Studies on the first component of the bovine complement system.

Anthony Shing-Duen. Pang  

University of Windsor  

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STUDIES ON THE FIRST COMPONENT OF THE BOVINE COMPLEMENT SYSTEM

BY

ANTHONY SHING-DUEN PANG

A Thesis
Submitted to the Faculty of Graduate Studies through the Department of Chemistry in Partial Fulfillment of the Requirement for the Degree of Master of Science at the University of Windsor

Windsor, Ontario
1973
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ABSTRACT

Bovine CI was prepared by precipitation at low ionic strength (μ = 0.030 - 0.040) and at pH 7.5, and it was further purified by chromatography on Sephadex G-200.

Those inhibitors, phenylmethyl sulphonyl fluoride, diisopropylfluorophosphatase and 3-(3,4-dichlorophenoxyacet-amido)-N-(3, chloro-2-fluorosulphonylbenzyl) pyridinium bromide, which inhibited CI esterase activity also inhibited the ability of CI to form EAC_{Bov}^{142} from the EAC_{Bov}^{42} intermediate. It was suggested that the esterase active site might be involved in the active CI^{142} enzyme or part of the CI molecule might be involved in somehow maintaining in active conformation of the lytic intermediate.

From the studies of the uptake of the active CI prior treatment of cells with CI-DFP and the uptake of CI-{³H}DFP suggested that this molecule was not taken up by the EAC_{Bov}^{42} complex. It was postulated that the EAC_{Bov}^{142} complex was compact structure in which CI has an intimate relationship with either or both of the C4 and C2 components.
ACKNOWLEDGEMENTS

I wish to express my deepest gratitude to my advisor, Dr. W.P. Aston, for his patient encouragement and expert guidance through this research work.

I also wish to express my appreciation and thanks to Dr. D.E. Schmidt Jr. for his valuable criticisms and his gift of PABPB compound. I am indebted to Dr. T.K.S. Mukkur of the Department of Biology, University of Windsor, for donating the guinea pig cells.

Finally the author wishes to express his gratitude to the Red Cross Transfusion Service, Windsor and Windsor Packing Co. for supplying human blood and bovine blood respectively.
DEDICATION

TO MY PARENTS

敬獻雙親

兒端
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ABBREVIATIONS

E  Erythrocyte (sheep cell)
A  Rabbit antibody to sheep erythrocytes
    (antisheep haemolysin)
C  Complement
Cl, C2 ... 9  Complement components
EACl - n  Intermediate complexes formed by the reaction
          of the first n complement components
Ca  Complement component that has acquired
    enzymatic or other biological activity
EDTA  Trisodium Ethylene Diamine Tetraacetate
G-EDTA  EDTA-treated guinea pig complement
PBS  Isotonic phosphate buffered saline
GBS  Gelatin-Barbital-Saline  (μ = 0.15, pH = 7.35)
GBS++  GBS containing 1 × 10^{-3} M MgCl_2 and
       1.5 × 10^{-4} M CaCl_2
GBS-Sucrose  Sucrose-Gelatin-Barbital Saline
GBS-Sucrose++  GBS-Sucrose containing 1 × 10^{-3} M MgCl_2
              and 1.5 × 10^{-4} M CaCl_2
EDTA-GBS  GBS containing 0.01M EDTA
DFP  Diisopropylfluorophosphate
3H-DFP  Diisopropyl-3H-fluorophosphate
Cl-DFP  GT treated with DFP
Cl-3H-DFP  GT treated with 3H-DFP
PABPB  3-(3,4-Dichlorophenoxyacetamido)-N-(3, chloro-
       2-fluorosulphonylbenzyl) pyridinium bromide
PMSF  Phenylmethyl sulphonyl fluoride
TLCK  N-α-Tosyl-L-lysylchloromethane HCl
TPCK  N-Tosyl-L-phenylalanyln Chloromethane
MeOEtOH  Ethylene glycol monomethyl ether (methyl
          cellusolve)
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<tr>
<td>N-Z-L-Tyr-p-Np</td>
<td>N-Carbobenoxyl-L-tyrosine-p-nitrophenyl ester</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>O.D.</td>
<td>Optical Density</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>d.p.m.</td>
<td>Disintegrations per minute</td>
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CHAPTER I

INTRODUCTION

The term complement refers to eleven serum proteins which together account for about 10% (w/w) of the globulin fraction of human serum (1). They are activated characteristically by antigen-antibody interaction and subsequently mediate a number of biologically significant consequences. It has become customary to define complement on the basis of its membrane-damaging or cytolytic capacity. In immune cytolysis, antibody plays the role of an adaptor which directs the action of complement toward a specific target, the cell membrane, thereby increasing its cytolytic efficiency. In exceptional situations, complement can cause cell lysis by itself without the aid of antibody or immune complexes (2). Membrane damage by complement, although studied primarily in vitro, may be produced experimentally in vivo and is considered an important feature of the pathogenesis of a number of immune disorders. In addition to cytolysis, complement may cause the release of histamine from mast cells, contraction of smooth muscles, capillary permeability changes, directed migration of polymorphonuclear leukocytes, and enhancement of phagocytosis. It plays a role in host defence against infections, and is a mediator of inflammation and possibly of blood coagulation (1).

Complement is a non-specific substance and is not increased in amount as the result of immunization. It is a fact that complement from the same serum can often be used to activate a variety of reactions involving a number of different antibodies and antigens. Nevertheless, different complement sources vary greatly in haemolytic activity when tested with erythrocytes derived from different animals (3,4).
or with erythrocytes sensitized with antibody produced in
other species. The highest haemolytic activity with rabbit
antibody was obtained using guinea pig, goat, cat and dog
complement. Dog antibody is very effective with goat
complement (5). Thus, it is clear that the titer of a given
serum complement may vary greatly according to the antibody
used for sensitization.

All classes of immunoglobulins are not equally proficient
in 'fixing' complement after their union with antigen;
IgM is usually more effective in this respect than IgG, but
IgA does not fix complement (6). One of the most striking
things about complement is that heating to 56°C for half
an hour destroys its activity, although most of the serum
proteins resist this treatment. The activation may not be
entirely irreversible, for Gramenitzki (7) found a gradual
return to an active condition after moderate heating.
Complement is also inactivated by prolonged shaking and
its activity is permanently destroyed by the addition of
any considerable amount of acid or alkali, and in fact
complement seems to have maximal stability only within the
pH ranges 6.0 to 6.5 (8). Under ordinary conditions of cold
storage at 0 - 4°C, as much as 90% of the activity may
disappear in 3 to 4 days.

In their native form complement proteins are inactive.
Following activation they display at least two functional
sites, a combining region and a site through which a given
complement protein fulfills its specific role in the
complement reaction sequence (1).

The components of complement (C) are designated by
numbers (9), i.e., they are referred to as C1, C2, C3,
C4, C5, C6, C7, C8 and C9. Table I shows the physiochemical
characteristics of human complement components. C1 consists
of three subcomponents called Clq,Clr and Cls and they
TABLE I

Properties of Human Complement Protein (1)

<table>
<thead>
<tr>
<th>Complement Component</th>
<th>Serum Conc. (µg/ml)</th>
<th>Sed. Coeff. (S)</th>
<th>App. Mol. Weight</th>
<th>Electrophoretic Mobility</th>
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<td>C1q</td>
<td>190</td>
<td>11.1</td>
<td>400,000</td>
<td>γ₂</td>
</tr>
<tr>
<td>C1r</td>
<td>---</td>
<td>7.0</td>
<td>168,000</td>
<td>β</td>
</tr>
<tr>
<td>C1s</td>
<td>22</td>
<td>4.0</td>
<td>80,000</td>
<td>α₂</td>
</tr>
<tr>
<td>C2</td>
<td>20-40</td>
<td>5.5</td>
<td>117,000</td>
<td>β₂</td>
</tr>
<tr>
<td>C3</td>
<td>1,200</td>
<td>9.5</td>
<td>185,000</td>
<td>β₁</td>
</tr>
<tr>
<td>C4</td>
<td>430</td>
<td>10.0</td>
<td>240,000</td>
<td>β₁</td>
</tr>
<tr>
<td>C5</td>
<td>75</td>
<td>8.7</td>
<td>180,000</td>
<td>β₁</td>
</tr>
<tr>
<td>C6</td>
<td>?10 ?α50</td>
<td>5-6</td>
<td>95,000 (140,000)</td>
<td>β₂</td>
</tr>
<tr>
<td>C7</td>
<td></td>
<td>5-6</td>
<td>110,000 (140,000)</td>
<td>β₂</td>
</tr>
<tr>
<td>C8</td>
<td>10</td>
<td>8.0</td>
<td>(150,000)</td>
<td>γ₁</td>
</tr>
<tr>
<td>C9</td>
<td>10</td>
<td>4.5</td>
<td>79,000</td>
<td>α</td>
</tr>
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are held together by calcium ions. Intermediate reaction products of complement-dependent cytolysis consisting of cell (E), antibody to cell surface antigen (A), and complement components are symbolized by notations which indicate the components required for their formation. For instance, EAC142, EAC42 are notations referring to antibody-cell complexes which have reacted with C1, C4 and C2; C1 and C4 respectively. Complement-associated enzyme activity may be indicated by placing a bar above the numeral which refers to the component in which the activity resides: enzymatically active C1 may be written C1.

