Proceedings of the 2nd. Meeting of the Federation of European Biochemical Societies Vienna, 21-24 April, 1965.

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Antibodies to Biologically Active Molecules

Edited by

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PREFACE

THE neutralization of biologically active molecules has been a recurrent theme throughout the history of immunology and was studied in different problem formulations during successive eras of immunology.

The interaction between toxin and antitoxin was the first immunological process to be examined *quantitatively*. The complexity of the animal test system, however, did not allow detailed analysis of the events which result in neutralization *in vivo*. One consequence of this difficulty was the development of a simplified system, in which only two components (antigen and antibody) were involved, and this led to our present knowledge of the nature and specificity of antibody and of the forces which act between antigen and antibody. Recent advances in this area have resulted mainly from work with nucleic acids and globular-proteins; they are reviewed in the second and third chapters of this volume.

The mechanisms by which antibody interferes with the biological activity of an antigen had to await the development of a system of three components: enzyme, antibody and substrate. A detailed analysis of the reaction mechanism of such a three-component system and the present status of this field are given in the fourth to seventh chapters. Our insight into this problem has also gained much from modern studies of the reaction between toxin and antitoxin (eighth chapter).

During the last few years, considerable advances have been made in the analysis of systems of even greater complexity, in particular, those involving the interaction of antibody with toxins, hormones and viruses, and these will be reviewed (eighth to eleventh chapters).

For a full understanding of neutralization *in vivo*, a multi-component system involving a vast number of interlocking biochemical systems must be analyzed and the nature and interrelationship of a number of constitutive and induced factors must be taken into account (see first and twelfth chapters).

The relative importance of the different components of the defense system depends both on the structure of the biologically active molecules and of their host-targets. Each component such as antibody synthesis, complement activation, and phagocytosis, is therefore best analyzed in the particular neutralization reaction in which it plays a decisive role. In recent years, impressive advances have been made in the analysis of the processes underlying the functioning of certain of the above mentioned components and some of these advances are discussed in the first, eighth, eleventh and twelfth chapters. The ultimate synthesis of the contribution of all the separate

components to a particular neutralization reaction still remains a task of the future.

This book is based on a colloquium, organized in the framework of the second meeting of the Federation of European Biochemical Societies. It seems most fitting that a colloquium on the neutralization of biologically active molecules should have taken place in Vienna, the town in which Kraus, Landsteiner and Pirquet contributed so much to immunology. All of us, who were fortunate enough to attend the second meeting of the European Federation of Biochemical Societies, will have taken with them delightful memories of the lively and stimulating scientific sessions and the pleasant social occasions which our hosts provided. Our thanks are due to Dr. and Mrs. Hoffmann-Ostenhof and indeed all the members of the organizing committee, with a special note of appreciation for the efforts of Mrs. Hertha Dax who helped throughout the meeting with all our small and large problems. I would also like to express my personal thanks to Dr. A. Axelrad for editorial advice.

B. CINADER

CONTENTS

CHAPTER 1

THE NEUTRALIZATION OF BIOLOGICALLY ACTIVE MOLECULES

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THE biological activity of foreign macromolecules or of foreign cells, which have gained access to the biochemical interior of a vertebrate, is affected by a variety of constitutive and induced processes. It is the purpose of this book to examine some of the mechanisms which are involved in this reaction: specificity of antibody, regulation of quantity and quality of antibody output, phagocytosis and complement, all play a part, and an attempt is being made to present contemporary aspects of some of these processes.

Our survey beings with recent advances in the investigations concerned with the structure of determinants. Antibody combines with a volume of the antigen which does not exceed $34 \times 12 \times 7$ Å (Kabat, 1954, 1956, 1957, 1958; Kabat and Bezer, 1958; Nisonoff and Pressman, 1958; Cebra, 1961; and Anderer, 1963a and b) so that the reaction between antigen and antibody depends on the structure of relatively small portions of the antigen. The analysis of the interaction between determinant and antibody became the spearhead for the introduction of the techniques and concepts of chemistry and physics into immunology, and has been explored in considerable detail during the first four decades of the twentieth century (Landsteiner, 1936; see 1962 reprint of revised second edition; Marrack, 1938). In the last decade a renewed incentive to orient research in this direction, resulted from the unequivocal proof that antibodies can be formed to *nucleic acids* and from the subsequent analysis of the specificity of such antibodies (Levine and Van Vunakis, p. 25). During the same period, progress was also being made towards an understanding of the nature of the determinants of *globular proteins*. This is a problem presenting considerable difficulties, since antibodies to globular proteins are *not* directed to determinants consisting of the *linear sequence* of amino acids *but* to determinants whose configuration results from the *folding of polypeptides*, so that amino acid sequences, which are widely separated in the unfolded chain, may be contiguous in the native

protein. This may be illustrated by the absence of reactivity between unfolded ribonuclease and antibody to the native enzyme (Brown *et al.*, 1959).

