PHARMACOLOGICAL REVIEWS
Vol. 26, No. 2

Printed in U.S.A.

Activation, Activities and Pharmacologically Active Products of Complement

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I. Introduction

UNLESS mediated by immune-competent cells immunological reactions are triggered by antibodies which combine with the antigenic agent. In most instances the antigen-antibody complex activates the effector system, known since Ehrlich as complement. In recent years complement has been recognized as a system of at least nine components, eight individual serum proteins and one, a complex of three proteins. When triggered by an antigen-antibody reaction they react in a fixed sequence with each other. Typically the reaction takes place at and is directed towards cellular membranes. During the intermediate phase of the complement reaction the cell membranes are changed leading to the phenomenon of immune adherence. If free, these cells are also easily phagocytized. When all of the complement components have reacted the cells are usually lysed. When cells containing biologically active substances such as platelets or mast cells are the target of a complement reaction these substances may be released and produce secondary effects.

The fixation and activation of complement is, however, not restricted to cell surfaces. Wherever in tissues antigen-antibody complexes are deposited, and even in solution, they can activate complement. Blood vessel walls are the sites of predominant pathological significance (Arthus reaction, glomerulonephritis) in which deposition of immune complexes and fixation of complement have been demonstrated by histological techniques (54, 57, 177, 199, 328, 350). Only when there is very close contact between the deposited immune complex and adjacent tissue cells will direct cytotoxic effects of the activated complement system occur. However, complement may produce tissue changes through a different mechanism by the release of pharmacologically active products. The activation of some complement components leads to the cleavage of peptide bonds resulting in fragmentation. The larger part of the molecule usually represents the activated complement component which remains with the complex of components that have already reacted, while the smaller peptide fragment is released into the fluid phase. Three such cleavage products have been shown to be pharmacologically active products. They may act as humoral mediators of immunological, complement-dependent reactions. Complement may thus act by its activated complexes upon cells, and through its by-products, humorally.

While the classical complement reaction begins at the site of an antigen-antibody complex with the fixation and activation of the first component, followed by consecutive reactions of the other components in strict order, other pathways exist which by-pass some components and include factors not belonging to the classical complement system. These "alternate pathways" are related to the properdin system discovered by Pillemer *et al.* (260, 263).

Most information about complement has resulted from studies of human and guinea pig serum. In general, other species have comparable components. They are usually but not throughout interchangeable among species.

The complement system is of interest to the pharmacologist because complement activity may be affected by drugs, and fascinating inherited and acquired abnormalities are known. A brief survey of the classical complement reaction and the activities of intermediate complement complexes will be given. Then the alternate mechanisms of complement activation will be described. Finally the pharmacologically active peptide products, released from complement components will be discussed.

II. The Classical Complement Reaction

As a result of the efforts of many laboratories the nine components of complement, the sequence of their reaction, and to some extent the mechanism of their activation and action have been elaborated. Most of the factors of human and guinea pig complement have been characterized, and some factors have been isolated in pure form. Some general properties of the components, enumerated in the order of the reaction sequence are shown in table 1. For detailed descriptions of the purification, properties, and reactions of individual components see references 212a, 223, 224, 237.

Complement components circulate in an inactive state. Ordinarily the classical system *per se* does not react with cells, even those foreign to the host, and free antibodies do not activate it directly. By the combined action of specific, antigen-bound antibody and the complement components an active effector system for cytotoxic reactions is generated with the target being determined by the antibody attached to the antigenic site.

Most reactions and activity of complement have been studied in the system of sheep erythrocytes (E) coated with specific antibody (A) forming the cellular complex EA. The presence and activity of an individual complement component can be assessed by estimating the degree of hemolysis resulting from its addition to sensitized cells suspended in an incomplete complement system which lacks the factor under investigation but contains all other components in excess. When components are altered by their specific reactions during complement activa-

TABLE 1

*Some properties of human complement factors, properdin factors and inhibitors**

Component	Molecular Weight (Daltons)	Electrophoretic Mobility	Approximate Serum Conc.	Major Fragments (activated components *in italics*)
			μg/ml	
Complement components:				
C1q	400,000	γ_2	190	
C1r	168,000	β		
C1s	79,000	α_2	120	
C4, β_1E	240,000	β_1	430	C4a, *C4b*
C2	117,000	β_2	30	*C2a*, C2b
C3, β_1C	185,000	β_1	1300	C3a, *C3b*
C5, β_1F	185,000	β_1	75	C5a, *C5b*
C6	125,000	β_2	60	
C7		β_2	Trace	
C8	150,000	γ_1	Trace	
C9	79,000	α	Trace	
Properdin factors:				
Properdin	223,000	β–γ_2	10–20	
Factor A=C3				
Factor B, C3PA, GBG	80,000–100,000	β_2	225	GAG, *GGG*
Factor C=C3bINA				
Factor D, C3PA convertase, GBGase	21,000†–35,000	α	Trace	
Inhibitors:				
C1INH	90,000	α_2	180	
C3bINA, KAF	100,000	β_2	25	
C6INA		β_1		

* Most data from reference 286a, other from 178, † from 335a.

tion, they are said to be "activated." If their presence is then tested in a subsequent complement estimation they appear to be "inactivated" or "consumed." Thus activation and inactivation of a complement component proceed simultaneously: specific reactions producing the activated component with loss of the original complement factor as measurable by immune hemolysis. Individual components may also be inactivated by nonspecific means (*e.g.*, heating, low pH, chemical treatment) which do not produce active factors.

A. Binding and Activation of the First Complement Component[1]

When most antibodies of the IgG and IgM class combine with their respective antigens, the Ig molecules are modified to form a binding site for the first complement component. C1 is a complex of three globulins C1q, C1r, C1s, and Ca^{++} ions (190). It is fixed to the Fc portion of the immunoglobulins with C1q carrying along C1r and C1s. Then C1r is activated to an enzyme which transforms C1s into an active esterase (C1 esterase, $\overline{C1s}$) (234, 241, 269). The esteratic activity of C1 is thus fixed in the immediate neighborhood of the antigen-bearing site such as the cell membrane of the sheep erythrocyte coated with antibody, forming the EAC$\overline{1}$ complex.

The existence of the C1 complex in serum, its binding to antibody, and its activation depend on the presence of Ca^{++}. When Ca^{++} is chelated by the addition of ethylenediamine tetraacetic acid (EDTA), the complex dissociates, C1s is not activated and no C1 activity is fixed—only inactive C1q (47, 233, 234).

B. Formation of the Intermediate, EAC$\overline{142}$

Fixed C$\overline{1}$ or free $\overline{C1s}$ react with C4 and C2. Both proteins are cleaved (213, 250, 265, 318) and a small peptide fragment is released from each [C4a (250), C2-derived "kinin" (169)]. The larger fragment of C4 is bound to the cell surface next to the site of C$\overline{1}$ fixation or to the antibody which links C$\overline{1}$ to the cell membrane. The complex is now designated EAC$\overline{14}$. If binding does not occur immediately after cleavage, the major C4 fragment is inactivated to form C4i in the fluid phase.

The major fragment of C2 is bound to C$\overline{4}$ in a stoichiometric ratio of 1:1 forming C$\overline{42}$ on the cell surface. Its formation has also been observed in the fluid phase (230). C2 can be cleaved slowly by $\overline{C1s}$ alone, but the efficiency is greatly enhanced by the presence of C4 (113). Since native C2 can also bind to C4 to yield an inactive complex it is probable that under normal conditions C2 is first bound to cell-fixed C$\overline{4}$, then cleaved by $\overline{C1s}$ to form C$\overline{42}$. The binding of C2 to C$\overline{4}$ and its activation depend on the presence of Mg^{++} (300). Several functionally active C$\overline{42}$ complexes can be fixed around each C$\overline{1}$ site amplifying the efficiency of bound antibody and C$\overline{1}$ (243).

C. Cleavage of C3. Fixation of C$\overline{567}$

For further reactions, C1 is no longer essential (14). The complex C$\overline{42}$, "C3 convertase" (230) reacts with C3 which is cleaved (213, 226, 296) into a major fragment, C3b, some of which is attached to the cell surface to form EAC$\overline{1423}$ or EAC$\overline{423}$ (68), and a smaller fragment, C3a, which is a biologically active peptide (74). Most of the C3b escapes immediate binding, and is converted in the medium to an inactive protein, C3i.

The intermediate EAC$\overline{423}$ has C5 cleaving activity (297, 298). This may reside in the C$\overline{42}$ complex, where C$\overline{2}$ seems to represent the enzyme (120), which is altered to acquire the new affinity by bound C3b. A biologically

[1] In accordance with the proposed nomenclature (Int. Arch. Allergy Appl. Immunol. 37: 661–664, 1970) the first, second . . . ninth components of complement are designated C1, C2 . . . C9. Activated components and intermediate complexes are denoted by a bar over the component: *e.g.*, C$\overline{42}$ for the activated enzyme, C3 convertase, in contrast to C42 for the reversible complex between the inactive second and fourth components; C1s = inactive subcomponent (proesterase) of C1; $\overline{C1s}$ = active C1 esterase. Fragments of complement components produced during activation are denoted by additional letters: *e.g.*, C5a (anaphylatoxin), C5b (major fragment of C5, activated form).

active peptide fragment, C5a, identical with Friedberger's anaphylatoxin (97), is released from C5. The major fragment, C5b, is fixed to the cell membrane. If present, C6 and C7 are bound simultaneously in a stoichiometric ratio of 1:1:1 to produce $C\overline{567}$ (172) and to stabilize the binding of C5 (64), since the intermediate complex $C\overline{1-5}$ is extremely unstable (23, 64, 117, 130, 145, 240, 297). In contrast, $EAC\overline{1-7}$ is a stable cellular intermediate. For further complement reaction $C\overline{1-3}$ is no longer essential. The complex $C\overline{567}$ provides the binding site for the last components.

D. Reaction of C8 and C9

The eighth component is bound to the intermediate $EAC\overline{567}$ ($EAC\overline{1-7}$), probably in a stoichiometric ratio of 1:1 (172, 275). At this stage the erythrocyte is irreversibly damaged and eventually undergoes lysis (200, 294a, 316). C9 which is bound subsequently at the site of C8 (172, 316) appears to further enhance the membrane change which produces cytolysis. According to Kolb *et al.* (172) up to 6 molecules of C9 can be bound by each C8 attached to the $C\overline{567}$ complex. In one model of this decamolecular complex at least three C9 molecules have been calculated to be necessary for full cytotoxic activity (172). Alternatively, Rommel and Mayer (274) concluded that only one molecule of C9 is sufficient to produce the cell membrane lesion, in accordance with the one hit theory of complement action (212).

Cytolysis follows the binding of C9 to animal cells. Recently, however, an additional factor has been found necessary for complement to damage bacteria such as *Escherichia coli*, namely a serum β- or early γ-globulin whose site of interaction is still unknown (123).

III. Properties, Activities, and Control of Intermediate Complexes

In addition to participation in the sequential complement reaction described in the proceeding section, some components and complexes have other properties and biological activities. Alternate mechanisms of activation also exist, and inhibitors are known which control the activity of complement intermediates.

A. C1 and C1 Esterase, $C\overline{1}$ Inhibitor, Hereditary Angioedema (HAE)

Nonspecific activation. Purified C1 or C1s can also be activated to $C\overline{1s}$ by treatment with plasmin or trypsin (191, 268). This indicates that C1s like trypsinogen or other proteases and esterases, is a zymogen which becomes active after cleavage of a peptide bond. Pillemer *et al.* (262) reported activation by the plasminogen-activator, streptokinase, of C1 esterase activity in whole serum. However, Laurell *et al.* (182) suggested that contaminants of the partially purified streptokinase preparations used in the experiments of Pillemer were reacting with natural antibody, inducing C1 activation, independent of plasmin. Some streptokinase preparations contain a pyrogen, probably an endotoxic lipopolysaccharide and, unlike highly purified streptokinase, these preparations induce anaphylatoxin (AT) formation in rat serum (238). The findings that the release of AT in rat serum by an impure bovine plasmin preparation was not blocked by antifibrinolysin (200a), and that highly purified human plasmin was inactive as an AT liberator (238) further argue against activation of C1 by plasmin. On the other hand the lack of C1 activation in whole serum by highly purified streptokinase may have reflected inadequate concentrations of the activator (268). It is also possible that the activation of C1 by plasmin *in vitro* is of little significance because of the abundant plasmin inhibitors and C1 inhibitor in whole serum.

C1 esterase can also be activated by Hageman factor (HF), coagulation factor XII (79). Evidence for $C\overline{1}$, consumption of C4 or cleavage of N-acetyltyrosine ethyl ester, did not appear in normal serum after treatment with HF, but it was present in angioedema serum which lacked $C\overline{1}$ inhibitor. Donaldson (79) suggested that HF acts on C1 indirectly,

proceeding either by intermediate activation of plasminogen or of prekallikrein. The effect of HF links the coagulation, complement, and kinin systems permitting synergistic action in some of their biological reactions.

Purified C1 can become active spontaneously when brought to pH 7.4 at ionic strength 0.15 (192). The activation of C1 after binding to immune complexes may also be regarded as autocatalytic.

In serum C1 is activated by treatment with ether (78). This effect results from the inactivation of $C\bar{1}$ inhibitor (see below). This may indicate that spontaneous, nonimmune activation of C1 occurs in whole plasma, but the $C\overline{1s}$ generated is immediately inactivated by $C\bar{1}$ inhibitor.

C1 may be activated by a variety of macromolecules. Early reacting complement factors are consumed during incubation with polyinosinic acid (poly I), and complement-dependent lysis is induced when serum, red cells, and poly I are mixed (372–374). Cellulose sulphate has also been reported to activate C1 esterase (84) and complexes between polyanionic and polycationic high molecular weight substances (*e.g.*, heparin-protamine, lysozyme-DNA, poly I:C) can activate C1 in serum. Depending on the ratio of the two components forming the complex, C4, C2, and C3 are also consumed (104, 363). Since this activation is prevented by EDTA, it resembles that induced by immune aggregates. The shock and vasculitis produced by the intravenous injection of poly I:C may be related to complement activation (142).