Erythrocytes sensitized with anti-erythrocyte antibodies may be lysed by complement. Normal guinea pig serum usually possesses a high level of lytic activity and is commonly used as a source for complement study. Most investigators who have studied the fundamentals of immune cytolysis have used sheep erythrocytes optimally sensitized with rabbit anti-sheep cell serum as target cells and either guinea pig or human serum as the source of complement.

The first step in the complement reaction is thought to be uptake of the components Clq,Clr and Cls, in the form of the Cl complex. The uptake requires calcium ions. Then the proesterase Cls is activated, presumably by some mechanism involving Clq andClr (10). Activated Cl can move from site to site and from cell to cell and like an enzyme can react with many molecules and substrates (11). Although is is an enzyme, C1 esterase is not involved in the actual process of cell lysis. The natural substrate of C1 esterase is not the cell membrane but apparently C4 and C2. The next event in the complement reaction therefore seems to be the sequential uptake by the complex, BACT, of C4 and C2. Following the formation of the complex BACT42, activation of C3 and its subsequent binding to the cell membrane occurs.
The subsequent event of immune lysis are less well understood. The bound C3 is thought to modify the EAC42 enzyme so that it initiates the activation of the remaining five components with the assembly on the membrane of a C56789 complex. At this stage lesions occur in the cell membrane, perhaps as the result of a phospholipase action, and the osmotic balance between the exterior and interior of the erythrocyte cell is upset which eventually brings about lysis of the cell (51). Fig I shows the sequence of the immune lysis of a red blood cell.

Although bovine serum has a very potent bactericidal complement system and haemolytic complement activity against sensitized rabbit erythrocytes (13), its lack of haemolytic function in standard assays with sensitized sheep erythrocytes makes it difficult to carry out comparative kinetic studies of haemolytic complement activities among species (14). However, recently Barta and Barta (15) reported that the bovine haemolytic complement could be assayed using guinea pig erythrocytes sensitized with naturally occurring antibodies from bovine serum, as the target cells in Sucrose-NaCl Veronal Buffer (pH 7.0, ionic strength \( \mu = 0.094 \)) containing \( 1 \times 10^{-3} \text{M Mg}^{++} \) and \( 3 \times 10^{-4} \text{M Ca}^{++} \) ions.

Fong, et al (16), reported that although bovine serum was non-haemolytic in standard haemolytic complement assays it reacted with sensitized sheep erythrocytes, under conditions of carefully controlled concentration, temperature and ionic strength, to form an intermediate which could be lysed with EDTA-treated guinea pig serum (C-EDTA). The optimum pH for EAC_Bov formation was between 7.0 and 7.5. They concluded that the formation of a bovine lytic intermediate with sheep cells sensitized by rabbit antibody suggested that there was no incompatibility of interaction between early-acting bovine complement components and guinea pig
FIGURE I

SEQUENCE OF REACTION: OF COMPLEMENT COMPONENTS

EA

Clqrs
Ca++

EACT1

C4

EACT4

C2
Mg++

EACT42

G3

EACT423

C5, C6, C7

EACT423567

C8

EACT4235678

C9

EACT42356789

EA designates the reaction site between antigen and antibody.

C4 is important for immune adherence.

Immunoglobulin reacts to C4 and C3. C3 important for immune adherence and erythrocyte phagocytosis. Low molecular weight chemotactic factor released from C3.

Chemotactic factor is released from C5. Formation of ultrastructure lesions in cell membrane.

C9 is necessary for membrane-lysis. Formation of functional lesions in cell membrane.
terminal complement components or sensitized sheep cells. Subsequently the inability of bovine serum to lyse sheep erythrocytes probably resides in the difference in reactivity of the terminal bovine complement components. Thus the specificity of lytic complements of different species may be a function of their terminal complement components. They showed indirectly that the lytic intermediate was EAC42 (17). The intermediate was lysed completely by C–EDTA in six minutes of incubation at 37°C at ionic strength between 0.075 and 0.125. It was extremely stable in low ionic strength buffer (glucose–gelatin–barbital–buffered saline, GGBS++) with divalent cations. However when the intermediate was washed in 0.01 M EDTA and then twice in GGBS++, it became non-haemolytic with C–EDTA. But when the EDTA-treated-intermediates were incubated with bovine or human euglobulins in GGBS++ for 15 minutes at 37°C, the intermediates became haemolytic again with C–EDTA. Since the human euglobulin was functionally pure for Cl activity and contained no C4 or C2 activity, the identity of the decayed lytic intermediates appeared to be EAC42.

The first component of complement (Cl) is an euglobulin. Studies on the effect of the divalent cation chelator trisodium ethylenediaminetetraacetate (EDTA) on Cl led to the recognition that human Cl is composed of at least three fragments, Clq,Clr and Cls, separable by chromatography on DEAE cellulose (18). The complex reforms when the three proteins are mixed in the presence of Ca++. Affinity and specificity of Cl for immunoglobulins resides in the subunit Clq (19). Following binding of the complex to EA, a proenzyme (Cls) is activated through internal mechanism involving Clr (20); activated Cl triggers the complement chain reaction. It may be postulated that Clr is able to act on Cls through a distortion or spatial rearrangement of the Cl complex.
resulting from combination with antibody through Clq. Human and guinea pig Cl is a proesterase which becomes an esterase following its activation to CI (21). The proesterase and esterase activity has been identified with the Cls (22). It hydrolyzes p-toluene-sulphonyl-L-arginine methyl ester (TAME), N-acetyl-L-tyrosine ethyl ester (ATEE) (23, 24) and N-carbobenzoxy-L-tyrosine-p-nitrophenyl ester (25).

Becker reported that in the guinea pig system with the assembly of the C42 enzyme on the cell surface, CI had fulfilled its function and might be removed without impairing progress of the immune haemolysis reaction (26). But in the bovine system Fong et al found the presence of CI was required by EAC142 for it to function as a lytic intermediate (17). Therefore it is of interest to investigate the role of CI in activity of the EAC Bov 142 complex.

Studies on several inhibitors of haemolytic and esterase activities of the first component of complement can contribute to our knowledge of biochemistry of this component.

A naturally occurring serum protein found in the sera of rabbits, guinea pigs, and humans reacts with CI but not with Cl and blocks the esterase activity of both CI and the 4S fragment (Cls) derived from CI (27). That serum protein is called Cl-esterase inhibitor with a sedimentation constant of 3S. It also inhibits the haemolytic activity of CI. A relative high molecular weight substance, carrageenan, inhibits the haemolytic activity of Cl or CI (28) by preventing attachment of the complement to antigen–antibody complexes. But it does not inhibit the esterase activity of partially purified CI. These show that the site on CI that binds to antigen–antibody complexes is distinct from the site possessing enzymatic activity (26).

Human and guinea pig CI have several properties in common with other proteolytic enzymes such as trypsin and chymotrypsin (29). Most notable of these is its ability to
hydrolyze synthetic amino acid esters such as TAME and ATEE. One of the most effective esterase inhibitors is diisopropylfluorophosphate (DFP). Trypsin and chymotrypsin are both inhibited by DFP (1). The haemolytic and esterase activities of guinea pig C1s and C1 are inhibited by DFP (30). Since it has been proved that this compound attaches to serine in the active site of chymotrypsin and trypsin and inactivates them (31), it may be assumed that serine is part of the active centre of C1 and C1s.

However, in the absence of sensitized cells, DFP fails to inhibit the haemolytic activity of the whole guinea pig complement (32). The inert precursor forms of trypsin and chymotrypsin, trypsinogen and chymotrypsinogen, respectively, are not inhibited by DFP (33). Therefore one can postulate that C1 also exists in serum in an inactive precursor form resistant to the action of DFP (32).

