part of C′3. In carbonic acid precipitation, this soluble fraction contains at least four protein fractions as observed electrophoretically (71), two of which are albumin and gamma globulin. Eagle (19a) has attempted to characterize endpiece in the ultracentrifuge and his studies show that endpiece sediments with the same velocity as the serum albumins. Endpiece is non-dialyzable against distilled water and salt solutions (25). In contrast to midpiece it is relatively stable on standing, maintaining its activity for several days at ice-box temperature (3–5°C.). However, its activity is abolished in 30 min. at 54°C. and instantaneously at temperatures above 60°C. The presence or absence of salt appears to make little or no difference in its stability as long as the pH is maintained between 6.2 and 7. It is more stable in alkaline solutions than in acid solutions (70). Thus, whereas a pH of 10 or over is needed to inactivate endpiece totally, at a pH of 5.8 or under its activity rapidly disappears. Endpiece is inactivated by shaking, by proteolytic enzymes, and by organic solvents (66). Its activity is destroyed by ultraviolet rays (33, 84), although it is more resistant in this respect than midpiece and in the dry state is only slightly affected by ultraviolet

light. Endpiece is readily adsorbed when passed through a Berkefeld filter (87). Like midpiece, it is inactivated by treatment with highly acidic colloids and extremely basic proteins or colloids (23).

THE PURIFICATION AND CHARACTERIZATION OF GUINEA PIG C′2

Endpiece has been usually associated with the albumins or psueodoglobulins of serum (63). Ferranti offered the first evidence that it is not identical with the serum albumins, but that it is associated with the "albuminoids" (28). He claimed to have separated the active endpiece principle by adsorption with fibrinogen (29). However, because Ferranti did not characterize his preparations, their true identify remains obscure.

Recently (72), a mucoeuglobulin has been isolated from guinea pig serum which exhibits both C′2 and C′4 activity. This mucoeuglobulin, which separated after the dialysis against conductivity water of the serum proteins soluble at a concentration of 2.0 *M* but insoluble at a concentration of 2.2 *M* ammonium sulfate, has mainly an electrophoretic mobility of 4.2×10^{-5} in a phosphate buffer of pH 7.7 and 0.2 ionic strength. Ultracentrifugal analysis of this fraction revealed several boundaries. The sedimentation constants observed—17, 6.3, and 3.8×10^{-13} units (in amounts of 5, 35, and 60 per cent, respectively)—are close to those reported for purified alpha globulins from other species. A study to determine if the fraction containing these components of complement represents a single component in the ultracentrifuge, several independent components, or several components in equilibrium with each other is definitely indicated.

As stated above, the mucoeuglobulin possesses both C′2 and C′4 activity. This was not the first indication of the close relationship of these two fractions of complement. Whitehead, Gordon, and Wormall (96) suggested that these two components might be closely associated, since they had noticed that the fraction of serum soluble in carbonic acid generally contains these two components. Further studies by others (68) substantiated this hypothesis, and extended the view that the two components are a carbohydrate–protein complex. For a fuller understanding of the nature of C′2, the chemistry of C′4 should best be elaborated and the nature of the two components discussed simultaneously.

THE CHEMISTRY OF C′4

Complementary activity can be removed from blood serum by appropriate concentrations of ammonia in such a manner that the activity can be regenerated by the addition of serum lacking in either C′1, C′2, or C′3. Gordon, Whitehead, and Wormall (32), who first observed this fact, found that the ammonia effect is not due simply to alkalinity, but that it is a specific reaction involving a relatively thermostable fraction of complement. This component is characterized as being non-identical with the third component, as being non-dialyzable, and as being capable of destruction by dilute acids and alkalies at 37°C. These authors designated this fraction of complement as the fourth component. They also stated that, of the other amino compounds, primary methylamine and

ethylamine behave like ammonia in inhibiting the fourth component, while glycine, alanine, and urea do not. Others have claimed that viper venoms inactivate a similar factor (63). Attempts have also been made to associate the fourth component with lipids and bound calcium (63), but recent evidence appears to invalidate the view that lipids and calcium play a rôle in the structure of fourth component (69). In fact, it has been shown that the extraction of lipids from serum by numerous organic solvents does not specifically inactivate C'4 (66), and the weight of experimental evidence indicates that calcium is not involved in the immediate labile reactions of C'4 nor is it a part of its internal structure. Since inactivation of C'4 (34) is not associated with the protein constituents of serum or with any interferences with the phosphorus partition or with the lipids or calcium of serum, the hypothesis (68) has been offered that the ammonia inactivation of the fourth component is due to the conversion of the active carbonyl groups of the component to some less active structure. This hypothesis

TABLE 2*

Properties of a mucoeuglobulin possessing C'2 and C'4 activity

PROPERTIES	C'2 AND C'4
Mobility in phosphate buffer, at pH 7.7, 0.2 ionic strength	4.2×10^{-5}
Protein nitrogen, per cent	14.2
Carbohydrate, per cent	10.3
Phosphorus, per cent	0.1
Optical rotation, $[\alpha]_D^{25°}$	−192.5
Apparent isolectric point	6.3–6.4
Fraction of total serum protein, per cent	0.18
Heat stability of complement, activity (destroyed in 30 min. at tabulated temperature), °C	50†, 66‡

* Reference 72.