Inhibition. Both the activation of C1 as well as the activity of C1 esterase can be inhibited. Binding of C1q to antibody is inhibited by carrageenin (35, 72) and the initiation of the complement reaction is prevented. This seems to be a general property of sulphated polysaccharides since it is seen also with heparin and heparinoids (181, 342). Also poly I blocks Fc-binding of C1q (372–374) but it activates C1 in the fluid phase (374) as noted above.

ϵ-Aminocaproic acid (ϵ-ACA) interferes with activation of C1 (322) just as it blocks plasminogen activation. Both $C\overline{1s}$ and plasmin once active are not inhibited by ϵ-ACA (322). The synthetic substrate *p*-tosyl-L-arginine methyl ester acts as a competitive inhibitor of $C\bar{1}$ interfering with its action on C2 (317). $C\bar{1}$ activity is irreversibly blocked by high concentrations of diisopropylfluorophosphate (12, 267), but the nonactivated C1 molecule is unaffected. Unlike plasmin and serum kallikrein, $C\overline{1s}$ is not significantly inhibited by soybean trypsin inhibitor (79).

Serum contains a $C\bar{1}$ inhibitor ($C\bar{1}$ INH), an α_2-glycoprotein which blocks the esteratic, as well as C4 and C2 cleaving activity of $C\bar{1}$ in the fluid phase and in the intermediate $EAC\bar{1}$ (164, 253). It is identical with α_2-neuraminoglycoprotein (253a). $C\bar{1}$ INH is relatively inactive against plasmin, thrombin, trypsin, and chymotrypsin (253), but it inhibits serum kallikrein (156). $C\bar{1}$ INH is destroyed at temperatures above 48°C, and at pH below 5.5 to 6 (194, 253), or by treatment with ether (78, 164) or methanol (194). Inhibition of $C\bar{1}$ by $C\bar{1}$ INH results from enzyme-substrate interaction (203), and is irreversible.

$C\bar{1}$ INH serves to inactivate any $C\bar{1}$ released into the circulation. Genetic deficiencies of $C\bar{1}$ INH cause recurrent HAE (80) after any process which initiates C1 activation.

Enzymatic activity. The natural substrates for $C\bar{1}$ are C4 and C2. These two complement components are also cleaved by free $C\overline{1s}$ in the fluid phase. $C\overline{1s}$ affinity to C2 is weak unless C4 or C4i is also present (113). The susceptible bonds in C4 and C2 may be esters or peptide linkages. $C\overline{1s}$ is trypsin-like in its actions in that it cleaves esters of substituted amino acids (13, 202, 267) but no nonspecific proteolytic actions have been described.

The esterase activity of $C\overline{1s}$ is independent of Ca^{++} (13) suggesting that Ca^{++} in the C1 complex serves to keep C1s attached to C1q and C1r. The esterolytic activity is optimal between pH 7.5 and 8.2 at 41°C, with an ionic strength of less than 0.2 (267).

Biological activity. C1 activation may in-

duce inflammation even without completion of the complement reaction sequence. Intradermal injection of $C\overline{1s}$ in man or the guinea pig induces development of a local wheal and extravasation of previously injected Trypan blue dye (167, 168, 267a). The same signs of increased vascular permeability are seen when C1 is activated *in vivo*, by subcutaneous injection of polyinosinic acid (375). The increase in permeability occurs predominantly on the venous side of the capillaries and in the venuoles (267a). The vascular effect of C1 requires C2 and probably also C4, but no further factors. Patients genetically lacking C2 do not show a comparable vascular response to $C\overline{1s}$ (167, 168).

$C\bar{1}$ INH, but not soybean trypsin inhibitor prevents the vascular effect of $C\overline{1s}$ (267a). In man the permeability increase is not mediated by histamine. The wheal formation and blueing reaction are not prevented by antihistaminics and they develop in histamine-depleted skin (168). In guinea pigs, however, triprolidine was found to be inhibitory (267a).

In vivo activation of C1 must occur frequently, but, once activated, $C\bar{1}$ activity is controlled by $C\bar{1}$ INH. Direct evidence for the presence of active $C\bar{1}$ in guinea pig serum has recently been obtained by Loos *et al.* (201). Spontaneous activation is further suggested by the findings that a patient with C1r deficiency had elevated levels of C4 and $C\bar{1}$ INH (257), and that deficiency of $C\bar{1}$ INH can lead to hereditary angio (neurotic) edema[2] (HAE) which represents the uncontrolled effect of $C\overline{1s}$. This disease is clinically characterized by episodic attacks of nonpitting edema formation in local areas of the skin or mucosal surfaces including the gastrointestinal tract. When the latter is involved abdominal pain may result, and edematous swelling of glottis and larynx may be life-threatening. Attacks may last for up to two days and then slowly subside. The disease may result from complete absence of the $C\bar{1}$ INH protein, or from a structural defect causing functional deficiency, both genetically determined (215, 278).

Patients investigated by Donaldson and Evans (80) and Donaldson and Rosen (83) and by Granerus *et al.* (126) showed free C1 esterase activity in their plasma during attacks, no $C\bar{1}$ INH, and very low titers of C4 and C2. During clinical remissions, free esterase activity was absent or barely detectable, but C4 and C2 activity remained low. More esterase activity appeared after *in vitro* storage of the serum (80, 82, 126).

A patient of Lundh *et al.* (204) differed in that some functionally active $C\bar{1}$ INH was demonstrable in fresh plasma even when taken during attacks. The inhibitor level fell, however, when plasma was allowed to stand *in vitro*. Simultaneously, C1 esterase activity appeared. In the patient of Lundh *et al.* (204) an accelerated rate of activation of C1 may have produced the disease rather than lack of $C\bar{1}$ INH since treatment with ϵ-ACA prevented attacks in this patient. ϵ-ACA has not been therapeutically effective in all patients, however (204).

The basis for the intermittent nature of the clinical symptoms in HAE is not known. Attacks may not only follow local trauma, but may accompany psychogenic or physical stress. Donaldson and Evans (80) and Donaldson and Rosen (83) suggested that plasmin which can be activated by stress might cause C1 activation. Also kallikrein, activated by Hageman factor may play a role (79). HAE plasma was found to contain elevated kinin levels (319) which reflects the fact that $C\bar{1}$ INH is also an inhibitor for plasma kallikrein. Bradykinin could cause the capillary permeability associated with flare-ups of the disease. However, serum bradykinin was found to be elevated in HAE patients even in asymptomatic intervals (8) raising the question whether its appearance is only coincidental.

The increase in capillary permeability during HAE attacks could also be due to the action of a peptide fragment, "C2 kinin,"

[2] Since the cause of the disease was discovered it has been suggested to omit the term "neurotic."

released from C2 by $C\overline{1s}$, as will be discussed in section V A.

Whether C1 esterase is activated by nonimmunological means in normal individuals and has some physiological function, *e.g.*, control of vascular permeability, is not known. The finding that HAE patients have low C4 even when asymptomatic and in the absence of demonstrable $C\overline{1s}$ activity suggests that such activation does occur. If not spontaneous, the activation may be triggered by miniature immune reactions in blood, by local plasmin activation at sites of blood clotting, or by HF activation.

B. $C\overline{14}$ Intermediate

Other than efficiently cleaving C2, no specific enzymatic activities of $C\overline{14}$ are known. However, this intermediate has the ability to "neutralize" the infectivity of Herpes simplex after the virus particles have been coated with specific IgM antibody (71). In the presence of C1 and high concentrations of C4 these sensitized particles are inactivated. Later-reacting complement components are then not required for neutralization. When only low concentrations of C4 have been applied, C2 and C3 will increase the neutralizing effect. The inactivation may reflect covering of the virus surface with complement components thus impairing reactivity of the virus with its target cells. Whereas antibody and C1 probably are insufficient to block viral infectivity because of a limited number of antigenic sites, the many molecules of C4 fixed by one active C1 molecule could greatly increase the amount of surface covered.

Inactivation by this means may also provide protection from other viral infections and from adverse effects of foreign particles which cannot be lysed. When heavily loaded with C4 red cells show the phenomenon of immune adherence (60).

C. $C\overline{42}$ Complex

This complex consisting of activated $C\overline{4}$ and $C\overline{2}$ (C4b and C2a) is also known as "C3 convertase" (230). $C\overline{42}$ forms not only at the site of cell-bound $C\overline{1}$ but also in the fluid phase, when C1 esterase acts on free C4 and C2 (230). The soluble complex also cleaves C3, but the activated fragment C3b cannot then be transferred to normal cells although it may participate in further complement reactions in the fluid phase (117).

Though Mg^{++} is necessary for the formation of the $C\overline{42}$ complex, elimination of Mg^{++} by EDTA does not dissociate it or inhibit its C3-cleaving potency. The complex is, however, subject to spontaneous decay with time. In the fluid phase as well as on the cell, $C\overline{2}$ dissociates from $C\overline{4}$ and is irreversibly inactivated. Provided $C\overline{1s}$ and Mg^{++} are present, new C2 can be activated and combine with the C4 residue, regenerating the C3 convertase activity. In the case of human complement the stability of the convertase is greatly enhanced when the C2 used has been treated with iodine (264).

D. $C\overline{423}$ Complex, C3b Inactivator

With the attachment of the $C\overline{3}$ component, C3b, to membrane-bound $C\overline{42}$ the cells acquire new properties. They show immune adherence by sticking to normal erythrocytes, leukocytes, or bacteria. Close contact with leukocytes is essential for phagocytosis, and thus target cells bearing $C\overline{1423}$ (or $C\overline{423}$) are phagocytized (opsonized) more readily than normal cells (115, 138). Cell-fixed $C\overline{3}$ is sufficient for immune adherence and opsonization and $C\overline{1}$, $C\overline{4}$, and $C\overline{2}$ are dispensable after the generation of the C3b fragment on the cell surface.

The cell-fixed $C\overline{423}$ is an enzyme whose only known natural substrate is C5, the next complement component in the sequence. C5 is cleaved to yield a pharmacologically active peptide fragment, C5a (anaphylatoxin), and a major fragment, C5b, which represents the activated fifth complement component (55, 120, 297, 298). In addition, $C\overline{423}$ can split the synthetic peptides, glycyl-tyrosine and glycyl-leucyl-tyrosine (63). The whole complex $C\overline{423}$ is required for enzymatic activity. Neither $C\overline{42}$ nor $C\overline{43}$, remaining after spontaneous decay of $C\overline{2}$, can cleave C5 or gly-

tyr. The enzymatic site probably resides in the $C\overline{2}$ portion (120) which is altered by the presence of $C\overline{3}$ to acquire new specificity. For this reason, the complex has also been called "C3-dependent" peptidase.

The aromatic amino acid esters and peptides which are substrates for the peptidase inhibit C5 activation (11, 297, 352). This suggests that the complement function of $C\overline{423}$ is related to the hydrolytic activity. However, immune adherence and hemolytic activity of cell-bound $C\overline{3}$ are not always proportional to the peptidase activity (63), and once formed $EAC\overline{423}$ continues to cleave gly-tyr for hours although decay of $C\overline{2}$ and loss of hemolytic activity occurs much more rapidly.

$C\overline{42}$ produced by $C\overline{1s}$ in the fluid phase readily reacts with C3, but the resultant products do not cleave C5 efficiently (76). Additional factors such as those found in guinea pig serum (59) may be essential to enhance the peptidase activity. In principle cleavage of C5 by fluid phase $C\overline{42}$ and $C\overline{3}$ is possible (117), but cleavage by $C\overline{423}$ is obviously facilitated when the complex is fixed to a cell membrane. It is possible that $C\overline{42}$ and $C\overline{3}$ do not form a true complex in solution. A $C\overline{423}$ complex in solution has not yet been demonstrated. On a membrane many molecules of $C\overline{3}$ can be fixed by and near to one $C\overline{42}$ unit (226). This may provide better cooperation. Little is known at present about the exact nature of the functional intermediate $C\overline{423}$ or its relationship to the peptidase activity.

Inactivation. Although membrane-fixed $C\overline{3}$ is much more stable than C2, it is slowly degraded by C3b inactivator [C3b INA, conglutinin-activating factor, KAF; see also section IV E 7)], a β-globulin of plasma (176, 283, 287, 320). The phenomenon of adherence and the potency of the $C\overline{423}$ complex to cleave and activate the next complement component, C5, are then lost, but a new property is gained. Cells in the intermediate stage $EAC\overline{423}$ (or $EAC\overline{1423}$) and treated with C3b INA clump together in the presence of conglutininogen, a serum protein occurring in bovine serum but lacking in human serum (176). Conglutination indicates that $C\overline{3}$ is not detached by the action of C3b INA, but is altered to expose a new site. A fragment, C3c, is released from the cell-bound $C\overline{3}$ (C3b) whereas the other split product of C3b, designated C3d, remains fixed and confers on the cell the potency to react with conglutininogen (285). Fluid phase C3b is also cleaved by C3b INA into the same fragments.

E. $C\overline{567}$ Complex, Chemotaxis, Blood Clotting, C6 Inactivator

When C5 is cleaved by $C\overline{423}$, the major fragment, C5b, binds to C6 and C7 forming a trimolecular complex which fixes to cell membranes eventually leading to cytolysis (172). A stable $C\overline{56}$ complex can also form in the fluid phase and combines with C7 if present to give a soluble $C\overline{567}$ complex. Fluid phase $C\overline{567}$ can be transferred to red cells not carrying antibody or early reacting complement components. Such sensitized cells ($EC\overline{567}$) are just as susceptible to cytolysis by C8 and C9 as are cells prepared by the complete sequential reaction (117, 172, 179, 323). "Reactive lysis" (179, 323, 324) is induced when red cells, activated serum containing $C\overline{56}$ complexes in excess of $C\overline{567}$ and normal serum containing free C7 are brought together. Preformed $C\overline{567}$ rapidly loses the ability to fix to cell membranes (half-life less than 1 minute at 30°C), but the complex is otherwise stable; it is able to react with C8 and C9 in the fluid phase (121, 122, 179) and is chemotactic.

One of the most important biological functions of complement is the attraction of neutrophilic leukocytes to the site of an immune reaction. The peptides, C3a and C5a, and free $C\overline{567}$ all contribute to complement-derived chemotactic activity (352). Boyden (36) by using an *in vitro* assay first showed that serum in which complement had been activated, acquired chemotactic activity. Leukocytes suspended in the upper portion of a two-compartment chamber move actively through a Millipore filter

toward the lower compartment when the latter contains chemotactic factors. The number of leukocytes which appears on the lower side of the filter is counted. This method has allowed quantitation of the role of complement in leukocyte chemotaxis.