Since the shape of most determinants of globular proteins depends on the integrity of the major portions of the intact molecules, the identification of determinants by classical methods, such as precipitation-inhibition with synthetic compounds of low molecular weight, becomes extremely difficult. It will be seen that this problem is now being attacked in studies of several proteins, including myoglobin. (Crumpton, p. 61.)

After the examination of recent advances in two component immunology in the next two chapters (immunochemistry of nucleic acids p. 25 and globular proteins p. 61), the remainder of this book is devoted to various aspects of multi-component immunology in ascending order of complexity, starting with three component systems, that is with *in vitro* studies of the reaction between enzyme, antibody and substrate.

The *in vitro* combination of antibody with enzymes interferes with the interaction between the antigen and its substrate, partly because of *steric hindrance* of access of substrate to and egress of product from the catalytic site and partly because of antibody-imposed *conformational changes* (see Fig. 1).

Whereas in two component immunology determinants are operationally defined by their structure and the effect of antibody combination by insolubility, in three component immunology determinants are *initially* defined by function and the effect of antibodies is evaluated by the modification of function. In this way, certain determinants can be linked with the biological activity of the antigen and singled out for structural analysis. Tantalizing, but as yet ambiguous indications of this possibility can be found in the study of insulin (Wilson, p. 235). The hormonal activity of experimental hybrid molecules consisting of the A chain of Ox-Insulin and the B chain of Cod-Insulin is inhibited by antibody to Ox-, but not to Cod-Insulin. It is tempting to conclude that the A-chain contains the site which acts on the "substrate" of insulin. However, we cannot exclude the alternative possibility that the A-chain of Ox-Insulin imposes a configuration on the Cod B-chain which enables its determinants to combine with antibody to Ox- B-chain. (Wilson, Dixon and Wardlaw, 1962.) That this latter possibility must be seriously considered is indicated by the report of Pollock and his associates (p. 139) that the enzyme activity of a mutant penicillinase was changed to that of the wild-type by antibody to wild-type penicillinase. Experimental discrimination between these two possibilities should open a fascinating field for further study.

Our first objective in analyzing the interaction between biologically active antigen and antibody concerns the mechanism by which the antibody modifies biological activity. Steric hindrance (Arnon, p. 153; Cinader, p. 85) and configurational changes (Crumpton, p. 61; Pollock, p. 139; Cinader, p. 85)