† For endpiece activity.

‡ For fourth component activity.

was tested by extending the work of Gordon and his coworkers to include various amino compounds of two general classes: namely, those which are known to react readily with aldehydes and those which do not. The results obtained are summarized in table 2, which reveals that only amino compounds capable of reacting with carbonyl groups specifically inactivate C'4. Simple alkalinity of the amines is not the cause of the inactivating property. In water, the effective compounds dissociate to form bases of moderate strength, but it is readily seen that many of the inactive compounds dissociate in the same manner. In fact, the dissociation constant for tetramethylammonium hydroxide or triethylamine is greater than that of ammonia or phenylhydrazine. The $—NH_2$ group is necessary for inactivation of the fourth component. Hydrazine, with two $—NH_2$ groups, requires only one-half the concentration of ammonia to inactivate C'4. The type of substituent group replacing a hydrogen atom of ammonia to produce the amino compound has a pronounced influence on the

capacity of the compound to inactivate the fourth component. A positive substituent (simple alkyl), as in methylamine, continues to act as does ammonia. A negative substituent (phenyl group) diminishes the amino effect on C'4. Polar groups which potentially lend acidic or redox properties to the amino compounds completely abolish the effect on the fourth component. This is seen in such compounds as the carbazides, urea, glycine, and hydroxylamine. Amino compounds which have been treated previously with aldehydes do not inactivate the fourth component. The fourth component of complement cannot be reactivated by the addition of aldehydes to serums treated with amino compounds. This indicates that a firm union exists between the amino compounds and the reactive group of the fourth component. Further, the union is sufficiently stable to resist dialysis against 0.9 per cent sodium chloride. These results suggest that the amino compounds act upon a reactive carbonyl group of serum which is most likely associated with a glucoprotein. Further studies have shown that the fourth component is relatively stable in alkaline solutions, but unstable in slightly acid solutions. Its pH range of stability is of the same order as that of C'2 (70). C'4 is relatively stable on standing; at 1°C. its activity remains unimpaired for several days. It is non-dialyzable and retains its activity after heating at 56°C. for 30 min., but is readily inactivated at temperatures above 60°C., being inactivated in a few minutes at 65°C. (21). C'4 is quite resistant to non-specific adsorption. Indeed, twelve consecutive filtrations through a Berkefeld filter are required to remove this component from serum (87). C'4 is far more resistant to ultraviolet light than are the thermolabile components of complement (33, 84).

The above-mentioned properties of C'4 may explain past failures to purify complement by fractionation with ammonium sulfate, because unless care is taken to maintain slightly acid pH and low temperature during purification, inactivation of C'4 readily occurs. In this respect, Tokunaga (89) has shown that ammonium sulfate inactivates complement, the rate of inactivation increasing on raising the temperature or on dilution of the serum. He further found that a large excess of ammonium sulfate fails to inactivate complement. Apparently, the acidity of the excess ammonium sulfate is sufficient to suppress the ionization of reactive amino groups.

As stated above, C'2 and C'4 were found to be present in the same mucoeuglobulin of guinea pig serum. Treatment of this protein at 50°C. for 30 min. removed all of its endpiece activity, but did not inhibit C'4. This euglobulin then retained two functions, one by virtue of a relatively heat-labile, and the other by virtue of a relatively heat-stable component. Its carbohydrate content was 10.3 per cent and it reduced Schiff's reagent. These findings are not inconsistent with the hypothesis that C'4 is a carbohydrate with reactive carbonyl groups and that it is associated with C'2 as a complex. The heterogeneity of the sedimentation patterns of the mucoeuglobulin makes it advisable, however, to postpone a definite assumption as to its exact nature until more detailed study of this protein is undertaken. The properties of the mucoeuglobulin are given in table 3. It is noted that the yield of this protein from 1070 ml. of serum was

120 mg., or less than 0.2 per cent of the total serum proteins. The mucoeuglobulin is characterized by an extremely high levorotatory optical activity and by low phosphorus content. It exhibits full activity at dilutions of 0.01 per cent and shows no inhibitory action on the addition of excess quantities, as was found for C'1.

TABLE 3*

The effect of amino compounds on the fourth component of complement

SUBSTITUENT TYPE	REAGENT	FORMULA	MINIMAL MOLARITY	MAXIMAL MOLARITY	pH	INACTIVATION OF FOURTH COMPONENT
Positive.....	Ammonia	HNH_2	0.16		8.5–10	+
	Methylamine	CH_3NH_2	0.12	0.24	7.4– 8.0	+
	Dimethylamine	$(CH_3)_2NH$		0.72	7.0–10	–
	Trimethylamine	$(CH_3)_3NH$		0.72	7.0–10	–
	Tetramethylammonium hydroxide	$(CH_3)_4NOH$		0.96	7.0–10	–
	Ethylamine	$CH_3CH_2NH_2$	0.12	0.24	8.0– 9.0	+
	Diethylamine	$(CH_3CH_2)_2NH$		0.72	7.0–10	–
	Triethylamine	$(CH_3CH_2)_3N$		0.72	7.0–10	–
	Hydrazine	H_2NNH_2	0.08		7.2– 8.0	++
Negative.....	Phenylhydrazine	$C_6H_5NHNH_2$	0.24	0.36	7.0– 7.4	+
	Aniline	$C_6H_5NH_2$		0.64	7.0–10	–
Polar.....	Urea	NH_2CONH_2		0.95	7.0–10	–
	Acetamide	CH_3CONH_2				
	Glycine	$HOOCCH_2NH_2$		0.96	7.0–10	–
	Semicarbazide	$H_2NCONHNH_2$		0.72	6.0–10	–
	Thiosemicarbazide	$H_2NCSNHNH_2$		0.72	7.0–10	–
	Hydroxylamine	$HONH_2$		0.72	6.0–10	–
Blocked polar...	*O*-Methylhydroxylamine	CH_3ONH_2	0.16	0.24	8.5– 8.5	+
Indifferent.....	Methenamine	$(CH_2)_6N$		0.96	7.0–10	–