At first the evidence for $C\overline{567}$ being chemotactic was indirect. Normal rabbit serum, but not serum from animals deficient in C6, developed chemotactic activity when incubated with immune complexes or zymosan (351). C6-deficient serum could be reconstituted by addition of purified human C6 which was inactive by itself. Chromatographic fractions of normal rabbit serum or euglobulin fractions, rich in C5 and C6 and containing C7, developed chemotactic activity when treated with stromata of $EAC\overline{1-3}$. Ultracentrifugation separated chemotactic activity into those fractions containing C5 and C6 with a high rate of sedimentation compatible with a heavy complex (351, 359). Under conditions causing dissociation of protein complexes the chemotactic activity was lost (352). Not only anti-C5-, but also anti-C6-serum reduced the chemotactic activity (359). Since in human serum $C\overline{56}$ readily takes up C7, it is probable that the chemotactic factor contained C7 in addition to activated C5 and C6.

Red cells coated with antibody and reacted sequentially with purified complement components formed chemotactic activity in the supernatant only when the intermediate stage $EAC\overline{1-7}$ was reached. After ultracentrifugation of the supernatant, the activity was recovered in a rapidly sedimenting high molecular weight fraction (349, 352). Although the leukotactic activity of $C\overline{567}$ was questioned by Keller and Sorkin (162), direct evidence was obtained by Lachmann *et al.* (175) who prepared $C\overline{567}$ from purified $C\overline{56}$ and C7 and demonstrated its chemotactic activity.

Chemotactic activity has been detected in plasma after intravenous injection of complement-activating substances such as zymosan or aggregated human γ-globulins (352). The action of $C\overline{567}$ is probably very important for the accumulation of polymorphonuclear leukocytes at the site of immune complex-induced lesions. It is clear, however, that $C\overline{567}$ is not the only complement-derived chemotactic agent. Peptides derived from C3 and C5 have similar properties, and may also contribute to immunologically-induced leukocyte infiltration *in vivo*. Except where stated, the cause of the following complement-related phenomena may not be limited to $C\overline{567}$.

The role of complement in leukocyte infiltration of immune disorders has been studied in two experimental animal models and one human disease: the vasculitis of the reversed passive Arthus reaction in rats and guinea pigs, characterized by leukocyte infiltration at the site of a subcutaneous injection of antibody subsequent to intravenous injection of antigen; the nephrotoxic nephritis of rabbits, rats or mice, resulting from injection of antibody directed against the host's basement membranes of the glomerular vessels; and the acute inflammatory exudate in synovial fluids of patients with human rheumatoid arthritis. The perivascular leukocytic infiltration in these three disorders is associated with complement activation. Complement has been detected histochemically in the vascular walls where immune aggregates are deposited (54, 350). Depletion of complement in the blood inhibits leukocyte infiltration and other inflammatory symptoms, but not the deposition of γ-globulin aggregates. The inhibition lasts about 6 hours. After this time the complement titer in plasma again rises (350). Extracts of vasculitis lesions have chemotactic activity for neutrophils which is blocked by anti-C5- and occasionally by anti-C6-serum. Ultracentrifugal analysis and studies with labelled C5 indicate that the chemotactic activity is present in a low molecular weight factor, C5a, and a heavier complex, probably $C\overline{567}$ (356).

Nephrotoxic (Masugi) nephritis develops in two phases. The first is the consequence of deposition of the antibody injected which

binds to the glomerular basement membrane leading to complement fixation. After about 1 week, the host's newly-synthetized antibody then appears and is bound to the γ-globulin which had been injected and was deposited, and now reacts as fixed antigen in the glomerular vessels. At both stages inflammation occurs, being particularly severe during the second stage when leukocytic infiltration is extensive (281, 328). Elimination of neutrophils strongly reduces the severity of the inflammatory reaction (57). In rats depletion of complement components largely prevents leukocyte accumulation (57). It is, however, doubtful whether $C\overline{567}$ or even C5a are essential for the leukocyte chemotaxis of Masugi nephritis in all species. Rabbits deficient in C6 react to nephrotoxic serum with as much leukocyte infiltration in glomerular vessels and similar nephritis as do normal animals (281). C5 deficient mice also develop nephrotoxic nephritis with leukocyte accumulation. The overall nephritis is similar, or only a little less severe, than that obtained in normal mice (197, 328).

Joint fluids taken from patients with rheumatoid arthritis contain the chemotactic factors C5a and $C\overline{567}$ (380). Rheumatoid synovia produces γ-globulins and the synovial fluid of rheumatoid factor-positive patients contains reduced titers of complement as well as inactivated complement components. In addition, Winchester *et al.* (363a) have shown the presence of a complement-activating complex in rheumatoid synovial fluids. These findings suggest an immunologically-mediated complement activation (380).

The leukocytes which have entered the joint space by chemotaxis may in turn release a lysosomal enzyme which cleaves C5 and generates further chemotactic activity (307, 321, 354). The C5 cleaving enzyme has also been detected in rheumatoid synovial fluids (359). It is inhibited by ε-ACA, soybean trypsin inhibitor or *p*-tosyl-L-arginine methyl ester but not by N-acetyltyrosine ethyl ester (354). Thus the inflammatory process may continue, independent of the original immunological inducing factor.

Synovial fluids from some other types of arthritis contain C5 cleaving and chemotactic activity less frequently. It was absent in synovial fluids from four patients with systemic lupus erythematodes and in fluids from patients with osteo-arthritis. In the joint fluid from patients with lupus arthritis the leukocytes present were predominantly monocytes, with few neutrophils and no detectable C5 cleaving enzyme (359). The absence of both neutrophils and chemotactic activity in lupus joint fluid emphasizes the probable role of antibody, complement-related chemotaxis, and neutrophils in the pathogenesis of rheumatoid arthritis.

DeShazo *et al.* (73) have induced an experimental arthritis in rabbits by injecting antigen intravenously after an injection of antibody into the knee joint. A two-phase increase in local vascular permeability and leukocyte infiltration were observed which depended on complement, since the severity of the symptoms was reduced markedly in animals with C3 and/or C6 deficiency. The increase in permeability was absent when leukocytes had been eliminated from the blood indicating that chemotaxis was a prerequisite event.

Thus the ultimate effect of $C\overline{567}$ and other chemotactic factors is to bring neutrophils with their biological potencies to the site of the lesion. They phagocytize or adhere to the immune aggregates in the vessel walls in a reversed Arthus reaction or in nephrotoxic nephritis. In both cases the leukocytes release lysosomal enzymes which in turn are responsible for tissue necrosis and other pathological events (139).

The mechanism by which $C\overline{567}$ induces positive chemotaxis is unknown. Ward and Becker (347) have detected two esterases in neutrophils which cleave aromatic amino acid esters and are significant for the chemotactic response. One is active *a priori*, and is inhibitable by alkyl phosphonates which block serine esterases. Phosphonate-treated neutrophils do not respond to chemotactic

stimuli. The other esterase is present as a proenzyme, and is activated by exposure of the cells to $C\overline{567}$. It is confined to neutrophils (349). This activatable esterase, later called esterase 1, is blocked by phosphonates only after activation (16, 349).

Leukocytes which have been pretreated with $C\overline{567}$ are deactivated, and no longer respond to further chemotactic stimuli. Substrates of the enzyme, aromatic amino acid esters, protect against deactivation. On the other hand, these esters reversibly inhibit chemotaxis itself. These findings suggest that activation of esterase 1 and its action on an exhaustible substrate are significant steps in induction of the chemotactic response (348, 349). Activation of esterase 1 is also involved in neutrophil chemotaxis induced by C3a, C5a and bacterial factors (15).

The plasma of rabbits genetically deficient in C6 shows impaired blood clotting with prolonged clotting times and reduced prothrombin consumption (377). The defect is corrected by the addition of small amounts of purified C6. Inulin, endotoxin, and other agents known to activate complement without significantly involving the early components C1, C4, and C2, accelerate the clotting of normal, but not of C6-deficient rabbit plasma. Acceleration of clotting by kaolin is normal in C6-deficient rabbit plasma and then no further acceleration is induced by inulin in normal or C6-deficient plasma (378). The results indicate that in the rabbit complement activation promotes blood clotting, independent of Hageman factor, and that C6 is involved. This function of complement may enhance the intravascular clotting produced by the repeated injection or prolonged infusion of endotoxin (generalized Shwartzman-Sanarelli reaction).

A C6-inactivating factor (C6 INA) has been described and purified from serum of various species (320). It is a rather heat-labile protein, possibly an enzyme, which does not attack native C6 in plasma but inactivates it in $EAC\overline{1-6}$ so that hemolysis cannot be brought to completion by subsequent addition of C7–C9. Whether C6 INA inactivates soluble $C\overline{567}$ is unknown.

IV. Alternate Pathways to Complement Activation

Complement can be activated without following the classical, antibody-initiated sequence described in section II. The early reacting components, C4 and C2, may be largely by-passed, and complement reaction begun at the level of C3 activation. These alternate pathways depend upon separate enzymatic activities which cleave C3 replacing the C3 convertase, $C\overline{42}$. Because of the dispensability of the early factors, some natural processes were, at one time, considered not complement-dependent.

Several by-pass systems triggered by different agents seem to be related since they involve factors known to participate in the properdin system. As their exact relationship and complete reaction sequence is not yet known, these alternate complement activation systems will be described separately. Then the factors involved will be discussed, and finally an attempt will be made to interrelate the different pathways.

A. The Properdin System

1. Original description. About 20 years ago Pillemer and his colleagues discovered that the long-known inactivation of serum complement by yeast cells, zymosan,[3] or bacterial cell walls was not due to mere adsorption of C3t,[4] as had been assumed since the study of Coca (53), but to a specific inactivation of C3t by a complex system (259, 260, 263). A new serum factor, properdin, was recognized and partially purified. Properdin

[3] Insoluble cell wall polysaccharide preparation of yeast.

[4] When the action of yeast and of properdin was discovered the late complement components C3 and C5 to C9 had not yet been recognized as separate entities but appeared as "the" third component. This group as a whole is now called C3t ("terminal"). Although the properdin system inactivates mainly C3 and C5, it also inactivates later complement components.

was fixed from human serum by zymosan and other polysaccharides at temperatures above 15°C. Incubation with zymosan at 17°C depleted human serum of properdin but C3t activity was not lost at that temperature. At 37°C C3t was inactivated. Properdin-depleted serum (RP) retained its C3t activity even when incubated at 37°C with a fresh portion of zymosan. The lost inactivating potency was restored when properdin preparations obtained by elution of serum-treated zymosan were added to RP. Zymosan treated with human serum at 17°C (or higher temperature) had C3t-inactivating potency when washed and added back to RP. These findings indicated that properdin formed a C3 cleaving complex adherent to zymosan, called PZ which was essential for C3 depletion. Additional findings revealed that properdin was different from known complement components and that two other serum factors called A and B, and Mg^{++}, but not Ca^{++}, were essential for properdin to be fixed and to form the active complex (260, 261). Factor A was a serum protein resistant to heating at 56°C and was inactivated by hydrazine. Factor B was extremely heat-labile being inactivated at 50°C (261).

Pillemer suggested that properdin and its co-factors represented a system whose activation was independent of specific antibodies against zymosan or bacterial cell walls which acted directly on C3 without participation of the early complement components. Its assumed significance was the elimination of bacteria, other micro-organisms and abnormal blood cells such as paroxysmal nocturnal hemoglobinuria (PNH) erythrocytes, by a nonspecific activation of the complement system (260). Correlations were found between the fall and rise in the serum properdin titer, *in vivo*, and resistance to bacterial infections. Endotoxins, known to unspecifically increase the defense capacity against infections were shown to increase the properdin concentration in serum after an initial fall (259, 260). Increased resistance was associated with the ability to maintain a high properdin level (180).

A different interpretation of the zymosan-induced complement consumption was given by Nelson (235). He proposed that properdin was a natural antibody against zymosan, and that active PZ complexes consisted of antibody-bound $C\overline{14}$ and $C\overline{142}$ which would act on C3 analogous to the red cell intermediate $EAC\overline{142}$. A review of the original properdin system and possible interpretations has been given by Lepow (185).

Only in recent years have unequivocal new findings clearly shown that properdin really exists as an entity and is involved in an alternate mechanism for activation of the late complement components. Studies on C3 inactivation and on anaphylatoxin formation in serum by endotoxins, zymosan, and other polysaccharides and by a factor of cobra venom have proved that properdin and its co-factors A and B are specific proteins differing from antibodies, C4 and C2, and that the C3 cleaving complex of the activated properdin system cannot be equated with the C3 convertase $C\overline{42}$ of the classical complement system.

2. Recent investigations of complement activation by zymosan; anaphylatoxin (AT) formation. When introduced into rat, guinea pig or hog serum, polysaccharides, such as inulin, starch, dextran or zymosan, induce AT formation. Several findings suggested that the release was the consequence of complement activation. Other observations, however, were at variance with this assumption. It was long known that Ca^{++} ions are not essential for AT formation in serum by polysaccharides (111). Later it was shown that C4 and antibodies against the triggering polysaccharide were probably not involved, and that C2-deficient human serum possessed the potential for normal AT formation (39, 40, 339).

The serum factors involved in AT release were fixed to zymosan upon its incubation with whole serum. For the formation of these active zymosan complexes, a factor resembling properdin in its properties was found to be essential. Properdin-depleted serum was unable to coat zymosan with an AT

releasing complex unless reconstituted with properdin. The formation and maintenance of activity of the active complex required Mg^{++}, but not Ca^{++}, and an unidentified heat-labile factor (41) later identified functionally as properdin factor B (205). Thus the properdin system was involved in AT formation by zymosan and probably other polysaccharides. Evidence obtained from studies of other systems indicates that it is the activated factor B in the complex which cleaves C3 and C5 (see below and sections IV A 3 and IV C); from the latter the spasmogenic principle known as classical anaphylatoxin is released.