The intermediate product EACI42 in human and guinea pig system is unstable, i.e. it loses its ability to react with C3 (34). Its half-life is about eight minutes at 37°C, about twenty minutes at 30°C, and less than ten hours at 0°C. The rate of decay is not affected by EDTA. The decayed EACI42 can be lysed by treatment with purified C2, followed by C-EDTA. This means that the decayed cells are in the state EACI4 (35). On the contrary, EACBovI42 was reported to be extremely stable in low ionic strength buffer (GGBS++, \( \mu = 0.075 \)) with divalent cations: up to 18 hours at 37°C and 6 days at 2°C (16). The rate of decay of EACBovI42 is accelerated by EDTA and leads to another stable intermediate EAC42 (17). The reactivity of the decayed intermediate can be restored by either bovine or human C1 (17). This provides a convenient assay for C1. The EACBovI42 may be conveniently prepared by treating sensitized sheep erythrocytes with bovine serum at 2°C for 10 minutes in a low ionic strength buffer (GGBS++, \( \mu = 0.075 \), pH = 7.3 containing \( 1 \times 10^{-3} \) M
Mg$^{++}$ and 1.5 x $10^{-4}$M Ca$^{++}$). In contrast to the corresponding human and guinea pig intermediates the maximum formation of the bovine lytic intermediate is inversely associated with incubation temperature and is enhanced to a greater extent by magnesium ions.

As yet most studies on the function of complement have been investigated using guinea pig or human system. To fully understand the role of complement in the immune response of animals and to gain an insight into its evolution, the complement system of various species must be investigated.

The roles of the early acting components of bovine complement in the assembly and activity of EAC$^{142}$ complex seem different in many aspects from the corresponding components of the guinea pig and human complement systems. Therefore it is of great interest to examine these bovine components carefully and to compare them in their physiochemical and functional properties with their human and guinea pig counterparts.

This manuscript presents an investigation of some of the characteristics of bovine Cl. A method is presented for its functional purification. Its role in the EAC$^{Bov}_{142}$ complex has been investigated by subjecting the protein to various inhibitors and examining their effects on the enzymatic activity of Cl and its capacity to form the active intermediate EAC$^{Bov}_{142}$ from EAC$^{Bov}_{42}$.

The selective inhibition of enzymes of the serum complement system may prove a useful means of controlling many of the pathological states in animals promoted by complement, e.g. inflammation and rejection of tissue and organ transplants. The selective inactivation of Cl, the component which sets in motion a chain of events whereby the other complement proteins enter into their characteristic interactions and functions, would have a profound effect on the whole complement system. It is
FIGURE II

Proteolytic Enzyme active-site-directed Irreversible Inhibitors

Type I

Examples

\[ \text{R}_1 \]

3,4-Cl\textsubscript{2}C\textsubscript{6}H\textsubscript{3}OCH\textsubscript{2}CONH \hspace{1cm} 4-SO\textsubscript{2}\textsubscript{F} \n
\[ \text{R}_2 \]

3,4-Cl\textsubscript{2}C\textsubscript{6}H\textsubscript{3}OCH\textsubscript{2}CONH \hspace{1cm} 2-SO\textsubscript{2}\textsubscript{F} \n
Type II

\[ \text{m-CH}_2\textsubscript{6}H_4\text{NHCONHC}_6H_3-3-\text{Cl}-4-SO_2F \]

Type III

\[ n \]

3 \hspace{1cm} \text{m-NHCONHC}_6H_4\text{NO}_2-p \n
\[ n \]

3 \hspace{1cm} \text{m-NHCONHC}_6H_4\text{SO}_2F-p \]
not surprising that other inhibitors of trypsin and chymotrypsin inhibit C1 and other components of complement since some are 'tryptic' or 'chymotryptic' or both in character (29). Baker showed that there were two types of chymotryptic inhibitor (Type I and Type II) and one type of tryptic inhibitor (Type III) which inhibited whole guinea pig complement (38). Figure II shows the structures of the three different types of inhibitor. Type I is a series of substituted pyridines quaternized with fluorosulphonyl-benzyl bromide. The good correlation in irreversible inhibition of C1 and inhibition of whole complement by analogs of Type I suggests that the main site of action by compounds of Type I is the inhibition of C1. The removal of the sulphonylfluoride group from Type I and Type II compounds results in loss of their activity, indicating that this group is necessary for activity (38).

Benzamidine itself, though a strong trypsin inhibitor (37,39) is a weak inhibitor of complement (40). But the introduction of m- (p-nitrophenylurea) or m- (p-fluoro-sulphonylphenylurea) in the phenoxy group will enhance its inhibition on the whole complement. However the lack of correlation of irreversible inhibition of C1 and inhibition of whole complement by Type II and Type III suggest the main site of action of the Type II and Type III compounds is one of the other components of complement between C2 and C9 (38).

These inhibitors may find a role controlling complement function in tissue transplants, inflammation etc. This manuscript describes a study of the effect of 3-(3,4-dichlorophenoxyacetamido)-N-(3-chloro-2-fluoro-sulphonylbenzyl) pyridinium bromide (PABPB), its structure is shown in Figure III, on bovine whole complement and on the haemolytic and enzymatic activities of bovine C1. This compound was chosen as representative of the Type I
FIGURE III

Structure of 3-(3,4-Dichlorophenoxyacetamido)-N-(3-chloro-2-fluorosulphonylbenzyl)
Pyridinium Bromide (PABPB)
inhibitors which have been shown to have marked inhibitory effect on guinea pig Cl (38). It is hoped that the studies with PABPB on bovine Cl will give helpful information concerning the function of Cl, as an esterase and its capacity to form EACT42 from EAC42.
CHAPTER II
MATERIALS AND METHODS

Glycerinated antisheep haemolysin (an antibody preparation to sheep erythrocytes raised in rabbits) and lyophilized guinea pig complement were purchased from Difco Laboratories, Detroit, Michigan. The reconstituted complement was stored at -20°C and was apparently stable for several months. A weekly supply of sheep red cells (10% suspension in acid citrate dextrose) was obtained from Becton Dickinson and Co., Canada Ltd. and stored at 4°C. Guinea pig red cells were kindly provided by Dr. T.K.S. Mukkur, Biology Department, University of Windsor and were stored in Alsevers solution at 4°C. Fresh bovine whole blood was obtained from Windsor Packing Company. Outdated human blood in acid citrate dextrose was kindly supplied by the Canadian Red Cross Blood Transfusion Service, Windsor.

The substrate, N-carbobenzoxy-L-tyrosine-p-nitrophenyl ester (N-Z-L-Tyr-p-Np) for the esterase activity of C1 was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.

The inhibitor, 3-(3,4-dichlorophenoxyacetamido)-N-(3-chloro-2-fluorosulphonylbenzyl) pyridinium bromide (FABPB) prepared by the method of Baker and Hurlbut (41) and was a gift of Dr. D.E. Schmidt Jr., Chemistry Department, University of Windsor. A 25mM stock solution of the compound was prepared using ethylene glycol monomethyl ether (MeOEtOH) as solvent and stored at 4°C.

N-α-Tosyl-L-lysyl chloromethane hydrochloride (TLCK) and N-tosyl-L-phenylalanyl chloromethane (TPCK) were purchased from Calbiochem, California. Phenylmethylsulphonyl fluoride (PMSF) and 5m diisopropylfluorophosphate (DFP) in isopropyl alcohol were purchased from Pierce Chemical,
Rockford, Illinois.

Diisopropyl-1-\textsuperscript{3}H(N)-fluorophosphate, with a specific activity of 0.9 curies/mM and packed at a concentration of 0.0513 mg in 0.25ml isopropyl alcohol in a sealed ampoule, was obtained from New England Nuclear, Boston, Mass.

All solutions were prepared using distilled water which had been passing through a Bantam Demineralizer (Barnstead Still and Sterilizer Co., Boston, Mass.). Solutions which were not used immediately were protected from bacterial degradation by addition of sodium azide to a final concentration of 0.005M.

The diluent used in standard haemolytic assays of complement was isotonic Barbital Buffered Saline (GBS++) at pH = 7.35 and ionic strength $\mu = 0.15$, containing 0.1% gelatin, $1 \times 10^{-3}$M MgCl$_2$ and $1.5 \times 10^{-4}$M CaCl$_2$. This was prepared either according to the method of Rapp and Borsos (36) or from tablet form supplied by Oxoid Ltd., London.

A 0.2M stock solution of EDTA was prepared by mixing equal volume of 0.2M Disodium EDTA and 0.2M Tetrasodium EDTA.

EDTA-GBS was prepared by diluting the stock EDTA solution to a final concentration of 0.01M with GBS as diluent.