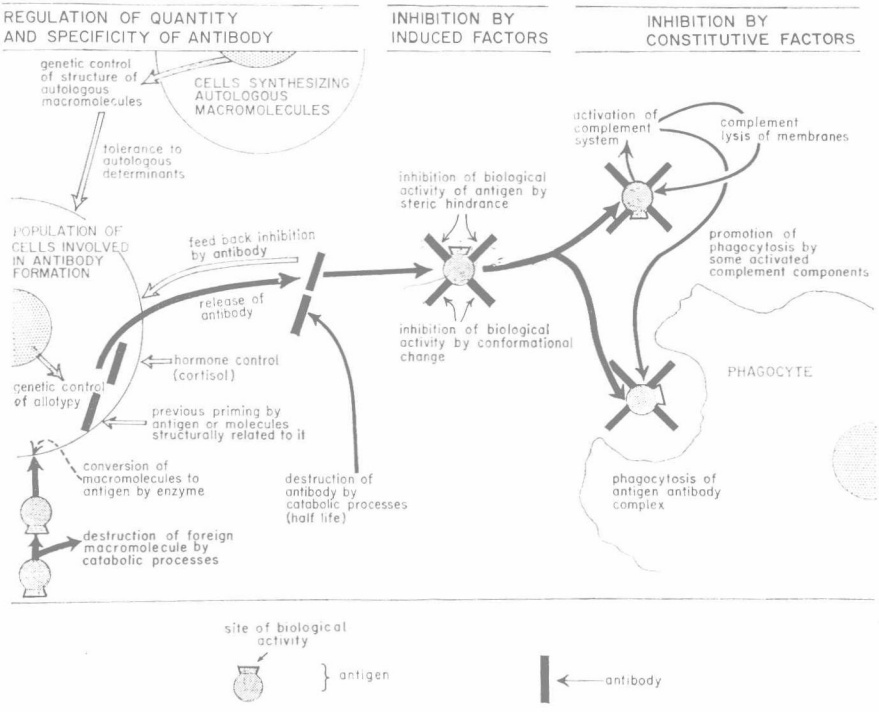

FIG. 1. Schematic representation of the processes involved in neutralization; for explanation, see text.

play an important part in the modification of the catalytic activity of enzymes, which is being examined in four chapters of this book (Arnon, p. 153; Cinader, p. 85; Pollock *et al.*, p. 61; and Uriel, p. 181). We can distinguish three functional types of antibody in the analysis of the *inhibitory* function of antibody. The first of these types is inhibitory, the second, though not inhibitory, competes with inhibitory antibody for sites on the enzyme and the third type of antibody combines with enzyme (or toxin), but does not affect its activity (Cinader, p. 85; Raynaud, p. 197).

Since both steric hindrance and conformational changes depend on the determinants with which antibody combines and hence on specificity of the antibody, we must examine the underlying mechanisms which contribute to immunogenicity of a molecule as a whole and of individual determinants, respectively. The immunogenicity of the molecule, as a whole, depends on its persistence in the body fluids and hence on its molecular weight, on the presence of enzymes which can prepare the macromolecule and possibly on certain structural requirements such as "rigidity". In addition special processes may affect the immunogenicity of individual antigens, such as that of adrenocorticotrophic hormone which liberates cortisol and thus mediates suppression of antibody formation (Felber and Micheli, p. 277).

The relation between *molecular weight* and immunogenicity is clearly seen amongst hormones and enzymes: molecules such as growth hormone (M.Wt. 45,000) are strongly immunogenic; whereas, such molecules as adrenocorticotrophic hormones (M.Wt. 4600) elicit detectable quantities of antibody only after prolonged immunization (Felber and Micheli, p. 277).

The implication of *catabolic enzymes* in immunogenicity has been postulated to interpret the finding that certain synthetic polypeptides and hapten conjugates of polypeptides, consisting of *laevo* rotatory amino acids, were immunogenic whereas corresponding compounds of *dextro* rotatory amino acids were *not* immunogenic (Maurer, 1963a, b; Levine, 1964; Parker and Thiel, 1963; and Benacerraf *et al.*, 1963). It might be assumed that the necessary enzyme systems are available for the catabolism of the vast majority of antigens and that catabolic enzymes, therefore, do *not* constitute the limiting reaction by which the decision between "good" and "bad" antigens is being made. Indeed, Levine and Benacerraf (1964) have investigated the ability of responding and non-responding guinea-pigs to degrade succinilated and non-succinilated poly-L-lysine conjugates and have found that animals capable of forming antibody to these substances, and animals unable to do so, degrade the polypeptides in the same way.

Attempts have been made to explain differences between "good" and "bad" antigens in terms of, for instance, *rigid areas* and presence of *aromatic amino acids*. (Sela, 1962; Sela *et al.*, 1962.) However, so far none of these features has shown consistent general correlation with immunogenicity (Maurer, 1963a, b). Perhaps the problem cannot be resolved in terms of

structure of antigens alone, and can only be resolved in terms of the relation between the composition of antigen and the composition of the autologous macromolecules of the animal which is being immunized (Cinader, 1960, 1961). In other words it may be that the problem of the immunogenicity of the molecule should be considered in terms of the differences between individual determinants of the antigen and of autologous macromolecules. This view is adopted because of the fact that of all the "bad" antigens, the autologous antigens are the worst, so that autoimmunity, if it occurs, is a rare exception to the normal suppression of synthesis of antibody to autologous macromolecules. It can be assumed that the suppression of autoantibody formation is due to *naturally acquired immunological tolerance to autologous macromolecules*. This inhibition would not only affect the response to the autologous molecule itself, but also to any foreign macromolecules which share determinants with it. The amount of antibody formed in response to any foreign protein would depend on the number of determinants on the foreign macromolecule that differ in structure from all autologous determinants (Cinader and Dubiski, 1963; and Cinader, 1963).