Further proof for the existence of the properdin system as different from classical complement has come from the demonstration that a typical classical complement complex which cleaves C5 and releases AT can only be fixed on zymosan under clearly different conditions (39). The formation of the classical complex first requires fixation of antizymosan antibodies, and acquires activity during further incubation with human serum only in the presence of Ca^{++} and C2. During the incubation of antibody-coated zymosan with human serum large amounts of C2 are consumed, in contrast to the formation of the complex, in which properdin and factor B are essential. It is possible to experimentally distinguish between the two zymosan-bound systems: 1) Addition of ethyleneglycol-bis-(aminoethyl-ether)-N,N′-tetraacetic acid (EGTA) to serum inactivates the classical complement system by selectively binding Ca^{++}, but does not block activation of the properdin system; 2) heating human serum for 30 minutes to 50°C inactivates the properdin system because its factor B is heat-labile. The classical complement activity is retained although some C2 is lost.

Zymosan-bound properdin complexes undergo spontaneous decay. Whereas decay of classical complement complexes is related to loss of C2, it is activated factor B which dissociates from the properdin complex when it becomes inactive. Incubation of decayed zymosan complexes with additional factor B regenerates the capacity of the complex to cleave C3 and C5 (38, 41, 205).

Factor B cannot be fixed directly to zymosan or other polysaccharides. Properdin and at least two other noncomplement components, the already mentioned factor A and a recently discovered factor D are necessary for its fixation and activation (38a, 125, 144, 228, 238a, 277). (For synonyms see table 2.) These factors are also activated and fixed to zymosan during its incubation with serum (38a, 238a). Slow release of factor D from the complex contributes to the decay of activity (38a).

Kinetic studies of Brade *et al.* (38) have shown that addition of factor B to decayed zymosan complexes regenerates C5 cleaving activity much more readily than C3 cleavage. Apparently, the enzyme complex which cleaves C3 requires different structural characteristics than that cleaving C5. In this contest it may be relevant that zymosan complexes from C2-deficient human serum show only minimal C3 cleaving activity (114), although their C5 cleaving potency, measured as AT release, is normal (39).

Attempts to sequentially fix components of the properdin system to zymosan have not been successful. Activated factor B would appear to be the last reactant in the generation of the complex, which cleaves C3, but it seems also to be involved in the fixation of other factors since treatment of zymosan with serum lacking factor B does not lead to substantial uptake of properdin (187), and if followed, after washing, by incubation with purified factor B gives only poor C3-cleaving activity (205). The exact sequence of events during the generation of C3-cleaving activity on zymosan is still unknown. Additional information about the properdin system has come from other investigations described below.

3. The C3-activator system. Götze and Müller-Eberhard (118) purified a human serum protein which could be activated to produce an enzyme which would cleave C3 to form C3b. They called the serum proen-

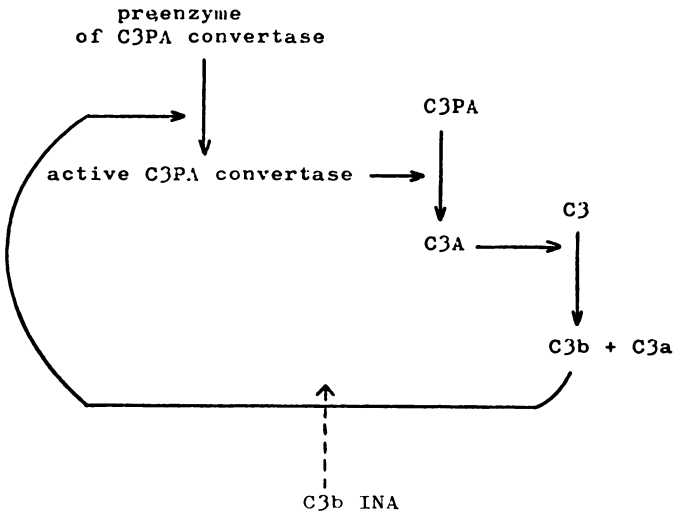

FIG. 1. Reaction scheme of the C3 activator system (118), feedback mechanism of C3b (228), and control of the system by C3b INA (239). The relationship of the components to properdin factors is shown in table 2.

zyme C3 proactivator (C3PA). This factor was discovered and originally studied as a serum co-factor necessary for the complement-consuming effect of cobra venom (229, 236) (see section IV E 7). However, it was then found that the same principle was involved in complement reactions activated by inulin. Since the relationship between C3PA, properdin factor B and serum co-factors for the cobra venom factor has been controversial, the C3PA system will be described separately.

The conversion of C3PA to the actual C3 activator, C3A, is accompanied by cleavage of the molecule (118). An activating enzyme, C3PA convertase, proved to be an α-globulin which was partially purified from human serum (228). C3PA convertase became active only after reaction with C3b. C3b is, on the other hand, the main product of the C3 activator system when it cleaves C3. Therefore, a positive feedback mechanism involving C3b exists (fig. 1) which causes increasing activation of the C3 activator system once traces of C3b are present (228). In fact, elimination from serum of the protease (C3b INA) which normally inactivates C3b once released is sufficient to activate the system (178, 239).

Recent evidence indicates that the C3PA system is identical with the properdin system, as far as its activation by polysaccharides or microbial cell walls is concerned (119). C3PA is both activated and fixed to yeast cell walls with no requirement for specific antibody to the activating agent, or for the participation of the early classical complement components (118). C3PA has been functionally identified with factor B of the properdin system and immunochemically with glycine-rich β-glycoprotein (GBG) (118, 124). The activation of C3PA by polysaccharides requires additional serum factors and divalent cations such as Mg^{++} (118). C3b, essential for the activity of C3PA convertase, is the activated form of Pillemer's factor A (228). C3PA convertase is probably the same as properdin factor D (see section IV E 3). Synonyms of the various factors are collected in table 2.

4. Activation by lipopolysaccharides. Spink and Vick (311) discovered that the vasoconstrictor and shock effects of Gram-negative bacterial endotoxins, the lipopolysaccharides (LPS) of bacterial cell walls, are dependent upon the activation of a heat-labile serum factor. They suggested that complement was the mediator of shock, and later a

TABLE 2
Factors involved in the properdin system, synonyms, and identity with known serum proteins

Factor	Synonym	Identical with
Properdin		
Factor A	Hydrazine-sensitive factor (HSF)	C3
Activated factor A	HSFa	C3b
Factor B	C3 proactivator (C3PA), unknown factor (UF), heat-labile factor (HLF)	Glycine-rich β-glycoprotein (GBG)
Activated factor B	C3 activator (C3A)	β_2-glycoprotein II, glycine-rich γ-glycoprotein (GGG), a-fragment of GBG
Factor C		Conglutinin-activating factor (KAF), C3b inactivator (C3b INA)
Factor D	C3PA convertase, C3PAase, GBGase	

reduction of complement titers in serum after contact with LPS was demonstrated, both *in vitro* and *in vivo* (116, 173, 217, 231, 251, 310). LPS can also generate anaphylatoxin (129, 195, 238) and chemotactic activity (303) in serum. Whole serum can cause lesions in LPS-containing membrane preparations which resemble the cytotoxic effects of complement on antibody-sensitized red cells (24).

Early investigators assumed that LPS triggered a classical complement reaction by first reacting with specific natural antibody. Complement was *not* consumed by LPS in precolostral piglet serum devoid of antibodies, unless antibody to the LPS used was supplied (173, 217). However, complement consumption induced by LPS is not entirely due to fixation of complement components in the classical sequence to LPS-antibody complexes (259, 263). The minute quantities of LPS necessary to produce profound complement reduction *in vivo* are not believed to be sufficient to form enough immune complex with natural antibody to explain the depletion observed (231, 251). Further, complement activation in preabsorbed serum free of detectable antibodies and in antibody-lacking piglet serum by LPS has been reported (109, 184, 216, 231, 301).

Strong evidence for an alternative mechanism came from the finding that LPS considerably reduces the titers of the late acting complement components, C3-C9, without significantly affecting C1, C4, and C2 (108). This is characteristic of the properdin system as activated by zymosan. Like zymosan, LPS forms a complex with serum factors, termed LPS-X. LPS-X, separated from the serum by centrifugation, acts directly on purified C3 (209, 299). C3 cleavage does not occur with untreated LPS. The active principle, probably an enzyme fixed to the LPS particles, is not C3 convertase, $C\overline{42}$, since it is unaffected by treatment with anti-C2-serum (209). This finding provided the first proof for an alternate mechanism for C3 activation by LPS. It does, however, not exclude the participation of early complement factors and specific antibody in the *formation* of LPS-X.

In addition to the early finding of Pillemer (259) that properdin was fixed to LPS, it has been shown that factor B is cleaved during treatment of serum with LPS in a similar manner as occurs with zymosan (118). The cleavage does not require Ca^{++}, but needs Mg^{++} (334a). Complement consumption by *E. coli*, probably triggered by their content of LPS also does not require Ca^{++} ions (89). All these findings point to the properdin system which is activated by LPS.

B. Complement Activation by Guinea Pig γ_1- and γ_2-Immunoglobulins; the "C3 Shunt"

The immunoglobulins, apart from their antigenic specificity differ in molecular size, electrophoretic mobility, chromatographic behavior, and in their functional involvement in various immunological reactions.

The 7S immunoglobulins of guinea pig serum have been separated into γ_1- and γ_2-fractions by electrophoresis and/or DEAE-cellulose chromatography (17, 242). γ_1-Globulins are fixed to tissues and mediate passive cutaneous anaphylaxis (PCA) but when combining with specific antigen do not ordinarily activate classical serum complement. In contrast, the γ_2-globulins consume complement but are unable to elicit a PCA reaction when used as the locally injected antibody (25, 242, 248). γ_1-Antibodies can consume complement under special conditions, *e.g.*, when present as preformed immune precipitates (246). Even then the γ_1-mediated complement consumption differs markedly from that triggered by γ_2-immune aggregates. The latter consume the early reacting components C1, C4, and C2 while these factors are hardly affected by γ_1-complexes which predominantly deplete the late acting components, in particular, C3 (293). Apparently γ_1-antibodies lack suitable sites for C1 fixation and such sites are lacking even when the γ_1-globulins are aggregated by antigen to form immune complexes (291, 293). Since C1 is bound to the Fc portion of IgG, one functional difference between γ_1- and γ_2-antibodies must reside in this part of the molecule. Differences in the Fc structure are probably also the reason for the different capacity of γ_1-, γ_2-antibodies to fix to tissues. At least in rabbit antibodies it is the Fc region which provides the link to tissue constituents (249).

The peculiar activation of complement by γ_1-aggregates which by-passed the early components markedly depleting C3, was reminiscent of the properdin system and was termed the "C3 shunt" (292, 293). Pepsin digestion of γ_1-globulin, to eliminate the Fc portion, revealed that this pathway was triggered by the $F(ab')_2$ fragments. $F(ab')_2$ fragments of γ_2-immunoglobulins also consume C3 and later acting complement components although their capacity to react with C1, C4, and C2 is lost after the destruction of the Fc part of the molecule (291).

Like the properdin system the C3 shunt requires Mg^{++}, but not Ca^{++}, it is blocked by EDTA but not by EGTA (292), and it is activated in C4-deficient guinea pig serum that does not support the classical complement reaction (294). The relationship to the C3PA system and the properdin system became apparent when it was found that complement activation by γ_1- and also γ_2-immunoaggregates and their $F(ab')_2$ fragments was accompanied by cleavage of C3PA (factor B of the properdin system) (290). The opsonization of pneumococci by immune guinea pig serum also proceeds *via* the C3 shunt, since it utilizes γ_2-antibodies and C3, but not C1, C4, or C2 (364).

The earlier discovery of atypical complement inactivation by pepsin-digested rabbit and sheep antibodies (295) was corroborated by these findings concerning the activation of an alternate pathway by $F(ab')_2$ fragments of guinea pig immunoglobulins.

C. Complement Activation by a Factor from Cobra Venom

Since the work of Ritz (271) it has been known that cobra venom can inactivate C3t (then called "the third component"). Klein and Wellensiek (165) found that both C3 and C5 (then called fractions a and b) are depleted. Nelson (236), and Müller-Eberhard *et al.* (229) characterized the component of the venom responsible for this effect, and discovered that it acts on C3 in the presence of a serum co-factor. Independently, a factor of cobra venom was purified which generated anaphylatoxin in guinea pig, rat, and hog serum, and which also required a serum cofactor (338, 340). Both of these systems later proved to be the same and the active principle is now called cobra venom factor (CVF). CVF has been isolated and characterized (222, 227).

CVF is not the only complement-consuming factor in whole venom. Ballow and Cochrane (9) have separated another fraction which has a higher molecular weight and reacts predominantly with C1 and possibly other early complement components. It contributes little to the anticomplemen-

tary effect of cobra venom, and is not believed to constitute a significant new pathway for complement activation.

The serum co-factor for CVF, C3 proactivator (C3PA) was purified from human (118) and guinea pig serum (22, 37). It reacts with CVF generating a stable, C3 cleaving enzyme complex (22, 37, 62, 77, 118, 144, 205, 335a). Suggestions about the formation and composition of this complex have been controversial and will be discussed in section IV E 2. As has been mentioned already, the co-factor of CVF, C3PA, can now be equated with properdin factor B. Another component of the properdin system, factor D, is also essential for the formation of the CVF-dependent C3-cleaving enzyme (62, 144, 335a), whereas properdin itself and factor A are not required (119, 286).

When red cells are incubated with CVF and serum they are lysed (9, 258). The phenomenon occurs in the absence of antibody to the cells, without participation of C1, 4, or 2, and even in serum autologous for the red cells used. This hemolysis requires complement components C5 through C9, and the serum co-factors essential for C3 cleavage by CVF.

By separation of the process into two stages it has been shown that at first an active CVF complex forms which acts on C3, then C5 allowing fixation of $\overline{C567}$ to the cells followed by cytolysis with C8 and C9 (9, 43). The mechanism of this passive hemolysis (258) is thus related to the phenomenon called reactive lysis by Lachmann and Thompson (179). The second step appears to proceed in the absence of divalent cations. CVF-induced hemolysis can be used as an assay system to test the activity and consumption of the properdin system (42, 43).

D. The C1 By-pass Activation Pathway

Red cells do not normally activate the properdin system. Apparently appropriate binding sites are lacking at their surface. However, when coated with 20 to 40 times the amount of antibody required to optimally fix complement, they can be lysed by an alternate pathway which does not involve C4 and C2, but requires C1, Ca^{++}, and properdin. During the process C3PA is cleaved (91, 209a). Detailed study of this pathway is not yet complete.