C-EDTA was obtained by mixing 1 volume of guinea pig complement with 9 volumes of 0.01M EDTA-GBS.

In the haemolytic assays of C$_1$, EAC$^{Bov172}$ and EAC$^{Bov42}$ the diluent used was GBS-Sucrose++ at pH 7.35 and ionic strength $\mu = 0.075$. This was prepared by mixing equal quantities of GBS++ and 9% sucrose and adjusting the final concentration of gelatin to 0.1% and divalent cation concentration to $1 \times 10^{-3}$M MgCl$_2$ and $1.5 \times 10^{-4}$M CaCl$_2$.

The diluent used in haemolytic assays of bovine complement using guinea pig erythrocytes as the target cells was GBS-Sucrose++ at pH 7.0 and ionic strength
\[ \mu = 0.094 \] containing 0.1\% gelatin, 1 \times 10^{-3} \text{M MgCl}_2 and
3 \times 10^{-4} \text{M CaCl}_2. It was prepared by mixing 3 volumes of
GBS++ with 2 volumes of GBS-Sucrose++ (\( \mu = 0.075 \)) (36).

Ten times concentrated stock Phosphate Buffered Saline
(PBS) was prepared by dissolving 80 gm NaCl, 2.0 gm KCl,
11.5 gm anhydrous Na_2HPO_4 and 2.0 gm anhydrous KH_2PO_4 in
1 liter of distilled water. Its pH was adjusted to 7.35 ± 0.05.
It was diluted 1:10 for normal use. PBS++ was prepared by
adding 1 ml of 0.15M CaCl_2 for every liter of PBS.

The scintillation fluid used for liquid scintillation
counting was prepared by adding 40 gm 2,5-diphenyloxazole
(PPO) and 600 mgm p-bis(2-(5-phenyloxazoly1))-benzene (POPOP)
in 600 ml toluene as stock solution. When in use, 1 liter
of the above solution was mixed with 700 ml ethylene glycol
monoethyl ether (58).

All tritium labelled radioactive samples were counted
using a Unilix II Scintillator Counter (Nuclear-Chicago
Corp.). The efficiency of counting was determined using the
Barium-133 external standard and a reference barium quench
curve (see Appendix).

All the O.D. data were obtained on a Gilford Model
200 absorbance recorder attached to a Bechman DU Monochromator.

A. Preparation of Cl

Bovine Cl was prepared initially according to
the method described by Tamura and Nelson (44) with some
modifications. Fresh bovine blood, 5 - 6 liters, was allowed
to clot at room temperature for 2 hours and then at 20°C for
six hours or overnight. Very little retraction of the clot
was observed and the serum had to be harvested by gentle
squeezing the clot. The serum was then cleared by
centrifugation at 8,000 RPM (10,400 g) for 20 minutes.
Usually a volume of 500 ml serum was used to prepare Cl. The
remainder was stored at -20°C where it remained stable with
respect to its capacity to form EAC\textsuperscript{Bo} cells for about 3 months. The pH of the serum was adjusted to 7.5 by dropwise addition of \(0.1\text{M} \text{K}_2\text{HPO}_4\) while stirring and then dialysed in 1/8 Visking tubing overnight against distilled water brought up to pH 7.5 by addition of \(0.1\text{M} \text{K}_2\text{HPO}_4\). For every liter of dialysing fluid, 1 ml of 0.15M \text{CaCl}_2 was added. The resulting precipitate was centrifuged at 12,000 RPM (173,000 g) for 20 minutes. The supernatant was a R1 (i.e. it contains all the other complement components except Cl) and the precipitate (containing Cl) was washed twice with distilled water and then dissolved in 1/10 of the original serum volume in 0.3M NaCl buffered at pH 7.5 with 0.01M phosphate and containing \(1 \times 10^{-3}\text{M} \text{MgCl}_2\) and \(1.5 \times 10^{-4}\text{M} \text{CaCl}_2\). The solution was centrifuged to clear at 12,000 RPM for 20 minutes. It was dialysed in 27/32 Visking tubing against PBS diluted 1:4 with distilled water and containing \(1 \times 10^{-3}\text{M} \text{MgCl}_2\) and \(1.5 \times 10^{-4}\text{M} \text{CaCl}_2\) for 4 – 6 hours. The precipitated Cl was then harvested by centrifugation at 12,000 RPM for 20 minutes and then dissolved in 1/10 of its original serum volume in 0.3 NaCl buffered at pH 7.5 with 0.01M phosphate and containing \(1 \times 10^{-3}\text{M} \text{MgCl}_2\) and \(1.5 \times 10^{-4}\text{M} \text{CaCl}_2\). The resulting solution was centrifuged to clear. The preparation was stored at -20°C.

Further purification of bovine Cl was achieved by molecular sieve chromatography at 2°C on Sephadex G-200 (Lot No. 5112, particle size 40 – 120 u, Pharmacia, Uppsala, Sweden). A glass column 2.5 x 100 cm. fitted with flow adaptors (Pharmacia, Uppsala, Sweden) was packed with Sephadex G-200 equilibrated with GBS++ (without gelatin). The final bed volume was 2.5 x 72 cm. The column was primed by passing through it 5 ml of bovine serum and subsequently washing with GBS++ (without gelatin) until no further protein was detectable in the effluent.

Sucrose was added to 5 ml of the Cl, previously obtained
by precipitation, to give a final concentration of 3% sugar. This was carefully applied to the surface of the Sephadex, overlaid with GBS++ (without gelatin) buffer, and then eluted by gravity with the same buffer. The flow rate was 30 ml/hr. Fraction of 10 ml were collected and read for protein content by reading O.D. at 280nm in 1 cm glass cuvette. The CI haemolytic activity in each sample was estimated in the following way. To 0.2 ml of a 1/30 dilution of an aliquot of each sample was added 0.2 ml of 1% EAC\textsuperscript{Bov}42 cells. The diluent was GBS-Sucrose++. After 20 minutes at 37°C the samples were each treated with 0.2 ml of C-EDTA for 30 minutes at 37°C. The degree of lysis, which was relative to the active CI, was determined by measuring the optical density at 412nm of the haemoglobin released into the fluid phase. Fractions containing CI haemolytic activity were pooled. The CI was precipitated by dialysing GBS++ diluted 1/5 (μ = 0.03) overnight at 2°C. The CI was harvested by centrifugation at 8,000 RPM for 20 minutes and washed twice with the diluted GBS++ (μ = 0.03). The CI was then dissolved in 2 ml of GBS++ to which was added 0.15M NaCl and stored at -20°C.

B. Preparation of EA, EAC\textsuperscript{Bov}42 and EAC\textsuperscript{Bov}42 cells

1. a. Sheep EA

Ten per cent sheep cells were diluted to 2% with GBS++ and mixed an equal volume of haemolysin diluted 1/250 in GBS++ (the amount of haemolysin calculated to optimally sensitised the red cells). After stirring for 15 minutes at 2°C the sensitised cells (EA) were washed with GBS++ twice and then suspended in GBS++ to give an appropriate cell suspension. One ml of an erythrocyte suspension containing 1 x 10^9 cells when lysed with 14 ml of 0.1% Na\textsubscript{2}CO\textsubscript{3} (w/v) gives an O.D. of 0.7. at 541nm in 1 cm. glass
cuvette. Using this information the number of cells per ml of a particular erythrocytes suspension may be calculated (35). Lysing 1 ml of a 5% (v/v) suspension of EA with 14 ml of 0.1% of Na$_2$CO$_3$ resulted in an O.D. of 0.504 at 541nm, therefore the number of cells was calculated to be $7.2 \times 10^8$.

b. Guinea Pig EA

Guinea pig erythrocytes were washed twice with PBS++ and then twice with GBS-Sucrose++ ($\mu = 0.094$, pH 7.0). Naturally occurring sensitizing antibodies were obtained from bovine serum after heating at 56°C for half an hour to destroy its complement activity. Equal volumes of a 1% suspension of guinea pig cells and heat-treated bovine serum (diluted 1/40 in GBS-Sucrose++ $\mu = 0.094$, pH 7.0) were mixed and stirred for 15 minutes at 2°C. The sensitized guinea pig cells (EA) were washed with GBS-Sucrose++ ($\mu = 0.094$, pH 7.0) twice and then resuspended in the same buffer to give an appropriate cell suspension.