* Reference 68.

THE CHEMISTRY OF C'3

Although the inactivation of complement by yeast cells was described by von Dungern as early as 1900 (97), the nature of the phenomenon remained obscure until Coca (10) established that the inactivation by yeast is due to the removal or destruction of a relatively heat-resistant portion of complement, and not to the introduction of inhibiting substances. Whitehead, Gordon, and Wormall (96), employing a yeast powder, found that the third component combines either physically or chemically with the yeast powder. It has also been found that cobra venom (79) and weak solutions of formaldehyde (11) inactivate the

third component. Certain other agents which inactivate complement appear, in part, to exert their effect on C'3, i.e., bacterial suspensions (79), hexoxidases (20), and strong reducing agents (69). The divergent qualities of these inactivating agents make it difficult to draw conclusions as to the chemical structure of C'3. An insoluble fraction (67) from fresh yeast also adsorbs C'3 from fresh serum. This insoluble material is composed of 94 per cent carbohydrate and is insoluble in hot water, salt solutions, and organic solvents. The yield from fresh yeast is about 2 per cent. The insoluble carbohydrate inactivates the third component specifically in an amount only one twenty-fifth of the required amount of fresh yeast. None of the soluble fractions from yeast inactivated C'3. Since the anticomplementary factor in fresh yeast is undoubtedly this insoluble fraction, the inactivation of C'3 appears to be due to the adsorption of this relatively heat-stable component from serum. Recently, the amount of nitrogen removed from serum by the insoluble carbohydrate has been measured (26). It was found that the removal of the third component from 1 ml. of guinea pig serum is accompanied by the uptake by the insoluble carbohydrate of about 0.02 to 0.03 mg. of nitrogen. Little or no phosphorus is adsorbed. Elution studies on the adsorbed material are in progress. The results of such studies should do much to clarify the constitution of this hitherto unidentified component. Attempts to purify C'3 by the classical methods of ammonium sulfate fractionation have been unsuccessful. In these experiments, third component is present in small quantities in nearly every fraction of serum, especially in the euglobulins.

C'3 is resistant to inactivation at a temperature of 56°C., but is destroyed in 30 min. time at 62°C. (21). C'3 is the first component of complement to disappear on standing at room temperature. Its stability to changes in hydrogen-ion concentration is of the same order as that of C'1, since it is relatively stable in weak acid solutions and very unstable in weak alkaline solutions. It is non-dialyzable against distilled water, hypertonic or hypotonic solutions, or weak acid solutions.

Hypertonic salt solutions exert a protective action on C'3, in that C'3 becomes more resistant to inactivation on standing, and in that it cannot be removed from guinea pig serum by yeast, zymin, or the insoluble carbohydrate from yeast in the presence of 5 per cent or more of sodium chloride. The inactivation of complement on dilution with distilled water appears to be due to the destruction of C'3 (63). On the other hand, dilution of serum with saline is said to protect the third component from heat inactivation (7).

THE CHEMISTRY OF COMPLEMENT FUNCTION

General considerations

It was originally supposed by Ehrlich that the nature of complement action is similar to the mechanism by which living protoplasm obtains its nutrition; when complement combines with the sensitized cell, it was thought that a catalytic digestion follows. Others have also viewed complement as a catalyst or enzyme. Scheller (83), influenced by Pfeiffer's opinion as to the mechanism

of bacteriolysis, put forth the view that complement acts as a catalyst to the antibody, the latter then exerting an enzymic action. Hill and Parker (45) state that complement, during the interval between its combination and its entrance into the cell, acts as a catalyst and allows the release of hemoglobin by the associated antibody. On the other hand, McKendrick (56) concluded that the action of complement is lytic *per se*, the antibody acting as a catalyst to complement after fixation. Ponder (76) questions the interpretations of Hill and Parker and of McKendrick and offers experimental evidence to disprove their hypothesis. Furthermore, complement has not been identified with serum proteases and lipases, and there is insufficient evidence that complement produces enzymic decomposition such as is brought about by recognized ferments. In further attempts to define complement chemically, it has been suggested that complement action is analogous to the action of soaps. Noguchi showed that the hemolytic organ extracts were soap-like in nature (60), and attempted to associate this observation with complement function. Subsequent studies have shown that the hemolytic action of soaps is not destroyed by heating and that they operate just as well on unsensitized as on sensitized cells.