E. Factors Involved in the Alternate Pathways

Activation of components involved in alternate pathways may not produce direct biological reactions. Only when C3, C5, and later complement components are activated do cellular or humoral effects appear. Here only those factors involved in the alternate pathways will be described which react prior to C3 activation. In the subsequent steps there is no principal difference between the classical complement reaction and alternate pathways (see fig. 2).

1. Properdin. According to the original definition by Pillemer, properdin is a serum factor essential for C3 activation by zymosan and other polysaccharides which is fixed from serum to zymosan, *etc.*, at temperatures above 15°C, can be eluted from the zymosan complex thus formed, and is able to reconstitute deficient serum obtained by absorption with zymosan at 15 to 17°C.

Human properdin has been purified by elution of serum-treated zymosan (252), by affinity chromatography of serum on insolubilized anti-properdin-antibody (219), and by a series of chromatographic separations of human serum euglobulin (119). It is a euglobulin with β- to γ_2-mobility which is antigenically as well as functionally distinct from the immunoglobulins. It consists of four noncovalently linked subunits and is at its isoelectric point at pH 9.5 (119, 252). After isolation, properdin is present in an activated form. This is understandable for preparations obtained from zymosan eluates but it also is true of other preparations. Addition of purified preparations to properdin-depleted serum causes factor B and C3 activation even without the addition of a triggering compound such as zymosan. The presence of endotoxins as activating factors

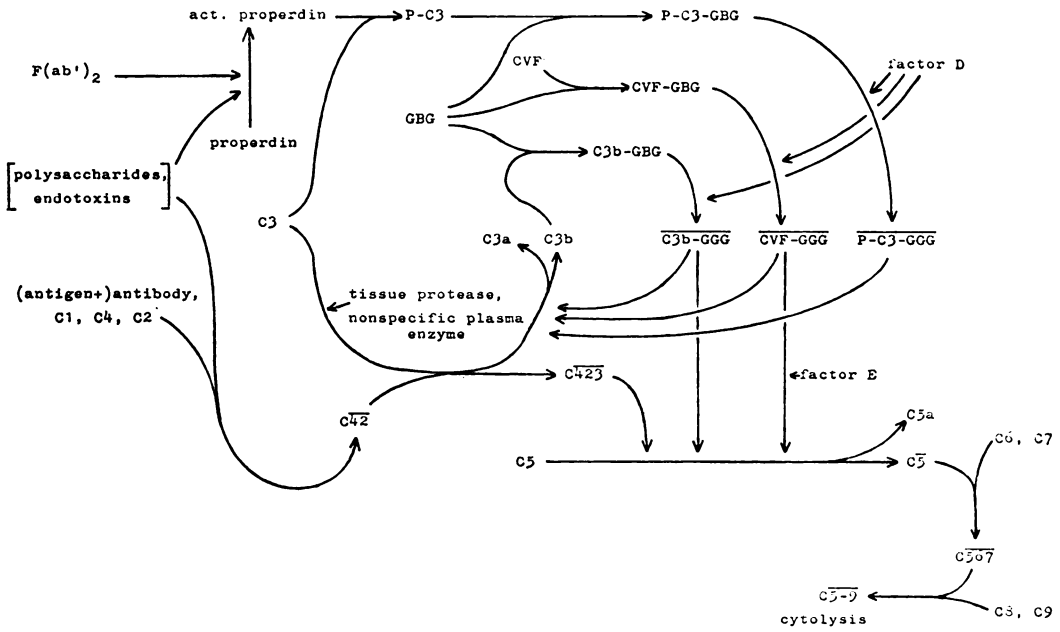

FIG. 2. Proposed scheme of the properdin system and its connections with the classical complement sequence.

in the preparations has not been ruled out, however (119).

A radioimmunoassay for properdin has been developed (218) which shows that properdin, even in its activated form, binds to zymosan only when additional serum factors are present (187, 252). It is also not capable of activating factor B or C3 directly but does so only in conjunction with other factors. Whereas properdin is essential for the activation of complement by solid surfaces such as bacteria and insoluble polysaccharides which process by-passes C1, C4, and C2, complement activation by CVF does not require properdin (119). Its role in the C3 shunt triggered by the $F(ab')_2$ portion of γ-globulin (292) is not known. Certainly, properdin acts at an early step of the system which eventually activates factor B (C3PA). It has been suggested that properdin reacts with native C3 conferring on it factor D (C3PAase) activating properties. The activated factor D would in turn activate factor B to form the C3 cleaving enzyme. With release of C3b the feedback cycle would be set in motion (119). The experiments supporting this concept have, however, been made with purified properdin preparations which were already active. A serum factor capable of activating properdin has recently been described (312). The question still remains, therefore, of exactly how properdin is activated.

2. Factor B of the properdin system. This factor was defined by Pillemer as an extremely heat-labile component in serum which was essential for C3 activation by zymosan. The protein having these properties (synonyms are given in table 2) has been isolated from human (118, 144, 205) and guinea pig (22, 37) sera. Factor B is found in the serum throughout the animal kingdom as illustrated in investigations of the cofactor for CVF (44, 105), which was later shown to be functionally identical with factor B. Chemically, human factor B is identical with the glycine-rich β-glycoprotein (GBG) (5, 32, 118, 144, 205). Guinea pig factor B is immunochemically related to the human protein (37). Human GBG has a molecular weight of 80,000 to 100,000, and is at its isoelectric point at pH 5.7. It shows

genetically determined molecular polymorphism (4).

During treatment of serum with polysaccharides or endotoxins GBG is cleaved into two fragments: a glycine-rich γ-globulin (GGG) identical with β_2-glycoprotein II (137), a basic protein of 60,000 molecular weight, and glycine-rich α-globulin (GAG), a smaller fragment (m.w. 20,000) more negatively charged than the parent molecule (3, 32, 118, 205). Each of the fragments carries different antigenic sites of GBG. Cleavage of the parent molecule thus can be well demonstrated by immunoelectrophoresis. Functionally the cleavage of GBG leads to its activation. The GGG fragment represents the activated form of factor B. It is the C3 cleaving enzyme of the properdin system, hence the name proposed by some authors, C3 activator (C3A). However, although C3 cleaving activity of purified C3A has been reported (118) other results indicate that the activity is only expressed (or preserved) when C3A (GGG) is bound to a properdin complex, *e.g.*, on zymosan, or to CVF (38, 335a). Whether the binding serves the cooperation with other factors or merely to protect from decay, is not yet known. Curiously enough, the pre-enzyme, GBG, has been shown to have enzymatic activity; it cleaves several amino acid esters (61).

Since factor B is involved in all known alternate pathways which activate the late complement components, but not in the classical complement activation, demonstration of the cleavage of GBG into GGG and GAG provides an important tool to study the participation of alternate pathways in biological reactions.

While the function of GBG as factor B in the properdin system is beyond any doubt, its role as the co-factor in the cobra venom-activated system has been a matter of controversy. This is partially due to the fact that protein complexes have been investigated and assumed to represent functional complexes. While the facts that CVF needs co-factors to become active, that GBG is essential and that CVF forms a protein complex in serum generally are accepted, suggestions about the nature of the CVF-binding protein and the role of GBG for its formation differ. According to Götze and Müller-Eberhard (118) the heat-labile human serum protein, C3PA, which they identified immunochemically with GBG was cleaved during incubation of human serum with zymosan or inulin and reconstituted serum lacking factor B. In this property C3PA was identical with factor B, functionally. In addition, the same preparation was reported to form an active C3 cleaving enzyme complex (1 + 1) with the cobra venom factor (CVF). This was not confirmed for GBG in other laboratories (5, 37, 144, 205), and thus the relationship of C3PA to GBG and factor B was unclear. Alper *et al.* (5) did not see any indication for interaction of factor B with CVF in human serum. According to Hunsicker *et al.* (144) factor B was essential together with factor D, but it was not found in any stoichiometric relationship in the CVF complex. Cooper (62) found factor B to form a reversible complex with CVF which had some C3 cleaving activity. When exposed to factor D this complex was stabilized and acquired strong C3 cleaving activity. Similarly Ruddy *et al.* (286) and Hadding *et al.* (131) found that factors D, B, and CVF generated an active complex.

According to recent results in the author's laboratory (206, 335a) the C3 cleaving CVF complex contains the GGG fragment of GBG, besides CVF. Cleavage of GBG has been observed in whole human serum on treatment with CVF, and in systems containing purified factor D, CVF and GBG. Since an active C3 cleaving system was also generated when CVF was incubated with GBG, Mg^{++}, and trypsin, but not when any of these constituents was lacking it has been concluded that GBG first reversibly binds to CVF, with the help of Mg^{++}, and that only in this association its cleavage (by *e.g.*, factor D) leads to the stable and highly active $\overline{\text{CVF, GGG}}$-complex which no longer

depends on Mg^{++}. This reaction scheme would be compatible with and extend the above mentioned observation of Cooper, (62) that CVF and GBG form a reversible and weakly active complex which is turned into a stable C3 cleaving enzyme by factor D. The activity ascribed in the early investigations (118) to a complex of C3PA with CVF then appears either to have represented the weak C3 cleaving potency of the loose CVF, GBG (Mg^{++}) complex, or it was due to highly active $\overline{CVF, GGG}$ formed by trace contamination of the preparations with factor D. In any case C3PA can now be equated with factor B and GBG.

3. Factor D. Factor D was recognized as a component essential for the development of C3 cleaving activity from mixtures of cobra venom factor and factor B (GBG) (144). All evidence indicates that it is identical with C3PA convertase (228) and GBGase (277), factors which in serum cleave factor B (C3PA) when the properdin system is activated.

Factor D is a protein of rather small size. The originally proposed figure, an apparent molecular weight of 35,000 for human factor D (144), may still have been too high. Recent results indicate 21,000 (335a), the same molecular weight as that found for guinea pig factor D (131). It is possible, therefore, to separate factor D from other serum proteins by molecular sieve gel chromatography, where it elutes after albumin. The sedimentation rate of factor D is 2–4 S, its isoelectric point (guinea pig) is at pH 9.5 and it has an electrophoretic mobility of an α- or β-globulin (131, 144, 228, 277). Factor D is precipitated from serum at low ionic strength at pH 5.4 with the euglobulins (144). However, this may be due to co-precipitation rather than to its own insolubility. It forms only a trace constituent of serum (62).

Factor D is involved in two reactions both depending on the presence of Mg^{++}: the cleavage, equal to activation of factor B in the classical properdin system, and formation of the C3 cleaving CVF-factor B complex. For the former effect factor D needs the cooperation of activated factor A, the C3b fragment of C3 (228). The factor B cleaving potential of polysaccharide-fixed properdin complexes resides in factor D rendered active by fixed C3b. Both C3b and factor D can be eluted from zymosan complexes and the elution mixture is capable of cleaving factor B (38a, 228, 238a, 335a). How factor D is activated by C3b is unknown. It is a reversible process since elimination of C3b from serum abolishes factor D activity and addition of C3b restores it (178, 239).

In the cobra venom system factor D activates factor B (GBG) in a way that it firmly binds to CVF (62). This reaction again depends on Mg^{++} and includes cleavage of factor B (335a) but factor A is not essential (178, 286). Factor D then appears to be a GBG cleaving enzyme which becomes active either in the presence of C3b or of cobra venom factor.

4. Factor A. This factor was first recognized by the Pillemer group as a hydrazine-sensitive principle required for the generation of an active properdin complex (254, 261). Müller-Eberhard and Götze (228) purified a hydrazine-sensitive protein from human serum which was essential for the expression of C3PA convertase (factor D) activity. They identified it with C3. Recently its identity with factor A has been established (125). C3 is inactive as such; the principle which is active in the properdin system is the C3 fragment, C3b (activated factor A). On the other hand, C3b is also the major product resulting from cleavage of C3 either by the complement intermediate $C\overline{42}$ (C3 convertase) or by the activated properdin factor B or by incubation of serum with CVF. Thus C3b is both a product and an activating agent of the properdin system, providing a positive feed back mechanism (178, 228, 239). Unlike its precursor, C3, it is not destroyed by hydrazine.

The C3b fragment functions as an activator of factor D, conferring to it GBG cleaving activity. Thus addition of C3b to normal human serum or to a solution of purified factor D induces GBG cleaving

potency (178, 228). The possibility has not yet been ruled out that the conformation of GBG is altered by interaction with C3b, rendering GBG susceptible to factor D which then might be active, *a priori*.

C3b is fixed to zymosan during its incubation with serum and probably has the same function in fixed properdin complexes as in fluid phases. In addition it appears to be significant for the assembly of active properdin complexes on zymosan (238a). This function requires that C3b is fixed to the zymosan, itself.

5. Factor C (C3b INA). This factor was discovered as a principle which enhances the hemolytic activity of CVF and its co-factors in crude mixtures (144). In systems of highly purified CVF, factors D and B, it had no effect. It was then found that factor C is identical with the C3b inactivator (C3b INA) (286).

C3b INA plays a regulatory role in the properdin system. As has been pointed out, C3b activates the GBG cleaving enzyme, factor D. The activated fragment of GBG, GGG, then cleaves C3 and C5, activating the remaining complement system, and at the same time yielding more C3b. Traces of C3 are cleaved in serum spontaneously by complement activation and/or by other enzymes (371) as is evident from the decay of C3 activity during storage of serum. The resulting traces of C3b are split by C3b INA which thus prevents an uncontrolled activation of GBG and complement. When the activity of C3b INA is blocked by adding specific antibody to serum, spontaneous activation of the properdin system ensues (178, 239). This also occurs in a patient with C3b INA deficiency (1, 2). On the other hand, in the CVF system C3b INA enhances the activity as the competition of C3b with CVF for the activated GGG fragment is decreased. This explains the findings of Hunsicker *et al.* (144) that led to the discovery of factor C (C3b INA) function in CVF systems.

The patient with C3b INA deficiency suffered from multiple infections and his plasma contained a very low level of C3 and an unusually large amount of C3b (2). GBG was also absent (6, 7). Since his plasma cleaved GBG into GGG and GAG spontaneously, it was assumed that it contained an active GBGase (factor D) (277). Further study revealed that the plasma lacked C3b INA (1) which, when added, blocked the GBG cleavage (7). It is conceivable that this blocking action of C3b INA is due to functional elimination of C3b which is essential for the GBG cleaving activity of factor D.