2. EAC$^{Bov}_{42}$

This intermediate was prepared according to the method of Fong et al (16). Using GBS-Sucrose++ as diluent, equal volumes of 5% sheep EA and a 1/5 dilution of bovine serum were mixed and stirred well at 2°C exactly 10 minutes. Then without delay the cells were washed twice with GBS-Sucrose++ at 2°C and finally suspended to an appropriate dilution in GBS-Sucrose++. In a good preparation of EAC$^{Bov}_{42}$ cells, 0.2 ml of a 1% suspension give 100% haemolysis with 0.2 ml C-EDTA in 5 - 6 minutes at 37°C. These cells were stable at 2°C for 7 days.

3. EAC$^{Bov}_{42}$

These were prepared according to Fong et al (17)
with some slight modifications.

Two volumes of Gelatin-Barbital-Saline (GBS) at pH 7.35 and ionic strength u = 0.15, containing 0.01M EDTA (EDTA-GBS) were added to 1 volume of 1% BAC<sub>Bov</sub>42 in GBS-Sucrose and stirred at 37°C for 15 minutes. The mixture was centrifuged, the supernatant discarded and the cells resuspended to 0.33% in a buffer containing one volume of GBS-Sucrose and two volumes of EDTA-GBS. Following further 15 minutes at 37°C the cells were washed three times with GBS-Sucrose++ and finally suspended to appropriate dilution in GBS-Sucrose++. The BAC<sub>Bov</sub>42 cells, which were not lysed by C-EDTA at 37°C for an hour, would give 100% haemolysis with C-EDTA at 37°C in 15 minutes after the cells had been incubated with CI (diluted 1/50 with GBS-Sucrose++) for 20 minutes (see haemolytic assay for CI). The cells were stable after a week at 2°C. The term stable refers to their capacity to form a lytic intermediate with CI.

C. Haemolytic Assays

1. Method For Determining CH<sub>50</sub> Unit

The haemolytic capacity of complement is usually estimated in terms of the quantity required to give a 50% haemolysis (35). The 50% haemolysis unit of complement designated CH<sub>50</sub> is the reciprocal of the dilution of complement required to give 50% haemolysis under standard conditions (for details, see Appendix).

2. Titration of Whole Human and Guinea Pig Complements

The diluent used was GBS++ and the target cells optimally sensitised sheep cells. The assay was performed in tubes 1 x 7.5 cm. Serial dilutions of complement were made in 0.2 ml aliquots and to each dilution was added 0.2 ml of 1% EA. After 30 minutes at 37°C 1 ml of ice cold EDTA-GBS was added to each tube to stop the reaction. The extent of
alysis in each tube was determined as a function of the amount of haemoglobin produced by measuring the O.D. at 541nm of the fluid phase after centrifugation. A control containing 0.2 ml of diluent and 0.2 ml of 1% EA was subjected to the same procedure.

The results were expressed in CH$_{50}$ units.

3. Titration of Bovine Whole Complement

The system employed was essentially the same described by Bart and Barta (15) using optimally sensitized guinea pig erythrocytes as target cells and GBS-Sucrose++ at pH 7.0 and ionic strength $\mu = 0.094$ containing 0.1% gelatin, $1 \times 10^{-3}$M MgCl$_2$ and $3 \times 10^{-4}$M CaCl$_2$ as diluent.

The assay procedure then followed that for human and guinea pig complement.

4. Titration of Bovine and Human C1

The diluent used was GBS-Sucrose++ at pH 7.35 and ionic strength $\mu 0.075$. Bovine EAC$^{42}$ cells were used for the assays of bovine and human C1. The assay was performed in tubes 1 x 7.5 cm. Serial dilutions of C1 were made in 0.2 ml aliquots and to each dilution was added 0.2 ml of 1% EAC$^{Bov}$42 cells. After 20 minutes at 37°C, the cells were centrifuged and washed twice with ice cold GBD-Sucrose++ and finally resuspended in 0.2 ml GBS-Sucrose++. To each tube was added 0.2 ml C-EDTA (guinea pig complement diluted with EDTA-GBS 1/10). After incubating at 37°C for 30 minutes, 1 ml of EDTA-GBS was added to each tube to stop the reaction and the degree of haemolysis was measured at 541nm. A blank containing no complement but GBS-Sucrose++ was subjected to the same procedure.

The results were expressed in CH$_{50}$ units.
D. Assay for the Enzymatic Activity of Activated C1 from Bovine and Human Species

The enzymatic activity is determined by measuring its capacity to hydrolyse the substrate N-Z-L-Tyr-p-Np (25). The buffer used throughout was prepared by dissolving NaCl to a final concentration of 0.15M in a solution of 0.01M Tris adjusted to pH 8.00 - 8.05 with 1 M HCl. The substrate was dissolved in acetone to give a final concentration of 1 x 10^{-3}M. The assay system consisted of 3 ml of buffer, 50 ul of C1 and 100 ul of substrate mixed in a 1 cm glass cuvette. The production of p-nitrophenol was followed at 410nm using a recording spectrophotometer for 6 - 10 minutes. A control containing no protein was performed with each assay to correct for the spontaneous hydrolysis of the substrate.

The activity of the enzyme was expressed as esterase units. One unit of enzyme was defined as that amount of protein which released 1 x 10^{-6}mM of p-nitrophenol in 5 minutes at 25°C from 3 x 10^{-5}M N-Z-L-Tyr-p-Np at pH 8.05. The molar extinction of p-nitrophenol at pH 8.05 was taken as 1.66 x 10^4 (43).

E. Inhibitor Studies on Whole Complement, C1 and Intermediate Complexes

1. Whole Complement

PARP: Inhibition of guinea pig and human whole complement was performed as described by Baker and Erickson (40) using sensitized sheep erythrocytes as target cells except that the diluent used was GBS++ pH 7.35 and ionic strength \( \mu = 0.15 \) containing 0.1% gelatin, 1 x 10^{-3}M MgCl_2 and 1.5 x 10^{-4}M CaCl_2. Inhibition of bovine complement was performed by the same procedure except that GBS-Sucrose++ at pH 7.0 and ionic strength \( \mu = 0.094 \) containing 0.1% gelatin,
$1 \times 10^{-3}\text{M MgCl}_2$ and $3 \times 10^{-4}\text{M CaCl}_2$ was used as diluent and the target cells were guinea pig erythrocytes sensitized with bovine natural antibody. In nine $1 \times 7.5\text{ cm}$ centrifuge tubes were placed $0.25\text{ ml}$ of $2.5\%$ EA cells. To each tube was added $50\text{ ul}$ of MeOEtOH plus or minus inhibitor. The final concentration of inhibitor in tubes 2, 5 and 8 was $1\text{ mM}$ while in tubes 3, 6 and 9 was $0.5\text{ mM}$. To tubes 1, 2 and 3 was added $0.2\text{ ml}$ of $1:50$ guinea pig complement; to tubes 4, 5 and 6 was added $0.2\text{ ml}$ of $1:10$ bovine complement. Control tubes containing only sensitized cells and MeOEtOH plus and minus inhibitor were incubated to determine the effect of solvent and or inhibitor on the sensitized cells in absence of complement. The tubes were incubated at $37^\circ\text{C}$ for 15 minutes, then lysis was stopped by addition of $1\text{ ml}$ EDTA-GBS. The cells were then centrifuged and the degree of haemolysis determined by measuring the O.D. at $541\text{nm}$ in a $1\text{ ml}$ glass cuvette. Inhibition of complement by PABPB was expressed as a fractional percentage of the O.D. observed in the presence of inhibitor over the O.D. in standard tubes where the inhibitor was excluded.