The observation that complement is apparently consumed in the process of hemolysis or bacteriolysis led workers to investigate the nature of this phenomenon. Liefmann and Cohn (53) believe that the disappearance of complement is due to several factors: first, to the fixation of complement by the products of the red blood cell after hemolysis; second, to the dilution of complement, thereby increasing its instability; third, to the weakening of complement because of prolonged exposure to dilute solution at 37°C. Eagle and Brewer (19) and Ponder (76), however, disagree with Liefmann and Cohn in that they believe that the disappearance of complement is due to the adsorption of complement by the sensitized cell previous to lysis. The general belief is at present in agreement with the views of Eagle and Ponder.

Konikov (48, 49) has reported some interesting observations on the nature of the mode of action of complement and hemolysin that bear reporting in some detail. Konikov believes that the combination of the red blood cell with anti-sheep red blood cell rabbit serum follows Freundlich's equation of physical adsorption only when there is an excess of antiserum. The exponent of the isotherm is between 0.22 and 0.35. In low concentration of hemolysin, a strictly chemical reaction between the erythrocyte and antiserum predominates. Konikov further showed that the combination of immune serum and red blood cell is dependent on pH, the optimal hydrogen-ion concentration lying between pH 6.0 and pH 6.7. Furthermore, this combination is dependent on the salt concentration, and the nature of the salt is not as significant as the concentration employed. The active concentrations of all salts are approximately equivalent. Konikov further assumes that an ionic reaction occurs in the process of sensitization with the formation of a complex combination of salt, red cell, and antiserum. Continuing, he states that the hemolysis of sensitized cells by complement occurs only in the presence of salts whose effective concentrations are directly dependent on their equivalence. He concludes that the combination between complement

and red cell is purely chemical and that osmotic pressure phenomena do not play a rôle in this sort of hemolysis. This is in good agreement with the fact that stroma is not destroyed in complement hemolysis and that when lysis has been effected by lytic serum the stroma remains practically unaltered in different concentrations of salt. Apparently the hemolysis of the cell by complement has so increased the red cell permeability that simple osmotic phenomena no longer play a significant rôle. To return to Konikov's experiments, he further found that pH 7 is optimal for complement action. He explains the relationship between hemolysis, hydrogen-ion concentration, and the nature and concentration of the employed salt on the assumption that complement concentrates on the surface of the cell, where it combines to form a layer of protein having an isoelectric point of 4.7. This creates between the cell contents and the surrounding complement layer, in accordance with Donnan's law, a potential difference and an electrical field. Water is then driven electroösmotically through the stroma pores of the red cell along the lines of force; this causes a drop in the osmotic pressure within the red cell and then hemolysis occurs.

Eagle (18, 19) has pointed out that the physical constants of complement fixation,—namely, temperature coefficient, velocity, and quantitative relationships between reactants,—are those commonly associated with adsorptive processes. Eagle also mentioned the possibility that the enormous avidity of antigen–antibody aggregates for complement may be due to a specific chemical affinity. This author has also reported interesting observations on the mechanism of immune hemolysis which demonstrate that sensitization of the red cell confers upon it the property of adsorbing complement, and that the degree of sensitization determines the amount of complement fixed. Eagle found that variations in the electrolyte and hydrogen-ion concentration markedly influence the fixation of complement and subsequent hemolysis. On the basis of his findings Eagle states, "if this mobilization of complement is the sole function of immune serum (and there is as yet no reason to assume any other) then the accepted terminology, in which amboceptor, immune body and hemolysin are used synonymously, is erroneous. The immune body would function only as an 'amboceptor' mobilizing the effective hemolysin, complement, upon the surface of the cell." Recent work discussed below has served to substantiate Eagle's views.

Continuing along similar lines, Brown and Broom (5) have reported some interesting experiments on the possible mode of action of complement. The authors noted that the negative charge normally carried by bacteria and red blood cells is reduced considerably by the action of specific immune serum in the presence of electrolytes, and that the negative charge on the red cell is reduced in proportion to the amount of antiserum used to sensitize the cell. In the absence of electrolytes they found no reduction in the cell charge, even though the cell had been heavily sensitized. Extending their experiments, they showed that complement behaves as though it is negatively charged at neutral pH, and that the effective action of complement is dependent on the valency of the salt, provided, of course, that the salt itself has no deleterious effect on the comple-

ment. The electrolyte apparently lowers the charge of the red cell in the presence of immune serum, and when the charge is reduced to the required potential the negatively charged complement is fixed. This hypothesis would explain the findings of Sachs and Teruuchi (81) and of others that the absence of salts inhibits hemolysis by complement, and would also explain the inhibition of complement action by polyvalent anions, since the work of Loeb (54) demonstrates that the negative charge of colloids is first increased when acted upon by polyvalent anions and is only decreased in high concentration of salt. It should be pointed out that Brown and Broom make no claim that the charge of the antigen–antibody complex is the sole factor in fixation and hemolysis, but maintain that it is an essential preliminary process. Hambleton's (36) results also indicate that the fixation of complement is only a secondary effect due to the surface properties of suitably modified antigens and not to a specific chemical affinity of the antigen for complement.