6. Factor E. This factor has been recognized by Hunsicker *et al.* (144) as a compound which enhances the cleavage of C5 by CVF complexes. Whereas factors D, B, and CVF interact to form a C3 cleaving enzyme complex, the resulting activity does not optimally extend to activation of C5 unless factor E is added. Accordingly factor E is also necessary for CVF complexes to cause significant passive lysis. Factor E is a euglobulin of about 160,000 molecular weight. It cannot replace factor D or B. It is able to bind CVF, but C5 cleaving complexes certainly contain more factors. Whether it is significant in the polysaccharide-activated properdin system is not known.

7. Cobra venom factor. This factor was recognized by its ability to induce cleavage of C3 and formation of anaphylatoxin in serum. CVF has been purified by various methods (9, 22, 227, 236, 338). It is a 6.7 S protein of 144,000 molecular weight (9, 227). The factor is not toxic when injected. Since it depletes the plasma of the third component of complement, *in vivo*, it has been used to study the role of complement and the effect of decomplementation in various immune phenomena (56, 90, 98, 158, 207, 236, 302).

As mentioned already CVF acts on C3 only in conjunction with additional serum factors, but without involvement of early complement components. CVF, interacting with serum factors, forms a complex. This is indicated by an increase of the sedimentation rate to 8.5 to 9 S corresponding to a molecular weight of about 220,000 (118, 144, 227). The C3 cleaving activity resides in the

complex. Suggestions about its formation and composition have been controversial as discussed in section IV E 2. According to recent results the C3 cleaving complex forms when CVF interacts with factor B (GBG), factor D and Mg^{++} (62, 131, 286, 335a), and it consists of CVF and the GGG fragment of GBG (335a). C3b is not essential for its formation (43, 178, 286, 335a). In contrast, it even competes with CVF (43, 286) since it also reacts with factor D and GBG cleaving the latter which then cannot be utilized by CVF. C3b and CVF are thus very similar, functionally.

C5 and the remaining complement components are efficiently activated only when the CVF complex is formed in the presence of factor E, in addition to the factors mentioned (144).

8. Early reacting complement components C1, C4, C2. There is no doubt that activation of C3 and C5 in serum by its incubation with CVF takes place without any involvement of the early complement components. The same cannot be said without reservations about the (lipo)polysaccharide-activated properdin system although it is generally described as a by-pass of early complement components. Pillemer already considered that C1, 4, and 2 might be involved (360). The problem is related to the question of whether antibodies specific for the triggering substances are essential for activation of the properdin system (see section IV E 9).

Although activation of the late acting complement components by the properdin system is accompanied by little if any detectable loss of C1, C2, and C4, these factors might be essential in traces. Some findings indicate that the early components are indeed nonessential, principally. C1 activation can be ruled out by adding EGTA to the system eliminating Ca^{++}. Under these conditions classical complement-induced immune hemolysis is not possible, even in undiluted serum (47), but C3 cleavage and AT formation in serum by zymosan and endotoxic lipopolysaccharides can still be demonstrated (89, 334a). The activation of the "C3 shunt pathway" by aggregated γ_1-immunoglobulins proceeds also in the presence of EGTA (292). Further, elimination of C1 by adsorption with specific antibody against Clq does not prevent opsonization of bacteria *via* an alternate pathway (148).

C2 is not essential because serum of patients with a genetic C2 deficiency is as active as normal serum in generating complexes on zymosan which release AT (39) and C2-deficient serum also has bactericidal activity although this is reduced when compared to normal serum (155, 167, 170). Inulin can induce the cleavage of GBG in C2-deficient serum. It should be noted that even homozygous C2 deficient serum contains 0.5 to 4 % of the normal content of C2 (65) but it is unlikely that these traces of C2 which are too small to support immune hemolysis participate in properdin activation because the activities of the alternate pathways diminish with dilution to the same degree in normal as in C2-deficient sera (39). Also, calf serum which has no detectable C2 and is hemolytically inactive, is bactericidal for rough forms of bacteria (315). In the C3 shunt pathway C2 is not involved because immune complexes formed with isolated $F(ab')_2$ fragments, not capable of activating early complement components or fixing C2, can consume C3 (291).

One strain of guinea pigs completely lacks C4 (85). These animals are as resistant to infections as normal animals, and are able to develop passive cutaneous anaphylaxis and hypersensitivity reactions (86, 276). Their serum is bactericidal because of the intact properdin system (276, 364) which is also significant for resistance of these animals against endotoxin (211). When incubated with aggregates of guinea pig γ_1-immunoglobulins or with endotoxic lipopolysaccharides, the late acting complement components in C4-deficient sera are inactivated and a chemotactic fragment is released from C5 (92, 294). Since C2 cannot be activated without C4, the existence of an activatable properdin system in C4-deficient serum once

more appears to rule out its dependence on C2.

On the other hand, C1 is involved in the C1 by-pass activation pathway (209a), and there are suggestions that traces of C2 and C4 are significant for maximum activation of the properdin system (106, 288). Plasma from patients with C1 deficiencies has reduced bactericidal activity despite an intact properdin system (209a). C4 considerably enhances the formation of active properdin complexes on zymosan, and opsonization of bacteria proceeds more rapidly in the presence of C4 than in its absence (38, 276). C4-deficient guinea pigs are less resistant to endotoxins than normal ones (211). When C2 is very low, as in genetic deficiency, the formation of the C3 cleaving complex on zymosan is impaired (114) although it is functionally detectable (209). Snyderman *et al.* (304) even found that in guinea pig serum from which C4 had been eliminated by a C4 inactivator (150) a C3 consuming system could not be activated by incubation with endotoxin, and no chemotactic activity was formed. This result seems at variance with the finding that serum from guinea pigs totally deficient in C4 has been found capable of activation of C3 after being exposed to endotoxin (92). In fact, some chemotactic activity was released, and C3 consumption occurred in the experiments of Snyderman *et al.* (304), and the differences may be quantitative rather than qualitative. There are indications that endotoxins are poor activators of an alternate pathway when present in small amounts but very efficiently activate the classical complement cascade by reacting with natural antibody. It would be of interest to know whether activation of the properdin system by zymosan occurs in serum treated with the C4 inactivator.

In conclusion, the properdin system probably can be activated by immune aggregates or zymosan without any involvement of early reacting complement components but under natural conditions these are involved to some extent. Minimal activation of early complement components insufficient to promote a classical complement reaction considerably enhance activation of the properdin system. Generation of traces of $\overline{C42}$ supplying small amounts of C3b may be needed to trigger the properdin system efficiently.

9. Immunoglobulins. Pillemer originally visualized properdin as a system which would nonspecifically attack bacteria without antibodies (259, 260, 263). This assumption became doubtful, however, as further results were obtained. The function of immunoglobulins in the classical complement reaction, once combined with antigen, is to fix and activate C1 (146). Since C1 is not essential for activation of the properdin system, immunoglobulins would appear to have no function in this system either. However, the $F(ab')_2$ portion of immunoglobulins can fix complement and activate the "C3 shunt" pathway without involving C1 (147, 270, 290, 291). Therefore, it still can be questioned whether immunoglobulins are essential factors in alternate pathways to complement activation even when they by-pass C1, C4, and C2.

Complement in sera which had been pretreated with (lipo)polysaccharides to adsorb all natural antibodies could still be activated subsequently by the same agent (40, 231, 301). Complement activation by endotoxins is also possible in γ-globulin-deficient serum (107, 216, 231). However, other findings suggest that at least traces of antibodies may be essential. In particular, activation of complement by endotoxins (103, 173, 231, 256) but also by bacterial cell walls and zymosan (26, 244, 252) in some assay systems may depend, at least partially, on specific antibody.

These divergent findings may be due to quantitative rather than qualitative differences, perhaps reflecting different or multiple pathways simultaneously active in a given system. Thus activation of the properdin system by endotoxin in sera naturally deficient in immunoglobulins (precolostral piglet serum) or depleted of antibody specific for the activating agent, was only reduced but not blocked in several systems (26, 107,

231). The bactericidal effect of such sera often differed depending on the character of the bacterial surface rather than on its antigenic specificity; R forms but not S forms were attacked (10, 315). Webb and Muschel (361) have, however, detected natural antibody even in precolostral piglet serum in very low concentrations and with some atypical properties. Thus experiments with these sera may not absolutely rule out participation of antibody.

Although early investigations have indicated a dependence on properdin (331), the phage-neutralizing potency of serum depends on specific antibody (66, 325), *i.e.*, on the classical complement reaction rather than the properdin system. Phage surfaces are not composed of polysaccharides. Sera from newborn precolostral piglets which cannot neutralize T2 phage unless antibody is added, can be decomplemented by zymosan without the addition of antibody (135).

Hemolysis of endotoxin-coated red cells by fresh guinea pig serum depends on naturally occurring γ_2-immunoglobulin antibodies in such serum and early reacting complement components (99, 256). Perhaps, the extremely small quantities of endotoxin present are insufficient to activate an alternate pathway, and may be able to generate enough C3-activating potency only by classical complement activation. The small amounts of endotoxin and natural antibody could also explain why only so little C1, C4, and C2 are consumed. The normal red cell surface appears to be inefficient in fixation of factors belonging to alternate pathways (210). Much larger amounts of endotoxin than present on the LPS-coated cells may be required for effective fixation and activation of properdin factors. For substantial generation of anaphylatoxin in rat plasma 100 μg/ml of Salmonella endotoxin have been found necessary (238). The simultaneous presence of antibody and early complement components may not be essential but may enhance the activation of the properdin system by LPS.

According to Lachmann (174), lipid A, the constituent of endotoxin responsible for its toxic, fever- and shock-inducing properties (100), activates complement *via* the classical pathway without activation of C3PA (factor B), while whole endotoxin, probably the polysaccharide portion, causes C3PA cleavage. In addition LPS and lipid A can nonspecifically consume complement by direct adsorption (99) and some endotoxins may have direct toxicity, not dependent on immune mechanisms (100, 163). Thus, there are several pathways by which endotoxin can act on complement, and the lack of a requirement for antibody in some endotoxin-activated systems does not rule out the possibility that others can maximally express their activity only if antibody is present. The final proof for or against an absolute requirement for antibodies in the activation of the properdin system will be possible only when all components are purified and can be fixed and activated in the correct sequential order.

F. Synopsis of Alternate Pathways

All alternate pathways share in common that they terminate with their entry into the complement sequence at C3, and that their C3 cleaving activity resides in the activated factor B. Therefore, these pathways seem identical completely or partially with the properdin system. Their terminal reactions are probably the same; at least analogies are evident and no contradictory results are known. The initial steps are less well known and appear to differ; properdin itself is not always involved (*e.g.*, in activation by CVF).

The C3 cleaving activity results from interaction of factor A (C3b), factor B (GBG), factor D (GBGase), and Mg^{++}. It resides in the GGG fragment of GBG provided this is bound to other factors. GGG is split from the parent molecule when C3b is present. C3b is generally assumed to activate factor D which would then be a proenzyme. However, the functional similarity of C3b and CVF (see section IV E 7) and the reversible activation of factor D (178, 239)

suggest a different mode of action. In the CVF system GBG is cleaved by factor D when bound to CVF (335a), and factor D appears to be active, *a priori*. In analogy, in the endogenous systems C3b might serve to bind GBG and thereby to expose its bond to be cleaved to access by factor D.

In any case, generation of C3b is one mechanism to initiate the terminal reaction of alternate pathways. The mechanism by which C3b is produced may differ in various systems and more than one mechanism may operate in a given system. In activation by endotoxins and polysaccharides traces of $C\overline{42}$ may be formed by an antibody-triggered complement reaction, and may contribute to C3b production. This would be in accordance with the significance of natural antibodies postulated by some authors and with the enhancing effect of early complement components on the generation of active properdin complexes. Other nonimmunological mechanisms might be activation of spontaneous C3 cleavage in serum by yet unknown processes or, *in vivo*, release of C3 cleaving tissue enzymes subsequent to injury (140).

Properdin possibly acts by providing initial GBG cleaving activity independent of C3b, but involving genuine C3 (119), as outlined in section IV E 1. In the properdin-C3 complex C3 may acquire a conformation which corresponds to that of C3b and which allows combination with GBG. In this complex GBG could then be cleaved by factor D in the same way as in the complex with C3b, suggested above. How properdin is activated to be able to combine with C3, is unknown.

An attempt to delineate the activation and reaction sequence of the properdin system along the lines described above, and its links to the classical complement system are shown in the scheme, figure 2.

V. Pharmacologically Active Peptide Fragments of Complement Components

The complement reaction not only yields cytotoxic complexes which are fixed to the target cell but also soluble products which are pharmacologically active and probably significantly amplify inflammation associated with immune reactions. Free $C\overline{567}$ complex, one of the chemotactic factors for neutrophils, has already been described in section III E. The other active products are peptide fragments cleaved from complement components C2, C3, and C5 during their activation. A C4 cleavage product has also been isolated but so far has not been found to have any biological effect.

A. C2-derived Fragment

Activation of C2 (m.w. 117,000) by $C\overline{14}$ involves cleavage of the molecule (213, 265, 318). The major fragment, C2a (m.w. 83,000) is active in the complement reaction (265). The remaining peptide fragment or fragments with a maximal molecular weight of 34,000 initially escaped detection until Donaldson *et al.* (82) discovered a dialysable, heat-stable principle in the plasma of patients with hereditary angioedema which increased vascular permeability. Several findings indicated that this compound was cleaved from C2 by the pathologically increased C1 esterase activity. When given by intradermal injections, incubated plasma from angioedema patients produced a wheal which showed blueing when Evans blue had been injected intravenously before. The formation of this factor required C1, C4, and C2 in the plasma. $C\overline{1}$ INH as well as anti-C2 or anti-C4 serum blocked the development of the active principle while anti-C3 serum did not inhibit (81, 82). Soybean trypsin inhibitor blocked the formation of the active principle but did not alter its action, once formed (82). A peptide having the same biological and chemical properties appeared in incubation mixtures of $C\overline{1s}$, C4, and C2 (169). Intradermal injection of C1 esterase increased vascular permeability in normal, but not in C2-deficient patients (167, 168). Thus the active principle was not $C\overline{1s}$, but a product of its reaction with C4 and C2. The peptide cleaved from C4 by $C\overline{1s}$, C4a, contains much more tyrosine than the vasoactive peptide of angioedema plasma (186).