2. **Active CI from Bovine and Human Species**

   a. PABPB: The NaCl concentration in the stock solution of CI was lowered to $0.15\text{M}$ by dilution with equal volume of $0.01\text{M}$ phosphate buffer at pH 7.3 containing $1 \times 10^{-3}\text{M MgCl}_2$ and $1.5 \times 10^{-4}\text{M CaCl}_2$. An aliquot of $0.9\text{ ml}$ of the CI enzyme was treated with $0.1\text{ ml}$ of various concentrations of PABPB ($2.5, 1.25, 0.25, 0.125$ and $0.0625\text{ mM}$) in MeOEtOH at $37^\circ\text{C}$ for 30, 60 and 90 minutes periods. Controls were performed using $0.9\text{ ml}$ of CI enzyme and $0.1\text{ ml}$ of either MeOEtOH or phosphate buffered saline at pH 7.3 and the ionic strength $\mu = 0.15$. The CI enzyme activity in $50\mu\text{l}$ aliquots of each sample was determined spectrophotometrically using N-Z-L-Tyr-p-Np as substrate.
Haemolytic assays employed bovine EAC\textsuperscript{Bov42} cells and GBS–Sucrose++ (μ = 0.075) as the diluent. For each interval of incubation, several dilutions of CT were in 0.2 ml aliquot and to each dilution was added to 0.2 ml of 1% EAC\textsuperscript{Bov42} cells. After 20 minutes at 37°C, the cells were centrifuged and washed twice with ice-cold GBS–Sucrose++. To each tube was added 0.2 ml C-EDTA. After 30 minutes at 37°C, 1 ml of EDTA–GBS was added to each tube to stop the reaction and the optical density of the haemoglobin in the supernatant was read at 541nm in a 1 cm glass cuvette. The amount of CT required for 50% haemolysis was expressed as CH\textsubscript{50} units.

b. DFP : The 5M stock DFP was diluted with GBS–Sucrose++ to different concentrations, namely: 1 x 10\textsuperscript{-3}, 5 x 10\textsuperscript{-4} and 1 x 10\textsuperscript{-4}M. The diluted DFP was used immediately. The methods used were same as those described for the inhibition by PABA of the haemolytic and esterase functions of CT.

c. PMSF, TLCK and TPCK : A stock solution (0.1M) of PMSF was prepared using isopropyl alcohol as solvent; TLCK and TPCK stock solutions were prepared at 5 x 10\textsuperscript{-3}M using GBS–Sucrose++ and methyl alcohol respectively as solvents. Only one concentration of inhibitor, namely: 1 x 10\textsuperscript{-3}M was used in the studies of their effects on active CT in haemolytic assays, while in esterase assays the final concentration of the inhibitors was 5 x 10\textsuperscript{-4}M. The methods employed were similar to those described in the PABA studies.

3. Effect of Inhibitors in EAC\textsuperscript{Bov142} and EAC\textsuperscript{Bov42} Intermediates
   a. EAC\textsuperscript{Bov142} : Stock solutions of the inhibitors, 1 x 10\textsuperscript{-2}M DFP in GBS–Sucrose++, 0.1M PMSF in isopropyl
alcohol, $5 \times 10^{-3} \text{M TLCK}$ in GBS-Sucrose++ and $5 \times 10^{-3} \text{M TPCK}$ in methanol were diluted to either or both $10^{-3}$ or $10^{-4} \text{M}$ in GBS-Sucrose++. EAC$^{\text{Bov}142}$ cells were suspended to 1% in each solution and the mixtures kept at 37°C. After intervals of 5, 10, 15, 30 and 60 minutes aliquots of 0.2 ml were removed and centrifuged in 1 x 7.5 cm tubes. The cells were washed twice with cold GBS-Sucrose++ and finally resuspended to 1% in the same buffer. After adding 0.2 ml of C-EDTA each tube was incubated at 37°C for 30 minutes and the reaction was quenched by the addition of 0.4 ml of EDTA-GBS. The degree of haemolysis was determined by measuring the O.D. of the fluid phase at 412nm in a 1 cm glass cuvette. Controls using solvents minus the inhibitors were performed at the same time.

b. EAC$^{\text{Bov}42}$ : The intermediate, EAC$^{\text{Bov}42}$ in 1% suspension was incubated with $1 \times 10^{-3}$ and $1 \times 10^{-4} \text{M DFP}$. The procedure of treating the cells with inhibitor was the same described above for the effect of various inhibitors on EAC$^{\text{Bov}142}$. For each interval of time the cells were used to titrate the activity of bovine CI as previously described (see p. 22).

F. Uptake of CI by EAC$^{\text{Bov}42}$ Cells

Bovine CI was diluted 1:20 with GBS-Sucrose++. Equal volumes of 1% EAC$^{\text{Bov}42}$ cells and diluted bovine CI were incubated at 37°C for 2, 5, 7, 10, 15, 20, 30 and 60 minutes. After each interval, 0.4 ml of the incubation mixture was pipetted and washed with cold GBS-Sucrose++ twice and finally resuspended as 1% cells in the same buffer. To each tube 0.2 ml C-EDTA was added and the mixture incubated at 37°C for 30 minutes. The reaction was stopped by adding 0.4 ml cold EDTA-GBS and the degree of lysis was determined spectrophotometrically at 412nm.
in a 1 cm cuvette. A control containing no bovine CT was incubated at the same time. Graph of O.D. against time of incubation were plotted.

G. Effect of Active CT on EAC BoV 42 previously treated with DFP-inactivated CT

Bovine CT (0.9 ml 2,000 CH$_{50}$ units/ml) was incubated with DFP (0.1 ml of 2 x 10$^{-3}$M) for 15 minutes at 37°C. The final concentration of DFP was 2 x 10$^{-4}$M. The mixture was diluted 1:15 with cold GBS-Sucrose++. Equal volumes of the diluted mixture and 1% EAC BoV42 cells in GBS-Sucrose++ were kept at 37°C. Aliquots of 0.4 ml were pipetted into duplicate sets after immediately washing with cold GBS-Sucrose++. To the first set of tubes was added 0.2 ml of C-EDTA and after 30 minutes at 37°C the reaction was stopped by addition of 0.4 ml cold EDTA-GBS and the extent of lysis in the fluid phase was determined spectrophotometrically. To the second set of tubes was added 0.2 ml of active bovine CT (diluted 1/20 in GBS-Sucrose++). After 20 minutes at 37°C the cells were washed twice with GBS-Sucrose++, resuspended in 0.2 ml of the buffer and then incubated at 37°C for 30 minutes with 0.2 ml C-EDTA. The reaction was stopped by the addition of 0.4 ml of cold EDTA-GBS. The extent of lysis was determined by observing the O.D. of the fluid phase at 412 nm.

The whole procedure was repeated for a sample of bovine CT incubated at 37°C with 5 x 10$^{-4}$M DFP for 30 minutes.

A control experiment was performed using bovine CT in the absence of inhibitor.
H. Stability of EAC<sup>Bov.142</sup> in Various Ionic Strength Buffers

The ionic strength of various GBS buffer mixtures were prepared according to Rapp and Borsos (36) containing 0.1% gelatin, 1 x 10<sup>-3</sup>M MgCl<sub>2</sub> and 1.5 x 10<sup>-4</sup>M CaCl<sub>2</sub> from GBS++ and 9% sucrose containing 0.1% gelatin, 1 x 10<sup>-3</sup>M MgCl<sub>2</sub> and 1.5 x 10<sup>-4</sup>M CaCl<sub>2</sub>.

<table>
<thead>
<tr>
<th>Ionic Strength</th>
<th>Volumes of GBS++</th>
<th>Volumes of % Sucrose++</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.150</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>0.135</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>0.120</td>
<td>8</td>
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</tr>
<tr>
<td>0.105</td>
<td>7</td>
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</tr>
<tr>
<td>0.090</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>0.075</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>0.060</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

EAC<sup>Bov.142</sup> cells were made to 1% suspensions in different ionic strength buffers and maintained at 37°C for 5, 10, 15, 50 and 60 minutes. Then the cells were centrifuged and washed twice with GBS-Sucrose++ (µ = 0.075) and resuspended to 1% in the same buffer. To 0.2 ml of each cell suspension was added an equal volume of G-EDTA, the mixture was incubated at 37°C for 30 minutes. The reaction was stopped by adding 0.4 ml EDTA-GBS to each tube and the degree of lysis was determined at 412 nm.

I. Stability of EAC<sup>Bov.142</sup> in EDTA-GBS-Sucrose and EDTA-GBS

Different concentrations of EDTA in GBS and GBS-Sucrose solutions were prepared. The intermediate, EAC<sup>Bov.142</sup>, was suspended to 1% in the above solutions and aliquots of 0.2 ml maintained at 37°C for 1, 2, 3, 4, 5, 7, 10, 15, 30 and 60 minutes. The cells were then washed with GBS-Sucrose++ and resuspended to 1% in GBS-Sucrose++. To
each tube was added an equal volume of C-EDTA and incubated at 37°C for 30 minutes. The reaction was stopped by adding 0.4 ml ice cold EDTA-GBS. The degree of lysis was determined by measuring the O.D. of the fluid phase at 412nm. Graphs of O.D. against time of incubation were plotted.

J. Uptake of Cl-H³-DFP by EA, EAC⁹Bov142 and EAC⁹Bov42 cells

A reaction mixture containing 0.675 ml of bovine 
CT (14,400 CH₅₀ units), 0.675 ml of GBS++, 0.075 ml of 
H³-DFP (i.e. 0.075 mCi) and 0.075 ml of 2 x 10⁻² M cold DFP 
diluted with GBS-Sucrose++) was incubated at 37°C for 
60 minutes. After which time the CT was completely inactivated 
with respect to its capacity to form EAC⁹Bov142 from EAC⁹Bov42. 
The final concentration of DFP in the resulting mixture 
was 1.05 x 10⁻³ M with a specific activity of 71.5 µCi/mM. 
The mixture was then dialysed in 8/32 Visking tubing against 
two changes of two liter volumes of cold GBS++ at 4°C for 
a period of 18 hours.