While it is agreed that the electrical state of the reactants,—complement, antigen, and antibody,—is an important factor in the fixation of complement, the composite nature of complement and the multiple functions of its components do not allow an elementary explanation such as simple adsorption for this action. On an adsorption basis, alone, it would be difficult to explain the selective fixation of C′1 by immune compounds while other serum euglobulins of similar isoelectric points and net charges remain unadsorbed. Furthermore, the attraction of C′3 to non-specific agents such as inorganic adsorbents and unsensitized bacteria, and not to immune aggregates, and the avidity of C′4 and C′2 for antigen–antibody compounds and not for non-specific adsorbents cannot be explained by simple adsorptive phenomena. While it is the general belief that electrical phenomena and other physical factors enter into the fixation of complement by immune aggregates, recent evidence also indicates that a certain degree of specificity accompanies such manifestations, and that such specificity involves chemical phenomena.

In the foregoing considerations, complement has been treated as a single entity, the final test for its presence being the occurrence of hemolytic or bacteriolytic action. However, as shown in this review, complement is composed of several components, the absence of any one of which leads to the inactivity of the total complement complex. Consequently, it is obvious that the fixation of complement may depend on the removal or inactivation of only one or more of the components of complement, and that hemolysis must be the result of the interactions of all of the components of complement. As will be seen later, this is true, and the varied results obtained by different workers have been due for the most part to their reluctance to accept a composite nature of complement. Furthermore, failure to distinguish between the primary stage of complement action (fixation) and the secondary stage (lysis, etc.) has also greatly contributed to many varied views.

Recently, there has been an advance in the knowledge of the fixation of the components of complement. Of particular significance are (*1*) the identification of the combining components of complement (73), (*2*) the investigations on the

physical and chemical factors governing the fixation of the components of complement (73), and (*3*) the development of methods for the quantitative estimation of the combining components (39). In this section, an attempt is made to summarize especially these developments.

THE IDENTITY OF THE COMBINING COMPONENTS OF COMPLEMENT

Although most investigators have generally accepted the belief that midpiece is the combining component of complement, the adequacy of this belief has often been questioned. Brin (3) holds that the endpiece of complement is the combining component of complement. Others (12, 85, 86) assert that both midpiece and endpiece combine with antigen–antibody compounds. Still others (1, 15, 58, 91) have shown that C′4 is involved in complement fixation. The work of Nathan (59) and of others (88, 95) clearly shows that C′3 does not combine with sensitized cellular antigens such as red cells. There are several reasons for these varied views. First, with the exception of a few recent studies, investigations on the rôle of the complement components in immune fixation were performed before the discovery of the fourth component. Second, much of the past work was qualitative in nature with little consideration of the chemical and physical factors which affect the combination of the complement components with antigen–antibody aggregates. Third, the results obtained under one set of experimental conditions were frequently interpreted to fit all other experimental conditions. Fourth, the methods for the preparation and identification of the components of complement employed in the fixation studies were usually not uniform.

Deissler (15) first pointed out that C′4 combines with immune aggregates. His results show that sensitized sheep cells fix the fourth component of complement and that the fixation of C′4 appears to be a prerequisite for the fixation of midpiece or endpiece. C′4 is only slightly or not at all adsorbed from heat-inactivated serum. Other findings are in agreement with Deissler's. Misawa (58) and his associates have also shown that in the Wassermann reaction, while C′4 as well as endpiece and midpiece are fixed by strongly positive serums, C′4 does not always combine with the aggregates of weakly positive serums. On the other hand, these authors found that the aggregates of low-titer rabbit antiserums fix C′4 with little or no fixation of C′3, endpiece, and midpiece, and that the aggregates of high-titer rabbit antiserums fix all of the components. It appears from the results of these studies that the nature of the antigen or antibody, as well as the titer of the antiserum, influence both quantitative and qualitative fixation of the complement components. It has also been claimed (46) that only those guinea pig proteins which are soluble in one-third saturation but insoluble in one-half saturation of ammonium sulfate are fixed by antigen–antibody complexes.

The influence of physicochemical conditions on the fixation of the complement components have been recently studied (73). It was found that the amount of each component which combines with specific immune aggregates depends upon a number of factors, including the age of the complement, the concentrations of

antigens, antibodies, and complement, the hydrogen-ion concentration, the electrolyte concentrations, and the time and temperature of incubation for fixation. In more detail, it was shown that when serum had stood in the ice chest overnight and had lost 50 per cent of its complementary activity, C′4 became more resistant to fixation, more C′2 was fixed, and less C′1 combined. The combination of C′3 was unaffected. It was further shown that C′4 is fixed during the first 5 min. of incubation if a sufficiently high titer antiserum is employed. Under similar conditions, maximum fixation of C′1 occurs after 40 min. of incubation. The effect of temperature on the fixation of the components is striking, in that at temperatures of 37°C. C′4 is more resistant to fixation. These authors (73) also showed that, whereas complementary activity is removed over an an extremely wide pH range, there is, however, marked variation in the fixation of the individual components. At a pH below 7, less fixation of C′4 and C′1 occurs than at pH 7, while at a pH above 7.0 complete fixation of C′4 occurs, together with an increased fixation of C′1. Studies of the effect of salt concentration on the fixation of the complement components revealed that the combining components are fixed incompletely in slightly hypertonic solutions, while in hypotonic solutions little or no variation in the addition of the combining components occurs. It has been further demonstrated that dilution of serum previous to fixation results in less fixation of C′4 with an increase in the combination of C′1 and C′2. The concentrations of antigen, antibody, and complement employed in fixation experiments were shown to have a marked influence on the union of the combining components with immune aggregates. In great excess of antigen or antibody, the fixation of the combining components is greatly inhibited; this phenomenon is most marked in extreme antigen excess. Also, very dilute solutions of both antigen and antibody fix complement, especially C′4, incompletely. From the foregoing observations, the conclusions were drawn that *the fixation of each individual component of complement is both influenced by and dependent on definite physicochemical conditions.* This is not entirely unexpected when the marked variations in the chemical character of the components of complement are considered.