In addition to this tolerance mediated process, which leads to *recessive* inheritance of immunological responsiveness, there is also direct gene-control. This may be deduced from the *dominant* inheritance of antibody responsiveness, observed, for instance, in the response of mice to a polymer of L-glutamic acid and L-lysine, containing 5 per cent L-alanine (Pinchuck and Maurer, 1965).

We have considered the *inhibiting* influence on the antibody response of immunological "experience" with autologous determinants, however previous immunological experience, *resulting in antibody formation*, also steers the specificity of the immune response. This is well illustrated by the specificity of antibody response to various types of influenza virus, which depends mainly on the type of virus first encountered in life (*Original Sin*, Davenport and Hennessy, 1956; Svehag, p. 301).

In the foregoing discussion of neutralization, we have considered antibody specificity in relation to a unique site of biological activity. This point of view is probably also potentially useful in the analysis of neutralization of hormones and of bacterial toxins but not in the analysis of the inactivation of cells by antibody, as will become evident later. In the examination of virus neutralization, a definitive view on the relation between antibody specificity and inhibitory capacity cannot yet be formed. One of the central questions, which remains controversial, concerns the number of antibody molecules required for inactivation of virus activity and the exact nature of the reactions which follow the initial combination between virus and antibody. A unique specificity of antibody might be regarded as a prerequisite of neutralization-capacity, if *one* molecule of antibody were sufficient to inhibit biological activity. In fact, Dulbecco, Vogt and Strickland (1956) *have* suggested that a

single antibody molecule, bound at a critical area of the virus, can abolish its infectivity. However, other workers have obtained evidence that more than one combined antibody molecule may be required for neutralization (Burnet *et al.*, 1937; Kalmanson *et al.*, 1942; Lafferty, 1963). As a consequence we cannot dismiss the possibility that the decisive step in neutralization does not only depend on the specificity of antibody but on such factors as the charge of virus–antibody complexes which may, by electrostatic repulsion, interfere with attachment of virus to the cell. Whether a virus particle, complexed by antibody, is infectious depends on events at the surface of host cells. These events are determined by incompletely understood factors such as the number and distinctiveness of sites on the virus with which the virus contacts the host cells and the number and physical properties of combined antibody particles (Svehag, p. 301).

TABLE 1. TYPES OF HUMAN IMMUNOGLOBULIN

Type	Subtype	Heavy chain	Light chain	Molecular formula
γG (or IgG)	γGK	γ_2	κ_2	$\gamma_2\kappa_2$
	γGL	γ_2	λ_2	$\gamma_2\lambda_2$
γA (or IgA)	γAK	α_2	κ_2	$\alpha_2\kappa_2$*
	γAL	α_2	λ_2	$\alpha_2\lambda_2$*
γD (or IgD)	γDK	δ_2	κ_2	$\delta_2\kappa_2$
	γDL	δ_2	λ_2	$\delta_2\lambda_2$
γM (or IgM)	γMK	μ_2	κ_2	$(\mu_2\kappa_2)_n$**
	γML	μ_2	λ_2	$(\mu_2\lambda_2)_n$**

* There are also polymeric forms present.
** n is 5.
Based on: *Bull. World Health Org.* **30,** 447, 1964; *J. Exp. Med.* **121,** 185, (1965).

For a full understanding of interaction between antibody and antigen the heterogeneity of the antibody response must be analyzed, since a succession of immunoglobulins of different physical, chemical and biological properties is elaborated in the course of prolonged immunization; Table 1 lists some of the better characterized different molecular species (W.H.O. Bulletin). However, it is already evident that many additional types of immunoglobulins exist. The molecular diversity of immunoglobulin is being presented in chapters dealing with antibodies to toxins and to viruses (pp. 197, 301).