The Cl-H³-DFP was diluted with an equal volume 
of ice cold 9% sucrose solution containing 1 x 10⁻³ M MgCl₂ 
and 1.5 x 10⁻⁴ M CaCl₂. In three 1 x 7.5 cm centrifuged 
tubes were placed 0.4 ml of Cl-H³-DFP. To tube 1 was added 
0.4 ml 5% EA cells; to tube 2 was added 0.4 ml 5% EAC⁹Bov42; 
and to tube 3 was added 0.4 ml 5% EAC⁹Bov142 cells. Two 
control tubes were performed: to tubes 4 and 5 were added 
0.4 ml GBS-Sucrose++ and then equal volume of Cl-H³-DFP 
and 5% EA cells were added respectively. The tubes were 
kept at 37°C for 45 minutes with frequent shaking, and then 
were centrifuged at 3,000 RPM for 5 minutes. The 
radioactivity in 0.4 ml of the fluid phase from tubes 4 and 
5 was counted. The cells in tubes 1, 2, 3 and 5 were then 
washed well with 4 x 1 ml of ice cold GBS-Sucrose++, and 
were lysed with 0.1 ml distilled water, decolorized with 
0.1 ml of 30% hydrogen peroxide and solubilized with 0.8 ml
of hydroxide of hyamine (1 M solution in methanol). Aliquots of 0.8 ml of cell lysates (representing 8/10 of the total number of cells) were counted. The cell concentration in 5% EA, 5% EAC<sub>Bov</sub>42 and 5% EAC<sub>Bov</sub>142 cell suspensions was determined.

All samples were counted in 10 ml of scintillation fluid prepared by mixing a toluene solution of 4 gm/liter of PP<sub>6</sub> and 60 mgm/liter of POPP with methyl cellulose (MeOEtOH) in the proportion 1:0.7 by volume (58). Five counts of each sample were made. The counting efficiency of each sample was determined with reference external standard. A barium quench curve was prepared using a set of Nuclear-Chicago Model 180050 liquid scintillation tritium quenched standards (see Appendix).

The results were expressed in terms of uptake of radioactivity in d.p.m. .
CHAPTER III

RESULTS AND DISCUSSION

A. Preparation and Purification of Bovine Cl

Tamura and Nelson (44) found that at pH 7.5 and low ionic strength (μ = 0.03 - 0.04) guinea pig, human and canine Cl could be selectively precipitated from all the other serum complement components and the Cl inactivator. Thus Cl may be easily obtained in a functionally pure form. The major contaminants are being the serum γ-globulins.

The double-precipitation procedure of Tamura and Nelson (44) was used to prepare a functionally pure form of bovine Cl. Since little is known concerning the physiochemical properties and specific activities of all the bovine complement components it is not possible to say that the degree of purity of the bovine preparation is the same as the observed by Tamura and Nelson. It was found that the Cl thus obtained was capable of substituting for purified human Cl in its capacity to form EAC\textsuperscript{Bov}142 from EAC\textsuperscript{Bov}42. Further neither the bovine Cl nor the R1 were capable of forming EAC\textsuperscript{Bov}142 cells from EA cells. A typical preparation of Cl (50 ml) obtained by this method had an optical density at 280nm of 5.522 and containing 5,000 CH\textsubscript{50} units/ml and 5.64 x 10\textsuperscript{5} esterase units/ml.

As observed by other workers (44) during the preparation of Cl, by precipitation at low ionic strength, it becomes fully activated. Cl may be converted to Cl \textsuperscript{a} by warming it at 37°C for 15 minutes (59). Such treatment of the bovine Cl did not increase either its haemolytic or esterase activity. It had also been observed that though some human Cl preparations were haemolytically inactive, their esterase activities were still active. It was not surprising because that human serum was obtained from
out-dated de-calcified blood and the enzymatic activity may be associated with free CIs.

Rabbit and guinea pig Cl prepared by precipitation from whole serum at low ionic strength $\mu = 0.03$ and pH 7.5 have been further purified by gel-filtration using Bio-gel P-200 (52). The Cl thus obtained was completely free of all other complement components and contained 1 – 2 times as much Cl activity as could be measured in an equivalent amount of whole serum and only about 0.1% as much protein as determined by optical density at 280nm. Yields from Sephadex G-200 were reported to be disappointingly low (52). For bovine Cl the reverse was observed. Chromatography on Sephadex G-200 gave better preparation than on Bio-gel P-200. The new Sephadex G-200 column was first treated by passing 5 ml of whole bovine serum, made to 3% sucrose, through it, and washing with GBS++ (without gelatin) until no further protein was detectable in the effluent. Linscott (52) found such treatment improved yields of Cl.

Five ml of a sample of twice-precipitated bovine Cl which has activity of 908 CH$_{50}$ unit/ml/0.D. unit at 280nm was made to 3% sucrose and chromatographed on a column of Sephadex G-200 (bed volume 2.5 x 72 cm) using GBS++ (without gelatin) as buffer. The elution profile determined by measuring the optical density at 280nm of the collected fractions is shown in Figure IV. The haemolytic activity of the fractions containing protein was determined as described in Materials and Methods and in terms of optical density of haemoglobin released from the aleixed cells.

The Cl haemolytic activity was associated mainly with the exclusion protein peak and fraction 20 – 23 inclusive (40 ml total volume) were pooled. The protein material containing Cl was precipitated by dialysis at low ionic strength ($\mu = 0.03$) and pH 7.35. The Cl
Purification of bovine CT by Sephadex G-200

Legend

. . . . . Protein content of each fraction measured in terms of O.D. at 280nm

ooo ooo Bovine CT haemolytic function of each fraction determined by the O.D. at 412nm of haemoglobin released from alexinated cells

Aliquot of each fraction was mixed with an equal volume of 9% sucrose containing $1 \times 10^{-3}$M MgCl$_2$ and $1.5 \times 10^{-4}$M CaCl$_2$. The resulting solution was diluted to 1/15 with GES-Sucrose++ (i.e. the final dilution was 1/30). The haemolytic function of CT was assayed by its capacity to convert EAG$^{Bov}42$ cells to the lytic intermediate EAG$^{Bov}142$ which could then be lysed by C-EDTA.
precipitate was redissolved in 2 ml of GBS ++ to which had been added 0.15M NaCl to achieve solution of this relatively insoluble protein; this was the smallest volume in which CI could be dissolved. Titration of CI with EAC Bov 42 cells showed that it contained 2090 CH 50 units/ml and it had an optical density at 280nm of 1.597. Compared with the material applied to the column which 908 CH 50 units/ml/0.D. unit at 280nm the chromatographed CI had 1310 CH 50 units/ml/0.D. unit at 280nm. This represents a 1.45 fold purification. It is difficult to estimate the yield with respect to whole serum because CI can not accurately be titrated in whole serum. However the CI preparation contained only 0.001% as much protein, determined by optical density at 280nm, in an equivalent amount of serum. It is expected that further purification of the CI could be achieved either by sucrose gradient or zonal centrifugation procedure (53) or by affinity chromatography on γ-globulin linked to Sepharose (54).

B. The Stability of EAC Bov 142 in varying Ionic Strength Buffers

The specific interaction between CI and the antibody portion of immune complexes is more stable at low ionic strength than at physiological ionic strength (55). Fong et al found that EAC Bov 142 cells which were stable for 14 - 18 hours at 37°C at μ = 0.075 decayed about 50% in 4 minutes at 37°C when the ionic strength was raised to μ = 0.150 (17). In this laboratory it was not possible corroborate this. The lytic intermediate, EAC Bov 142 appears to be quite stable for up to 60 minutes at 37°C in ionic strength ranging from 0.06 to 0.15.
TABLE II

STABILITY OF EAC\textsuperscript{Bov-142} IN VARIOUS IONIC STRENGTH BUFFERS

<table>
<thead>
<tr>
<th>Ionic Strength</th>
<th>Time of Incubation in Minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>0.150</td>
<td>2.580</td>
</tr>
<tr>
<td>0.135</td>
<td>2.579</td>
</tr>
<tr>
<td>0.120</td>
<td>2.544</td>
</tr>
<tr>
<td>0.105</td>
<td>2.548</td>
</tr>
<tr>
<td>0.090</td>
<td>2.532</td>
</tr>
<tr>
<td>0.075</td>
<td>2.500</td>
</tr>
<tr>
<td>0.060</td>
<td>2.550</td>
</tr>
</tbody>
</table>
Table II. shows that EAC\textsuperscript{Bov}142 cells suspended to 1% in isotonic GBS–Sucrose++ buffers of different ionic strengths for various periods of time ranging from 5 - 60 minutes and then washing the cells twice with GBS–Sucrose++ and resuspending to 1% in this buffer, gave the same degree of haemolysis with C-EDTA.