Adherence to the immunochemical conditions of fixation described above has resulted in the exact identification (73) of the combining components of complement and in the determination of the amounts of their activity removed by combination with immune aggregates. These studies revealed that C′4 must be considered a definite combining component of complement, since it is invariably inactivated or adsorbed by immune aggregates. C′2, which is usually fixed along with C′4, must therefore also be considered a combining component. Midpiece, which contains C′1 and which is generally stated to be the single combining component of complement, is adsorbed by immune aggregates in varying amounts depending on the experimental conditions employed. C′3 is only partially or not at all fixed by soluble antigens in the presence of their specific antiserums, or is not fixed by sensitized red blood cells. No inactivation or adsorption of C′4 occurs when serum which has been heated at 56°C. for 30 min. is added to specific aggregates, nor is C′4 adsorbed from endpiece. Inactivation of C′4 by

ammonia or hydrazine or the removal of C'3 by the insoluble carbohydrate from yeast does not markedly influence the fixation of the other components of complement. Studies with various types of antigen–antibody aggregates show that the nature and molecular size of the antigen do not influence the qualitative picture of fixation of the complement components.

From the foregoing evidence, it is apparent that C'4, C'2, and C'1 should be considered combining components of complement. Furthermore, there are certain differences between the fixation of the components to specific aggregates and the union of these components to non-specific agents. For example, in non-specific adsorption both on inorganic adsorbents and on untreated bacteria, no fixation of C'4 and C'2 occurs, while C'1 and C'3 are adsorbed. On the other hand, it is now definitely established that immune aggregates combine with C'4, C'2, and C'1, while little or no fixation of C'3 occurs. It appears, therefore, that C'4 and C'2 are distinctly and immediately involved in specific immune fixation. The above facts may account for the observation, as yet little understood, that a very small amount of specific immune aggregate combines with a relatively large amount of complement, while a relatively large amount of a non-specific agent is required for the adsorption of only a small amount of complement. This difference in the combining activities of the two types of adsorbents may be due to the differential affinities of the specific immune compounds and the non-specific adsorbents for C'4. If this be so, then investigations with this component may do much to increase the sensitivity and specificity of the various diagnostic complement-fixation tests.

Meanwhile, the evidence derived from recent data appears to warrant the conclusion 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 reaction is dependent both upon the adjunctive action of the unbound C'3 and upon the nature of the substrate employed" (73).

Although it is now generally agreed that complement or, more strictly speaking, its combining components, combine with certain antigen–antibody precipitates, the actual addition of the combining components in weight units has not been measured until recently. Haurowitz (37), who could not detect any increase in the weight of the specific precipitates of ovalbumin or of azoproteins, when complement was added to them, nor any change in the content of antigen in the precipitates, assumed from his data that in complement fixation only a small group of the complement complex is bound to the antigen–antibody compounds.

On the other hand, Heidelberger (39), employing quantitative methods conforming to the criteria of analytical chemistry, was able to measure in milligrams of nitrogen a difference between the amounts of specifically precipitated nitrogen found in the presence of untreated guinea pig serum and in the presence of guinea pig serum which had been inactivated at 56°C. for 30 to 60 min. In a series of well-planned experiments he showed that this additive nitrogen is intimately associated with complement and is not due to non-specific nitrogenous compo-

nents of serum. He further stated that midpiece of complement added nitrogen to specific precipitates, while endpiece behaved like heat-inactivated serum and did not add nitrogen. On the basis of his findings and on the general belief that midpiece is the combining component of complement, Heidelberger provisionally designated the additive nitrogen as C'1 nitrogen. Later, Heidelberger (44) and others (75) showed that other components of complement contribute nitrogen to specific precipitates.