When the combination of antigen and antibody occurs *in vivo*, the neutralization of the antigen's biological activity depends on the relative ability of antigen and antibody to penetrate membranes, as already indicated in an earlier reference to virus-neutralization *in vitro*. As a rule, antibody

cannot penetrate to the biochemical interior of cells, that is, it apparently cannot come in contact with intracellularly located antigens. This has been shown by the inability of antibody to penetrate fibroblasts *in vitro* (Holter and Holtzer, 1959), and by the inability of neutralizing antibody to inactivate viruses which mature within the cytoplasm of infected cells (Howes, 1959, Kidd, 1961; and Metzgar *et al.*, 1962). Thus, poliomyelitis virus which belongs to the latter class is not affected by antibody when the virus is inside the cell, whereas viruses which mature at the cell membrane can be inactivated by antibody as in Newcastle disease virus (Rubin *et al.*, 1957), and Rous sarcoma (Vogt and Rubin, 1961).

If intact mammalian cells do, as a rule, not allow antibody to pass through the membrane, it is not surprising that membranes of unicellular organisms completely bar the entrance of antibody. Housewright and Henry (1947) showed that antipenicillinase increased the penicillin sensitivity only of those organisms that produce extracellular penicillinase and ascribed this to the inability of the antibody to pass the cell membrane. Miller *et al.* (1949), found that antiserum prepared against isolated yeast hexokinase completely inhibited the purified enzyme, but the same antiserum did not inhibit the activity in fresh whole yeast cells (Pasternak, 1951). Krebs and Wright (1951) have shown the influence on glucose fermentation of the antibody to triose phosphate dehydrogenase. There was a marked effect if the antibody was added to yeast juice. Antibody added to living yeast cells did not affect glycolysis.

The inability of antibody to penetrate cell walls is an important factor in the neutralization of antigens (such as viruses and certain bacterial toxins) which have an intracellular target; it also prevents the neutralization of intracellularly located enzymes or hormones. Furthermore, antibody can only affect the biological activity of that fraction of antigen which is outside the cell and the degree of neutralization will, therefore, depend on the relative proportion of antigen outside and inside the cell and on the relation between the period during which antigen and antibody are present.

The *in vivo* interaction of antigen and antibody thus involves many more than three components; in addition to membrane penetration, biosynthetic aspects of antibody formation and delicately balanced and interrelated constitutive factors come into play (see Fig. 1). We must take into consideration biosynthetic regulation of the *quantity* of antibody since antibody synthesis seems to be arrested when the level of circulating antibody reaches a critical concentration.* As a consequence, passive administration of antibody to an immunized animal may reduce rather than potentiate the effectiveness of the defense mechanism. (Uhr and Bowman, 1961a, b; Rowley and Fitch, 1964; Möller and Möller, p. 409.)

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* It must remain undecided to what extent this inhibition is caused by removal of the antigen, thus preventing it from reaching antibody-forming cells.

We must also consider qualitative aspects of the immune output since different types of antibody (19S alone, or 19S and 7S) may be produced, depending on the quantity of antigen administered, as was shown by Svehag and Mandel (1964) who found that a 50-fold increase in dose of polio virus changed a pure 19S to a 7S (and 19S) response. Since the half life and complement fixing properties of these two types of antibody are very different, a profound qualitative difference in neutralization-events may be caused by differences in the administered dose of immunizing antigen (p. 322). In the response to bacterial toxins, evidence may be found that there are also profound differences in the neutralizing capacity of γA (IgA) and γG (IgG) antibody and Raynaud suggests that these may be attributable to differences in the *number* of antigenic determinants to which antibody of different molecular species is directed (p. 197).

The relative importance of the above factors will not be the same whether the neutralization of self replicating (viruses, bacteria, tumor cells) or non-self replicating (toxins, hormones, enzymes) antigens is being considered. This is best illustrated by the relatively short persistence of antibody after vaccination with inactivated polio virus and the much longer persistence of antibody with live viruses.

The combination of antigen and antibody *in vivo*, implicates not only induced but also constitutive processes which contribute to the neutralization of the biological activity of the antigen. Antigen, combined with antibody, is more readily phagocytosed than antigen which is *not* combined with antibody (see Fig. 1). Thus in the case of toxins, for instance, there are at least two mechanisms of *in vivo* neutralization, one based on interference in the interaction between toxin and its "substrate" and the other based on the removal of toxin–antibody complexes by phagocytosis.

A second constitutive process, activated by the combination of antibody with antigen, depends on the components of complement. These participate in a number of interlocking reactions which result, in appropriate instances, in damage of the membrane with which the antibody is combined or in initiation of the inflammatory response. Elucidation of the mechanism of action and the physiological and pathological functions of complement is a frontier of immunological investigation which will not be treated elsewhere in this volume and a survey of this rapidly moving field, recently reviewed by Müller-Eberhard (1965), Nelson (1965) and Lepow (1965), will be presented here.