Hence, the angioedema factor probably originates from C2. This indirect conclusion is supported by the finding that brief incubation of purified C2 with trypsin releases a peptide fragment having similar biological and chemical properties (169).

Donaldson *et al.* (81, 82) have extracted and characterized the peptide increasing vascular permeability from angioedema plasma. It differs from both C4a and kinins in its low tyrosine content (186). It is less basic than bradykinin. Its activity is destroyed by carboxypeptidase B and, unlike bradykinin, by trypsin. Preliminary results on amino acid composition have been published (186). The C2 fragment obtained in a pure system of $C\overline{1s}$, C4 and C2 has an apparent molecular weight of less than 5000 (Sephadex G-25 gel filtration) (169). This suggests that more than one peptide is cleaved from C2 during activation by $C\overline{14}$.

Besides increasing vascular permeability the peptide from angioedema plasma evokes little pain when given by intradermal injection and contracts rat uterine smooth muscle without causing tachyphylaxis. Its vascular effect is not mediated by release of endogenous histamine (82).

The vasoactive peptide does not form spontaneously in normal plasma nor in plasma from angioedema patients obtained shortly after an attack when C2 is largely consumed. Since it causes effects similar to those seen in the disease during attacks it is probably the mediator of the pathological symptoms (81, 186).

The C2 fragment bears no relationship to plasma kinins chemically, yet it has been referred to as a kinin. It has not been established, however, that its biological properties conform to the definition of kinins as given in the "Reports of the Committee on Nomenclature for Hypotensive Peptides" (362).

B. C3-derived Fragments

1. C3a. This basic peptide, cleaved from native C3 during activation was discovered by Dias da Silva and Lepow (76) during studies on the formation of anaphylatoxin. It is often also called anaphylatoxin although it differs, chemically as well as biologically, from the substance for which this term had been used as a trivial name since about 60 years. For reasons to be presented in section V C the term will be reserved in this review for C5a which ultimately was identified as the original anaphylatoxin.

FORMATION. C3a is cleaved from C3 during incubation with mixtures of C1 esterase, C4, C2, and Mg^{++}, with preformed $C\overline{42}$ (55, 74, 76, 214), by the C3 cleaving cobra venom factor complex, by endotoxin-fixed properdin complexes, or by brief treatment of C3 with trypsin, plasmin, or thrombin (34, 55, 171, 299). Hydroxylamine cleaves a C3a-like peptide from C3 which is, however, slightly larger than that released by trypsin or $C\overline{42}$ (48, 49).

Complement activation in whole serum (human, pig, rat, guinea pig) by classical or alternate pathways does not lead to the appearance of C3a activity because C3a is rapidly destroyed once formed (189) by a peptidase similar to carboxypeptidase B in plasma, also called carboxypeptidase N or anaphylatoxin inactivator (33, 87, 88). C3a activity will form when serum is incubated with zymosan or inulin (330) in the presence of ϵ-aminocaproic acid (1 M), an inhibitor of carboxypeptidase (330, 376). It is unlikely, however, that ϵ-ACA acts only by inhibition of C3a destruction. It induces C3a activity in human serum even without addition of complement-activating agents (330, 334a), an effect not shared, but also not prevented by another carboxypeptidase inhibitor, 1,10-phenanthroline (376). ϵ-ACA may somehow trigger C3 cleavage, in addition to blocking C3a destruction. On the other hand, when the carboxypeptidase was eliminated by affinity chromatography Vallota and Müller-Eberhard (330) detected C3a activity in human serum even when incubated without any exogenous substance. This would suggest that C3a appearing in serum free of carboxypeptidase originates from the traces of C3 which are continuously cleaved spontaneously. Phenanthroline might inhibit

this spontaneous cleavage of C3, but not the additional release of C3a triggered by ε-ACA.

CHEMICAL PROPERTIES. C3a is a strongly basic peptide, moving toward the cathode at pH 8.6 (34). The molecular weight, calculated from the amino acid composition and measured by gel electrophoresis, is 7200 for the product of $C\overline{42}$ and trypsin action on C3, and 7800 for the hydroxylamine-cleaved C3-fragment (48). As in whole C3, the N-terminal amino acid is serine. Arginine forms the carboxyl terminal. Unlike native C3 and C3b, the major cleavage product, C3a is rapidly cleaved by carboxypeptidase B. These findings suggest that C3a represents the N-terminal peptide chain of C3 (48).

PHARMACOLOGICAL ACTIONS. C3a contracts isolated guinea pig ileum (49, 55, 74, 76, 171, 214, 299) maximally at a concentration of 0.1 μg/ml of medium (225) with very rapid tachyphylaxis. Only one or two contractions can be obtained in one strip of ileum. The spasmogenic effect is blocked by antihistamines. C3a releases histamine from rat and guinea pig mast cells, and its action on smooth muscle may be partially mediated by this agent (55, 76). However, in addition C3a probably has a contractile effect independent of histamine because it contracts rat uterus at estrus (33), and is fully active on a strip of ileum after tachyphylaxis to C5a, another histamine liberator (55, 76). The failure of cross-tachyphylaxis between C3a and anaphylatoxin (C5a) demonstrates that C3a acts on receptors different from those for anaphylatoxin.

Any systemic effects of C3a are not known. The lability of the spasmogenic action of C3a in plasma makes it unlikely that it would cause an anaphylactoid, lethal bronchospasm, the classical effect of anaphylatoxin (C5a).

Local increase in vascular permeability may be produced by injection of as little as 10 ng of C3a into guinea pig skin as demonstrated by the blueing reaction with previously infused Evans blue dye (55, 76). In man the increased permeability is observed as wheal and erythema formation (193, 225). Further, the intradermal injection causes pruritus. Human skin is much more sensitive than guinea pig skin. The effects are probably mediated by histamine because skin mast cells show degranulation (193) and antihistamines inhibit the reactions (55, 76, 193, 225).

Upon treatment of C3 with trypsin, chemotactic activity for neutrophils develops (34). The active principle has been identified as C3a since after gel chromatography it appears in the same region as the spasmogenic activity of C3a. Intradermal injection of C3a in man also attracts polymorphonuclear leukocytes (225). However, others have not demonstrated chemotactic activity *in vitro* with human, guinea pig, or hog C3a (70, 299, 309, 343–345). Further, the generation of only weak chemotactic activity by complement activation in human, mouse, and rabbit sera up to the reaction of C5 (351, 352) argues against major chemotactic activity of C3a. Tissue fluids from areas of inflammation such as the Arthus reaction, vasculitis, or rheumatoid arthritis (346, 356, 359) and supernatants from virus-infected tissue cultures (45, 353) contain chemotactic factors, but none corresponded to C3a.

The controversy about the chemotactic activity of C3a may reflect the widely different dose ranges tested. Definite chemotaxis has been reported with high doses (C3a from 1000 μg of C3) (34) while smaller doses (C3a from 75–150 μg of C3) have been found inactive (345). In contrast, C5a is definitely chemotactic at the lower dose range (345). The chemotactic activity of C3a obtained after cleavage of C3 by trypsin without further fractionation may have been due partly to other, acidic fragments (34).

2. Other C3-derived peptides. A number of proteases including plasmin (343, 344), the supernatant from virus-infected tissues (353), a rat tissue protease (140, 141), joint fluid of human nonrheumatoid arthritis (359), and bacterial proteases (52) liberate peptide fragments from C3 which are chemotactic for neutrophils. Most of these

peptides have not yet been purified or characterized chemically, and are probably heterogeneous. Their relationship to each other and to C3a are unknown. On a weight basis, some are much more active chemotactically than C3a (345, 359), but are less active than C5a (353). Smooth muscle-contracting activity among these peptides has not been observed. The plasmin-generated C3 fragment(s) is acidic (343, 344). Although brief treatment of C3 with plasmin and trypsin yields C3a, further prolonged incubation with trypsin cleaves C3a to release an acidic chemotactic peptide. Bokisch *et al.* (34) have suggested that the plasmin product of C3 obtained by Ward (344) is a fragment of C3a. The chemotactic fragments produced by tissue proteases from C3 are larger than C3a (140), however.

A different peptide fragment of C3, leukocyte mobilizing factor (LMF) has been described by Rother (280). It is formed by the incubation of whole serum of various animals with antigen-antibody complexes. C3-depleted serum will not release LMF, and it can be released from purified C3 by $C\overline{42}$. LMF is acidic and lacks chemotactic or spasmogenic activity. When perfused through isolated bones it releases mature granulocytes from the marrow into the effluent. The immediate nature of the effect indicates that no stimulation of cell division or differentiation is required for the cell release.

3. Pathophysiological significance of C3-derived peptides. Complement is certainly involved in local immunological reactions of the immediate type, in which antigen-antibody complexes are formed and deposited at the site of inflammation, *e.g.*, in vasculitis (Arthus reactions), certain types of experimental nephritis and rheumatoid arthritis. C3 derivatives may be extracted from affected tissues or detected by histochemical techniques in inflamed areas at the site of antibody deposition in experimental animals and in patients (54, 57, 177, 199, 328, 350, 352, 379, 380). The concentration of the early reacting complement components C1, C4, C2 and C3 is reduced in joint fluids from rheumatoid patients (284). Although these findings indicate complement activation and consumption, at least through C3 activation, no evidence of any significant participation of C3a in the production of clinical symptoms have been obtained. C3 cleavage by cellular enzymes may not always lead to release of biologically active fragments (321). Chemotactic activity detected in tissue extracts or fluids from sites of immunological reactions corresponded to other factors, *e.g.*, C5a or $C\overline{567}$ (346, 356, 359, 380). Mice deficient in C5 (B10D2 "old line") are able to develop Arthus lesions with leukocyte infiltration (199). This may suggest a chemotactic role for C3a in these mice, but chemotactic factors may originate from other sources (158, 161, 353). Comparatively little chemotactic activity can be generated in C5-deficient mouse serum (351).

However, C3-derived chemotactic peptides of unknown relationship to C3a, and probably released by tissue proteases, have been detected in nonimmunological inflammation such as myocardial infarction, and in synovial fluid from nonrheumatoid arthritis (140, 141, 346, 359). These peptides may account for the leukocytosis which follows infarction or other tissue damage. The C3 cleaving enzymes are probably released from damaged cells, since they can be extracted from normal tissue (140). In bacterial infections C3 cleavage may be produced by bacterial proteases, independent of immune processes (52), but their appearance at the site of infections has not yet been demonstrated.

Summarizing the available information, C3a is of doubtful significance in local immunological reactions whereas other C3-derived peptides probably act as mediators in nonimmunological inflammation. The leukocyte-mobilizing factor (280) may explain the general leukocytosis in infectious diseases, but contributes little to local leukocyte accumulation in the inflamed areas.

C. C5a, Anaphylatoxin

History. In an attempt to substantiate the hypothesis that anaphylaxis would be produced by toxic cleavage products of antigens, Friedberger in 1910 (97) incubated various antigens with specific antibody and guinea pig serum. The resulting mixture indeed produced lethal bronchospasm similar to anaphylactic shock when given by intravenous injection to normal guinea pigs. The toxic principle was called "anaphylatoxin" on the assumption that it was the mediator of anaphylaxis. Soon after its discovery it was found that the active substance did not arise from antigens, since it could be generated in their absence, and that it was of doubtful significance in producing true anaphylactic shock which results primarily from histamine release [for review of the earlier literature see (111, 332)].

Definition. In accordance with the historical development the term anaphylatoxin (AT) will be used in this review as the trivial name of the spasmogenic principle discovered by Friedberger. This principle was in recent years recognized as a peptide (314) and then identified as C5a, a cleavage product of C5 (149). In view of possible minor variations in the complete structure depending on the source and mode of formation of AT, the name will include all peptides biologically and chemically related to Friedberger's original substance, *i.e.*, all peptides acting on the same receptors (*i.e.*, anaphylatoxin receptors).

The term is not meant to denote a function. As pointed out above, "anaphylatoxin" in a functional sense would mainly be histamine, not C5a. After the discovery of C3a, as a spasmogenic principle some authors have extended the term AT to this peptide and the spasmogenic effects of both C3a and C5a have been described as "anaphylatoxic." Although a definition is lacking, it appears that the term AT is used by these authors in a functional sense to denote complement-derived mediators of anaphylaxis and to describe their effect on smooth muscle as related to a Schultz-Dale reaction. Such a definition would disregard the facts that complement is not involved in true anaphylaxis and neither C3a nor C5a contributes to its clinical signs (see below), and that the response of an isolated strip of guinea pig ileum cannot define, *per se*, a specific type of pharmacological agents. C3a and C5a clearly are different pharmacologically and chemically. Smooth muscle contraction with tachyphylaxis and histamine release is common to a variety of unrelated substances, *e.g.*, bradykinin and phospholipase A.

The use of the term AT as a trivial name is in accordance with common usage in pharmacology where names, originally coined with a functional meaning are strictly used to denote specific agents (*e.g.*, angiotensin and vasopressin which names have the same functional sense but mean different peptides and cannot be extended to other vasoconstrictors).

Origin and formation. Jensen (149) first demonstrated that guinea pig serum would form AT only when C5 was present. A principle which chemically and biologically resembled AT also was released when purified C5 from guinea pig serum was incubated briefly with EAC$\overline{1423}$ or trypsin. Similar results were obtained with human complement components (55, 188). Labelled, purified C5 treated with EAC$\overline{1423}$ yielded a labelled fragment with AT-like properties (298). When whole serum was incubated with yeast, added labelled C5 was also cleaved releasing a labelled product chromatographically identical with highly purified AT from hog serum (337). All these findings indicated that AT is the C5a peptide fragment of C5. It is the only spasmogenic principle formed when guinea pig, rat, or hog plasma is incubated with various polysaccharides or the cobra venom factor.