Heidelberger, Silva, and Mayer (41) further showed that the uptake of complement combining components by specific precipitates of S III was complete in 1 hr. at room temperature. This is in good agreement with the observation (73) that the fixation of the individual components as judged by functional tests is complete in 1 hr. These authors also showed that the combining nitrogen from guinea pig serum is the same regardless of whether the specific precipitates contain excess antibody or antigen. Of special interest was their finding that the amount of additive nitrogen combining with specific precipitates is independent of the weight of the specific antigen–antibody precipitates. The above information appeared to confirm Heidelberger's statement that the estimation of the combining nitrogen from fresh serum is a measure of complement or of its combining components in weight units. However (75), there is evidence that this measurement of complement nitrogen does not represent the total complement combining nitrogen present in serum unless data on the amount of activity of each individual component removed during fixation indicate that this is so. Heidelberger (39) noted that, although the absolute amounts of combining complement nitrogen are less when small samples of serum are used (5 ml. or under), the quantity of combining nitrogen per milliliter of guinea pig serum added is larger. Because of this fact, Heidelberger was unable definitely to determine the absolute amount of complement combining nitrogen per milliliter of guinea pig serum. Nevertheless, rough extrapolation of his data to zero volume gave 0.04 to 0.06 mg. of nitrogen or 0.25 to 0.40 mg. of protein as the amount of combining complement actually present in 1 ml. of guinea pig serum. Heidelberger (42) extended these studies to include human serum as the source of complement and found that human serum contributes from 0.03 to 0.05 mg. of complement nitrogen per milliliter of serum. However, even more so than in the case of guinea pig complement, the amount of fresh human serum nitrogen adding to specific precipitates bore little relation to the hemolytic titers of the various sera tested. Heidelberger (42) has called attention to the fact that the combination of appreciable amounts of complement components with antigen–antibody systems may complicate the interpretation of quantitative precipitin reactions in human sera. As this author states, "It is quite likely that small quantities of antibody, such as might occur during convalescence or after active immunization, could easily appear to be doubled by the C'N fixed during combination of the antibody with antigen and thus indicate too high an antibody content." . . . "Therefore it appears best, . . . to remove C' by the method used in the experiments referred to, namely, by adsorption with adequate amounts of an unrelated precipitating system. . . ."

Experimental evidence (75) has been presented which shows that the estimation of the fresh serum nitrogen which adds to specific precipitates is a measure of a variable portion of the total complement complex. The results of this study, which correlated the quantity in weight units of fresh guinea pig serum nitrogen adding to specific precipitates with a measurement of the residual activity of each complement component after this combination, have helped to clarify data on the quantitative complement component combining nitrogen. It has been found, when such parallel studies were performed, that C′1, C′2, and C′4 from whole fresh serum all contribute varying amounts of combining nitrogen to specific precipitates. In agreement with Heidelberger's data it was noted that midpiece also contributes combining nitrogen, while endpiece of complement does not add nitrogen to the specific aggregates. It was shown also that C′1 of midpiece or whole serum contributes combining nitrogen; C′2 and C′4 furnish no additive nitrogen in the absence of C′1; and C′3 contributes little or no nitrogen in any case. Heidelberger, Bier, and Mayer have also shown that C′4 adds nitrogen to immune aggregates, and that C′3 contributes little or none.

As stated before, unless very small amounts of serum are fixed under rigidly controlled conditions, the total weight of the combining components is not measured. It has been shown that this is due primarily to a diminution in the fixation of C′1 when large amounts of complement are allowed to fix.

When small amounts of serum (1 ml.) are allowed to combine with specific precipitates, these usually contribute from 0.04 to 0.08 mg. of combining complement nitrogen per milliliter of serum. Heidelberger, by extrapolation, obtained quite similar values of 0.04 to 0.06 mg. of combining nitrogen per milliliter of serum. Purified C′1, as determined by actual isolation, comprises about 0.6 per cent of the total serum protein of the guinea pig, and each milliliter of serum contains about 0.06 mg. of C′1 nitrogen. Endpiece (C′2 and C′4) accounted for about 0.02 mg. of nitrogen per milliliter of serum. It is obvious that small deviations in the fixation of C′1 would markedly influence the complement combining nitrogen adding to specific precipitates. On the other hand, relatively large changes in the fixation of C′2 and C′4 would be necessary in order to influence significantly the total amount of nitrogen adding. Thus, although C′1, C′2, and C′4 all contribute to the combining nitrogen, C′1 appears to contribute most of the additive nitrogen.

C′1 is strongly attracted to specific precipitates. Indeed, heat activation of this component of complement does not remove its total combining capacity. Also in whole serum, heat inactivation does not destroy the total combining capacity of the serum. This effect, first noted by Ehrlich, is termed a "complementoid" effect, and is probably due to the differential temperature stabilities of the combining and functional hemolytic groups of C′1. It has therefore been suggested that the true combining nitrogen is the total nitrogen added from a definite volume of serum under controlled conditions, rather than the value obtained from the difference between the combining nitrogen of untreated serum and that from an equal volume of heat-inactivated serum (75).

Furthermore, recent evidence indicates that the total combining nitrogen contributed from fresh guinea pig serum to specific precipitates is an estimate of the amount of complement components combining under the conditions of the experiment, and is therefore a measure only of a variable portion of the total complement complex, and does not always represent the total combining capacity or the hemolytic activity of the total complement present. If, however, experiments determining the amount of activity of each component of complement removed during the fixation accompany the estimation of the combining nitrogen, accurate data may be obtained on the weights of the individual combining components.

In any event, studies along this line have established a definite approach to a problem which the more conventional trends have failed to clarify. As Heidelberger pointed out (39), while the quantitative methods employed in such experiments are too laborious and require too large volumes of serum to expect their widespread use for routine purposes, the techniques employed are useful for the acquisition of precise data, and should serve as tools for research and for the establishment of reference standards.