The complement system comprises a group of normal serum proteins, present in trace amounts or in very low concentrations, which have the salient characteristic of interacting with certain antigen–antibody complexes and of mediating immunological injury. For reasons of convenience and precision, immune hemolysis has been most intensively studied as an example of red cell-membrane damage, effected by complement, and current concepts

TABLE 2. SEQUENCE AND MECHANISM OF IMMUNE HEMOLYSIS

E = sheep erythrocyte. E* = injured erythrocyte.

A = rabbit antibody directed against sheep erythrocyte.

C′1, C′4, etc. designate serum proteins interacting in a sequence of reactions resulting in immune hemolysis. C′1a, C′2a, etc. refer to enzymatically active forms of the corresponding component, either known or postulated.

C′1 = macromolecular complex, containing subunits designated C′1q (11S factor), C′1r and C′1s (C′1 proesterase). The intact complex of all these molecules functions as C′1.

Immunoelectrophoretic designation of some components of complement:
C′3 = β_{1C} globulin (converted in free solution to β_{1G} globulin under the influence of the C′4–2a complex).
C′4 = β_{1E} globulin.
C′5 = β_{1F} globulin.

Reaction	Biochemical event
$E+A \underset{Ca^{++}}{\rightleftharpoons} EA$	
$EA+C'1 \longrightarrow EAC'1a$	Attachment of C′1 at a site on C′1q to a receptor on A; activation of a catalytic center at a site on C′1s.
$EAC'1a+C'4 \longrightarrow EAC'1a, 4$	Enzymatic attack of C′1a on C′4, creating a site on C′4 which attaches to a receptor on E.
$EAC'1a, 4+C'2 \xrightarrow[\text{Thermal Decay}]{Mg^{++}} EAC'1a, 4, 2a$	Attachment of C′2 to a receptor on C′4; enzymatic attack of C′1a on C′2, creating a catalytic center on C′2; fulfilment of functions of C′1a.
$EAC'1a, 4, 2a+C'3 \longrightarrow EAC'1a, 4, 2a, 3a$	Activation of C′3 and attachment to a receptor on E.
$EAC'1a, 4, 2a, 3a+C'5+C'6+C'7 \rightarrow EAC'1a, 4, 2a, 3a, 5, 6, 7a$	Formation of a stable intermediate no longer susceptible to thermal decay: activation of C′5–C′6–C′7.
$EAC'1a, 4, 2a, 5, 6, 7a+C'8+C'9 \rightarrow E^*$	Unknown events leading to 80–100 Å holes in the erythrocyte membrane.
$E^* \longrightarrow ghost+hemoglobin$	Lysis of erythrocyte and release of hemoglobin.

of mechanism will therefore be discussed initially in terms of this experimental model (Table 2).

Prerequisite to the hemolytic action of complement is appropriate sensitization with antibody. In the case of sheep erythrocytes (E) and rabbit anti-Forssman antibody (A), the efficiency of sensitization is considerably greater with γM (IgM) antibody, than with γG (IgG) (Talmage *et al.*, 1956; Stelos and Talmage, 1957), despite the calculation that the maximum number of binding sites per cell is 90,000 for γM antibody and 600,000 for γG antibody (Humphrey and Dourmashkin, 1965). Whereas only 2–3 molecules of γM antibody are required to initiate the eventual production of a hole in the erythrocyte membrane, a number some 1000-fold greater is necessary to initiate the production of a hole with γG antibody (Humphrey and Dourmashkin, 1965). The explanation of these observations is not yet clear but is central to the more general question of why antigen–antibody complexes provide a structure which permits interaction with complement components. The ability of heat-aggregated γG globulin to fix complement in the absence of antigen (Ishizaka and Ishizaka, 1959; Christian, 1960) might indicate that the function of antigen with respect to complement action is to effect a state of aggregation of γ globulin molecules with the requisite structural features, a concept which derives from an earlier hypothesis of Heidelberger *et al.* (1941). In the same connection, it is of interest that heat-aggregated Fc-fragment (Porter Piece III) fixes complement (Ishizaka *et al.*, 1962), suggesting that the structural requirement for complement interaction may be localized in this portion of the antibody molecule. However, evidence for a role for other portions of the γG globulin molecule has also been presented (Shur and Becker, 1963a, b; Reiss and Plescia, 1963; Cebra, 1963).