Friedberger (97) assumed that complement was involved in AT formation. Osler *et al.* (247) found indirect evidence for complement-dependent AT release. Dias da Silva and Lepow (75) generated small amounts of AT activity by treating guinea pig or rat serum with C1 esterase. The lack of a Ca^{++}

and C4 requirement for AT formation by polysaccharides (110, 282, 339) however, initially seemed to be difficult to reconcile with complement-dependent AT release. Recently it has been shown that the polysaccharide-induced AT formation follows activation of the properdin system by-passing C1, C4, and C2, and independent of Ca^{++} (39, 41, 108, 118). The substances known to activate the properdin system induce AT release much more efficiently than immune complexes which follow the classical pathway of complement activation (39). C5 fragments having AT activity are also released by proteolytic enzymes such as lysosomal enzymes from leukocytes (307, 321, 354), bacterial proteases (52), an extract from the cercaria of *Schistosoma mansoni* (102), and trypsin (55, 149, 188).

Although AT is formed by incubation of guinea pig, rat, or pig sera with immune aggregates, polysaccharides or cobra venom factor, sera from man and some other species do not accumulate directly detectable AT activity under these conditions. Human AT can be generated in a system of pure complement components suggesting that it is inactivated in whole serum subsequent to its generation (33, 225). Purified human AT is also rapidly inactivated in sera from various other species (33). Probably the same carboxypeptidase (anaphylatoxin inactivator) is responsible which attacks C3a and bradykinin (33, 87, 88). On the other hand, AT *has* been detected in activated human and some other sera after separation and concentration by chromatography (335). These preparations were immunochemically identical with (336) and acted on the same receptors as hog AT (166) but the yield of activity was much less than from hog serum. The discrepancy between the two findings—demonstration of little AT activity, and inactivation of human AT in human serum—has recently been resolved by Vallota *et al.* (329, 330). They found that in hog and human serum the AT peptide which is cleaved from C5 contains lysine at the carboxyl terminal. The lysine is then cleaved by serum carboxypeptidase. The primary product is strongly spasmogenic while the secondary hog peptide has five times less activity and the secondary human AT has "no" activity. When human or hog serum is incubated with yeast in the presence of ϵ-ACA, an inhibitor of carboxypeptidase, active primary AT also accumulates (329, 330). The AT described by Friedberger thus represents a secondary peptide which is readily detectable only in some species, but in others can be detected only after concentration. Primary AT will be referred to as AT I and the secondary peptide, as AT II or simply (classical) AT where no special distinction is made.

Chemical properties. AT II from hog or rat serum is a slightly basic peptide, isoelectric at pH 9.5 (196, 314, 333, 367). Human AT I may be expected to be more basic than AT II but is much less basic than C3a (330). Values for the molecular weight of AT vary, ranging from between 9,000 and 11,000 (55, 196, 367) to 16,500 to 17,500 (298, 309, 330). An earlier value of 30,000 (313) probably reflected aggregation. Liefländer *et al.* (196) have analyzed the amino acid composition of hog AT and found 74 to 76 residues, the N-terminal, arginine and the C-terminal, leucine. As mentioned already human and hog serum AT I contain lysine at the C-terminal end which is cleaved by serum carboxypeptidase to give AT II (329, 330).

AT II is stable in serum, and resists boiling at low pH. Its biological activity is destroyed by trypsin, chymotrypsin (314, 333), and mercaptoethanol, compatible with functionally essential disulphide bridges (333, 367).

ATs from man, guinea pig, rat, rabbit, pig and the mouse generated by exposure to cobra venom factor or yeast are immunochemically identical in their reaction with a rabbit anti-hog AT serum (336).

Pharmacological actions. AT was first recognized because of its spasmogenic effect on the bronchi of guinea pigs. A sensitive and frequently used assay for AT is the

contraction of isolated strips of guinea pig ileum. Quantitative assay is hampered by the tachyphylaxis which develops after a few applications, but techniques have been developed to overcome this difficulty (166, 266, 273). On the other hand, the tachyphylaxis facilitates the differentiation of AT from other agents. Related substances act on the same receptors and show cross-tachyphylaxis. The lack of cross-tachyphylaxis indicates significant biological and chemical differences. For example Lembeck and Fischer (183), by cross-tachyphylaxis experiments, were able to predict that substance P is chemically related to physalaemin and eledoisin but not to bradykinin, later proved correct when the structure of substance P was established (326). AT from man, guinea pig, hog, or the rat, generated by incubation of serum with yeast, zymosan, or cobra venom factor, or C5a generated by the treatment of purified human C5 with EAC$\overline{1423}$ or trypsin, show cross-tachyphylaxis with one another (55, 149, 152, 154, 166, 333–335), but not with C3a preparations (55, 74, 76, 188).

Smooth muscle, other than ileum, of guinea pigs and some other animals is also contracted by AT II (95, 166, 341), but little, if any contraction is produced in the rat uterus (55, 95, 188, 189). AT II contracts the vascular smooth muscle of pig kidney (153) and guinea pig heart (21), and in intact guinea pigs AT II induces a secondary vasoconstrictor effect after an initial fall in blood pressure. This vasoconstriction, but not the initial drop in blood pressure, is subject to tachyphylaxis (28, 29). The contractility of isolated papillary muscles of guinea pig heart is increased by AT, but rat papillary muscle does not react (20). Spasmogenic effects of AT I have so far been studied only in the isolated guinea pig ileum. Concentrations of 10^{-9} M human AT I have been found to produce strong contraction (330).

Intradermal injection of AT in guinea pigs which have received intravenous injections of Evans blue dye increases local vascular permeability with dye extravasation (31, 55, 189). Human AT I produces a wheal and erythema reaction when 10^{-15} moles are injected in human skin (330).

Many tissues of guinea pigs and probably of man release histamine when exposed to AT (30, 96, 101, 132, 134, 188, 221, 272, 330), predominantly from mast cells (21, 188, 221). Rat mast cells react poorly (188, 220). This histamine liberation revived interest in AT (134, 272).

Since some of the effects of AT are inhibited by antihistamines, AT has been envisaged as a histamine liberator which acts by mediation of this amine, and the tachyphylaxis has been explained as due to exhaustion of histamine stores (282). However, contractions of guinea pig uterus, of rat and rabbit smooth muscle, and both the hypotensive and hypertensive effects in guinea pigs and cats are not counteracted by antihistamine (28, 29, 95, 341). Several of these effects are not subjected to tachyphylaxis. The constriction of coronary vessels is opposite to the effect of histamine. The increase in vascular permeability and bronchospasm are inhibited, but not blocked by antihistamine and may be viewed as enhanced, but not initiated by histamine (28, 29, 31, 289). Only the spasmogenic action of AT on the guinea pig ileum is blocked completely by antihistamine. But even this is largely independent of histamine, and the antagonism by antihistamine is due to blockade of AT receptors rather than of histamine receptors (30). These findings suggest that AT has a direct effect on smooth muscle and capillary walls in addition to its histamine liberating property. AT tachyphylaxis then cannot be due to exhaustion of histamine stores but could be the consequence of strong binding of AT to its receptors.

The hypertensive effect of AT in guinea pigs is blocked by the α-adrenergic blocking agent, dihydroergotamine, indicating that AT liberates noradrenaline in addition to histamine (29). Acetylcholine may also be released (27), but direct evidence is lacking.

AT is chemotactic for polymorphonuclear

leukocytes, *in vitro* and *in vivo* (69, 154, 159, 214, 298, 303, 329, 357, 358). The chemotactic activity is much less dependent on the intact peptide chain of AT I than is the smooth muscle activity. Human and rabbit serum, activated by immune complexes (305, 346), which is devoid of spasmogenic activity contains chemotactic C5a. Chemotactic C5a is also found in synovial fluid from patients with rheumatoid arthritis (359) although any spasmogenic activity would be expected to be destroyed by conversion of AT I to AT II by carboxypeptidase. Prolonged incubation of human C5 with trypsin abolishes the spasmogenic activity initially formed (55) but retains its chemotactic activity (357).

According to Wissler *et al.* (366, 369, 370) purified rat or hog serum AT is not chemotactic for neutrophils. It acquires this activity after interaction with another peptide, co-cytotaxin, which they have purified (368). Nucleotides, such as cAMP and ATP have also been reported to render AT chemotactic (370). These findings have not yet been confirmed in other laboratories. In fact, slight chemotactic activity is shown in a diagram published by Wissler *et al.* (370), raising the possibility that their results may represent only quantitative differences from other studies with highly purified preparations of AT which are chemotactic for neutrophils (69, 159, 329). The origin and relationship of co-cytotaxin to complement remains unknown.

Eosinophils are also attracted by C5a (157, 159). In the presence of the eosinophil chemotactic factor of anaphylaxis, EFC-A, the chemotactic effect of AT for eosinophils is selectively enhanced over that for neutrophils and even becomes predominant (159). EFC-A is unrelated to complement, is much smaller than C5a, and is specifically chemotactic for eosinophils (160).

AT is also chemotactic for monocytes (308). The available evidence indicates that the same molecular entity is responsible for the attraction of neutrophils, eosinophils, and monocytes (159, 308). Lymphocytes are not attracted by AT (358).

Biological significance. Originally, anaphylaxis was thought to be caused by AT, in particular bronchospasm and vasospasm. This is unlikely, for the following reasons. 1) True anaphylaxis results from the combination of mast cell-bound antibodies (IgE) with antigen, leading to the release of histamine and other intracellular mediators (8a, 46). The Schultz-Dale reaction, the classical model of anaphylaxis (contraction of, *e.g.*, an isolated ileum from sensitized guinea pigs on application of the antigen) can occur in the absence of serum without complement and AT formation. (The contraction produced by AT is not a Schultz-Dale reaction.) Monkey lung tissue may contain bound complement components even after washing (208) which are necessary for allergic histamine release but the same studies indicate that AT is not involved. 2) The Schultz-Dale reaction can still be induced after the isolated organ has been desensitized to AT (95, 127, 128, 221), and immunized guinea pigs insensitive to AT after prior injections still develop anaphylactic shock when given specific antigen (67, 95, 133). 3) Anaphylaxis is not accompanied by significant complement consumption or reduction of C5, the AT precursor protein (46, 112). 4) While antibodies inducing anaphylaxis do not activate the classical complement pathway, antibodies which fix complement do not provoke anaphylactic shock (46). Thus AT is not essential for anaphylaxis. It does not even contribute significantly to it because 1) injection of cobra venom factor does not cause serious symptoms although AT is generated in large amounts, and 2) humoral antibodies in the blood generate AT when the antigen is injected. By combining with antigen before it reaches cell-bound IgE, they rather protect from anaphylaxis than cause it. This argument was already proposed by Dale and Kellaway (67).

Passive cutaneous anaphylaxis (PCA) induced in rats requires complement (245),

and the increase in skin capillary permeability characteristic of this reaction may be mediated by AT. However, this reaction is partly an Arthus-type reaction rather than a true anaphylactic process (46). Certain types of passive cutaneous anaphylaxis can be elicited in mice deficient in C5, *i.e.*, without complement activation and AT generation (18, 51). In passively immunized animals AT may enhance anaphylactic shock since some (93, 136) but not other (94) investigators could protect such animals by previous desensitization with AT. Forssman shock which is clearly different from anaphylaxis (46) may also be augmented by AT since the capacity of the serum to generate AT and total complement activity were reduced after the induction of Forssman shock in guinea pigs (112). An inhibitor of C4 also protects guinea pigs from Forssman shock (151).

AT is probably involved in immune reactions which do require complement, especially in local inflammatory symptoms. In particular the accumulation of leukocytes in inflammation (58, 143) may be related to C5a which has been detected in tissues at the site of Arthus reactions (356), in synovial fluid from patients with rheumatoid arthritis (359, 380), and from rabbits with experimental immune arthritis (73). C5a has also been found prior to the accumulation of neutrophils in inflammatory peritoneal exudates produced by the injection of endotoxin or glycogen. C5-deficient mice fail to show significant neutrophilic extravasation (306). The initial step in such neutrophilic chemotaxis is formation of AT through complement activation by immune complexes or by agents such as endotoxin or glycogen which activate the alternate complement pathways. As soon as leukocytes have accumulated and deteriorate a vicious circle may develop since leukocyte lysosomes contain a C5-cleaving enzyme (307, 321, 354) capable of generating more C5a which would in turn increase vascular permeability and allow additional complement components to enter the local site, and prolong inflammation. Depletion of neutrophils can reduce vascular permeability in experimental arthritis, and this effect is reversed by the injection of neutrophils into the involved joint (73).

Nephrotoxic nephritis produced by the injection of antibody to glomerular basement membrane requires neutrophils and complement (327). C5a may well play an important role in this experimental disease related to poststreptococcal and lupus nephritis as well as in other forms of vasculitis. Proof for such a role, however, has not been obtained. It has been shown that C5-deficient mice can also develop nephrotoxic nephritis with leukocyte infiltration (197).

Gouty arthritis develops only after neutrophils enter the joint (255). The generation of chemotactic factors, including C5a, follows complement activation by the urate crystals deposited in the joint space (50, 232, 359).

Virus-infected cells release a C5-cleaving enzyme which releases chemotactically active C5a (45, 353). This process has been demonstrated in tissue cultures but it is likely that it also occurs *in vivo*. The inflammation following tick-bites or accompanying the development of cercaria of *Schistosoma mansoni* also seems to be produced by C5a (19, 102).

Jensen *et al.* (153) found that AT contributes to the rejection of xenografts by its vasoconstrictor effect. Pig kidneys perfused with dog blood develop severe vasoconstriction within minutes comparable to that observed in pig kidney transplants into dogs. This reaction is enhanced by leukocytes, but it is not entirely dependent on them. When the kidneys are perfused with cell-free dog plasma the vasoconstriction appears in about 30 minutes. It is mediated by natural antibody in the dog plasma combining with pig kidney constituents and activating complement. AT produces the same effects. Prevention of AT formation by heating the dog plasma to 56°C makes the heterologous plasma compatible (153, 198). In addition AT appears to contribute to xenograft rejection by its chemotactic effect since injection

of mouse anti-rat serum leads to acute damage of rat skin transplants in mice, and this effect is dependent on C5 and neutrophils (365). Of course the effects of AT are not the sole basis for xenograft rejection. but they may augment cellular immune reactions leading to histoincompatibility.

On a whole AT seems to be involved in a variety of local immunological reactions, though not in true anaphylaxis.

Acknowledgments. I wish to thank Dr. V. Brade and Dr. I. H. Lepow for sending preprints of publications, and to Dr. P. Ward, and two unknown referees for suggestions in completing the manuscript. Sincere thanks are also due to Mrs. H. Amort for her secretarial assistance.

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