IMMUNE HEMOLYSIS

The most clearly defined property of complement is the hemolysis of sensitized red blood cells. Nevertheless, until recently, the rôle and function of the components of complement have not been clearly understood. As stated earlier in this review, Liefmann and Cohn assert that the disappearance of complement in immune hemolysis is due to the effect of hemolysis rather than to the initial fixation of complement. Eagle and Brewer and also Ponder have since shown that complement must first combine with the sensitized cell before hemolysis can occur.

Employing experimental conditions which were found to be optimal for the fixation of the components of complement by immune aggregates, it has been shown recently (74) that if the immune aggregate employed in fixation is a substrate which upon resuspension and appropriate treatment shows a visible manifestation, such as hemolysis, the nature and the rôle of the fixed components can be determined. The results of such experiments have helped to clarify the function of the components of complement in immune hemolysis and have revealed the following information: At temperatures of 1°C., C′1, C′2, and C′4 combine with sensitized sheep cells, while C′3 does not combine; C′1, although combining with sensitized cells in the absence of C′4, is hemolytically inert unless C′4 combines previously or simultaneously. In other words, the hemolytic activity of C′1 after fixation is predetermined by the fixation of C′4. Furthermore, C′4 does not combine in the absence of C′1; and in agreement with Nathan it was found that C′3 is not fixed by antibody–sheep cell aggregates, but is essential for hemolysis, operating on the sensitized cell after the fixation of C′4, C′2, and C′1 and behaving as if it were a catalyst. It has further been shown that the amount of C′1 which combines with sensitized cells is determined by the amount of C′4 available for fixation, that is, the more C′4 fixed by the cell, the less C′1 is needed

to produce hemolysis and the less C′1 that is taken up by the sensitized cell. Indeed, only about 10 to 30 per cent of the actual amount of C′1 present in serum is utilized in the hemolysis of one unit of sensitized cells. The fixation of C′4 appears not only to predetermine the hemolytic activity of combining C′1, but also to determine the amount of C′1 that will fix and the amount that will be needed for hemolysis. This relationship, which has been hitherto unrecognized, indicates that a revision of existing thoughts on the interaction of complement, red cell, and antibody should be forthcoming.

Heidelberger (40) has attempted to trace the relationships of the combining complement to red cell and hemolysin in immune hemolysis. He was able to show that the complement combining components unite with antibody in equimolecular or even greater amounts. The interactions of sheep red cell stromata (43) with hemolysin result in the same amounts of complement being adsorbed as that taken out by other immune aggregates. The stroma combined with antibody takes up at least 80 per cent of its weight of complement combining components. Owing to the multiplicity of antibodies present in hemolysin and to the accessory components of complement which are not fixed but are utilized for hemolysis, Heidelberger concluded that his quantitative data have only limited application in the study of immune hemolysis. However, he was able to draw a reasonable picture of complement fixation. Accordingly, the combining components of complement are distinguished from normal serum globulins in that they possess one or more groupings capable of forming loose dissociable combinations with individual antibody and possibly antigen molecules, but which form firm combinations, dissociable with difficulty, when surrounded by great numbers of antibody or antigen molecules. Meanwhile, the following explanation of the action and rôle of the complement components in immune hemolysis appears the most plausible on the basis of existing data (74). Anti-sheep cell rabbit serum (hemolysin) by itself does not combine with or forms only loose dissociable unions with complement or any of its components, or at least does not inactivate them. Nevertheless, when the hemolysin combines with the red cell, the surface pattern of the antiserum molecule or the antiserum–cell aggregate changes and in turn different groups are in contact with the complement components, and firm combination then results between the specific aggregate and C′4, C′2, and C′1. C′4 combines first or simultaneously with C′1 to the red cell aggregates. The fixation of C′4 is accompanied by the fixation of C′2. The adsorption of these components in this definite order on the red-cell–antibody complex then renders the red cell amenable to the action of the unadsorbed C′3 and hemolysis results. Since C′3 is not taken up by the sensitized cell and is apparently not used up in the process of hemolysis, it appears to have certain enzymic qualities. This explanation, by no means complete, may serve as a working basis for continuance of studies along this line. Of course, actual isolation of all of the components of complement in sufficient quantities to subject them to thorough test will do much to clarify this as yet insufficiently explored field of immunochemistry.

SUMMARY

On the basis of the knowledge existing at this time, guinea pig complement can best be characterized as a composite of several serum entities: a euglobulin (C'1), a mucoeuglobulin which possesses both C'2 and C'4 activity, and a still uncharacterized component (C'3).

The interactions of these complement components with antigen–antibody compounds result in the combination of C'1, C'2, and C'4 with these compounds. In the red-cell–hemolysin system, C'4, C'1, and C'2 combine; after this combination, the red cell is rendered susceptible to the action of C'3, which behaves as if it were a catalyst, and hemolysis of the red cell occurs.

Recent advances in the study of the structure and function of the complement components served the purpose of elevating complement from the rank of a mysterious and elusive property of serum to the status of a composite of distinct chemical individuals, and may serve as a working basis for further exploration in this field.

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