The earliest event in complement action is an interaction between the immune complex and the first component (C′1), a protein which provides an interesting example of subunit structure. Chromatography of a euglobulin fraction of normal human serum on DEAE cellulose in the presence of EDTA yields three discrete activities all of which are required for reconstitution of C′1 function. These subcomponents are designated C′1q, C′1r and C′1s in the order of their elution (Lepow *et al.*, 1963). C′1q was shown to be identical with a previously described serum protein, named the 11S factor on the basis of its sedimentation constant (Müller-Eberhard and Kunkel, 1961; Taranta *et al.*, 1961). Subsequent studies demonstrated that C′1 is a 19S macroglobulin which dissociates in the presence of EDTA into the subcomponents C′1q, C′1r and C′1s, with respective sedimentation constants of 11S, 7S and 4S. Upon recalcification, the subunits could be reassociated to form a complex resembling the intact macroglobulin and possessing, as a single reactant, the biological and biochemical properties of C′1 (Naff *et al.*, 1964).

The sensitized erythrocyte (EA) reacts with C′1, in the presence of Ca^{++},

to form an intermediate complex, EAC′1. The biochemical event associated with this interaction is the conversion of an enzyme precursor (C′1) to an active enzyme, usually referred to as C′1a or C′1 esterase (Lepow *et al.*, 1956; Becker, 1956). In the course of immune hemolysis, C′1 is activated on the surface of the sensitized cell, yielding the functional intermediate, EAC′1a. The portion of the C′1 molecule from which the enzyme site is derived is C′1s and an enzymatically active subunit, corresponding to activated C′1s (C′1 esterase), may be highly purified by column chromatography of C′1a (Haines and Lepow, 1964a). This subunit, however, will not attach to EA unless C′1q and C′1r are also present to permit reassociation of the complete macroglobulin. Thus, the site of combination of C′1a with EA is on a different subunit from that which carries the catalytic center and present evidence strongly implicates C′1q. Intact C′1a and the activated C′1s subunit are characterized functionally by their esteratic activity on such synthetic substrates as N-acetyl-L-tyrosine ethyl ester and *p*-toluenesulfonyl-L-arginine methyl ester and their ability to interact with the fourth (C′4) and second components (C′2) of complement, properties which appear to be associated with the same catalytic site (Haines and Lepow, 1964b).

A normal serum inhibitor of C′1a has been described (Ratnoff and Lepow, 1957; Levy and Lepow, 1959) and partially purified (Pensky *et al.*, 1961). It is a heat-labile, acid-labile α_2-globulin, having a sedimentation constant of about 3S, which stoichiometrically blocks the various enzymatic activities of C′1a. Since the rate of reaction of EAC′1a with the inhibitor is slower than the rate of interaction of EAC′1a with C′4 and C′2, complement function can proceed in the presence of a potent inhibitor (Lepow and Leon, 1962; Leon and Lepow, 1962). However, the ease of dissociation of C′1a from EAC′1a and the role of this enzyme in initiating events of the inflammatory response (*vide infra*) demand a control mechanism for preventing dissemination of C′1a. It appears likely that the serum inhibitor serves this function, as well as participating in maintaining C′1 in its precursor form in normal serum (Lepow *et al.*, 1965a).

C′4, the next component of complement to enter into the mechanism of immune hemolysis, has been obtained from normal human serum as a homogeneous protein by Müller-Eberhard and Biro (1963). It is a β globulin with a sedimentation constant of 10S which fulfills all functional criteria of C′4. Antisera to purified C′4 give a single precipitin line (β_{1E}) upon immunoelectrophoresis of human serum and such antisera may also be used to detect or inhibit C′4 on sensitized cells.

Interaction of EAC′1a with C′4 yields the intermediate complex EAC′1,4 in the absence of a divalent cation requirement (Laporte *et al.*, 1957; Klein, 1960). Formation of EAC′1a,4 from EAC′1a will not proceed if the enzymatic activity of C′1a is inhibited by diisopropylphosphofluoridate (DFP) (Becker, 1956, 1960; Müller-Eberhard and Biro, 1963) or antibodies to C′1a