C. Stability of EAC\textsuperscript{Bov}142 cells in various concentrations of EDTA in either GBS–Sucrose or GBS

Becker (48) has shown that guinea pig Cl may be detached from EAC1, EAC14 and EAC142 by treatment with 0.01M EDTA at physiological ionic strength. It has been observed that the bovine lytic intermediate EAC\textsuperscript{Bov}142 decays very quickly i.e. more than 80% in less than 10 minutes, in 0.01M EDTA in GBS made to ionic strength \( \mu = 0.075 \) with glucose (17).

Figures V and VI show the effect concentration of EDTA ranging from 0.001M to 0.050M in either GBS–Sucrose (\( \mu = 0.075 \)) or GBS (\( \mu = 0.150 \)). After suspending the EAC\textsuperscript{Bov}142 cells in various concentrations of EDTA at the appropriate ionic strength for periods of time ranging from 5 to 60 minutes the cells were washed thoroughly with GBS–Sucrose++ and lysed with C–EDTA. The lowest concentration of EDTA used, 0.001M, had no apparent effect on the stability of the lytic intermediate at either \( \mu = 0.075 \) or \( \mu = 0.150 \). Higher concentrations of EDTA caused decay of the EAC\textsuperscript{Bov}142 cells and this decay was greater in the higher ionic strength buffer. The decay of EAC\textsuperscript{Bov}142 in EDTA concentrations of 0.01M and 0.05M at physiological strength occurred at a rate too rapid to follow accurately.

Fong et al (17) showed that decay of EAC\textsuperscript{Bov}142 at \( \mu = 0.15 \) and 0.01M EDTA resulted in the detachment of Cl from the complex. Since Cl is required for the action of EAC\textsuperscript{Bov}142 as a lytic intermediate (16,17) then it is highly
STABILITY OF EAC\textsuperscript{BovT42} CELLS IN EDTA-GBS-SUCROSE

Legend

One per cent EAC\textsuperscript{BovT42} cells were incubated with different concentrations of EDTA in GBS-Sucrose at 37\textdegree C. At various intervals of incubation, 0.2 ml samples were drawn and transferred to a test tube. The cells were then washed twice with GBS-Sucrose++ and finally suspended in GBS-Sucrose++. To the tube 0.2 ml 0.1 M EDTA was added. Following an incubation period of 30 minutes, the contents of each tube were then adjusted to 0.8 ml with ice-cold EDTA-GBS and the amount of haemolysis was determined spectrophotometrically at 412nm.

\begin{center}
\begin{tabular}{ccc}
\textbullet & \textbullet & \textbullet & \textbullet & \textbullet & GBS-Sucrose \\
\textbullet & \textbullet & \textbullet & \textbullet & \textbullet & 0.001M EDTA-GBS-Sucrose \\
\textbullet & \textbullet & \textbullet & \textbullet & \textbullet & 0.0025M EDTA-GBS-Sucrose \\
\textbullet & \textbullet & \textbullet & \textbullet & \textbullet & 0.005M EDTA-GBS-Sucrose \\
\textbullet & \textbullet & \textbullet & \textbullet & \textbullet & 0.010M EDTA-GBS-Sucrose \\
\textbullet & \textbullet & \textbullet & \textbullet & \textbullet & 0.050M EDTA-GBS-Sucrose \\
\end{tabular}
\end{center}
FIGURE V

STABILITY OF EAC$^{Bov}$142 CELLS IN EDTA-GBS-SUCROSE

Time in Minutes

O.D. at 412 nm
STABILITY OF EAG$^{\text{Bov}}\text{142 CELLS IN EDTA-GBS}$

**Legend**

Procedure was same as for Figure V except the GBS was used in place of GBS-Sucrose.

- △ △ △ GBS
- • • • 0.001 M EDTA-GBS
- △ △ △ 0.0025 M EDTA-GBS
- • • • 0.005 M EDTA-GBS
FIGURE VI

STABILITY OF EAC$^{Bov}_{142}$ CELLS IN EDTA-GBS

D. D. AT 412nm

TIME IN MINUTES
probable that the CI interacts in some way with C4 and/or C2. In buffers of ionic strength, $\mu = 0.075$, which favour the formation of EAC$^{Bov}$142 there is possibly a unique and rather firm association between the antibody molecule, Cl, C4 and/or C2. On raising the ionic strength to $\mu = 0.15$ then perhaps the association of CI with C4 and/or C2 is sufficient to maintain an active complex even though the association between CI and the antibody is weakened.

Treatment with EDTA in sufficient concentration to affect the integrity of Cl probably affects the interaction of CI with both antibody and C4 and/or C2 leading to the decay of the intermediate. The combined action of EDTA and high ionic strength causes sufficient breakdown of the interactions maintaining CI in the EAC$^{Bov}$142 complex allowing its rapid detachment and decay of the intermediate.

D. **Effect of Inhibitors on the Esterase and Haemolytic Functions of Bovine CI**

Since CI is required by EAC$^{Bov}$142 complex for its action on later components, it is possible to ask the following questions.

1. Is the active site of CI important for its function in the EAC$^{Bov}$142 complex?
2. Does CI act at a site or sites other than the active site by stabilising or modulating the C42 in an active conformation?

Human and guinea pig CI have esterase enzyme activities and both tryptic and chymotryptic substrate specificities can be demonstrated (25). It is expected that bovine CI would have similar properties.

Bovine CI is capable of hydrolysing H-Z-L-Tyr-p-Np in a manner similar to that described by Bing (25) for human CI and spectrophotometrically following the
liberation of nitrophenol from the substrate provides a convenient assay for the esterase activity of the CI.

Fluorosulphonate and fluorophosphate compounds can inhibit proteolytic enzymes and it has been shown that the DFP and PMSF are capable of inhibiting the esterase activities of human and guinea pig CI (49). The effect of DFP and PMSF on the esterase activity of both bovine and human CI is shown in Tables III and IV. At a final concentration of $1 \times 10^{-3} \text{M}$ DFP is quite potent and after 60 minutes at 37°C it inhibits almost completely the esterase activity of $4.26 \times 10^5$ units of enzyme/ml of bovine CI and of $1.515 \times 10^6$ units of enzyme/ml of human CI. The inactivation of human and guinea pig CI by DFP is believed to occur by attaching to a specific serine in the active site (36). The inactivation of bovine CI by DFP implies that it too is probably a serine protease. CI has both chymotryptic and tryptic specificities (25). The chloroketone derivatives of tosyl-L-lysine and tosyl-L-phenylalanine are capable of discriminating between trypsin and chymotrypsin in their capacities to alkylate a histidine essential for the activity of each enzyme (50). TLCK (Tosyl-L-lysine-chloromethylketone) specifically inactivates trypsin and TPCK (tosyl-phenylalanine-chloromethyl ketone) is specific for chymotrypsin. The effect of these inhibitors on the esterase action of bovine and human CI is seen in Table III.

At a final concentration of $5 \times 10^{-4} \text{M}$ TLCK is observed to inhibit the bovine CI enzyme ($6.84 \times 10^5$ units enzyme/ml) to an extent of between 14 - 17% during 60 minutes at 37°C. However there is significant inhibition of the CI by the isopropyl alcohol solvent used for TLCK i.e. 8 - 12% inhibition during 60 minutes at 37°C. Therefore it seems that the observed inhibition of bovine CI by TLCK is probably an effect of the solvent. No significant inhibition of bovine CI by TPCK at a final concentration
THE EFFECT OF DFP ON THE ENZYMATIC ACTIVITY
OF BOVINE AND HUMAN CT

Legend

One unit of enzyme is defined as the amount of protein
which releases $1 \times 10^{-6}$ mM of $p$-nitrophenol in 5 min. at
25°C from $3 \times 10^{-5}$M $N$-$Z$-$L$-$Tyr$-$p$-$Np$ at pH 8.05.

Inhibition is expressed as a fractional percentage
or the units of enzyme/ml observed after inhibition over
the units of enzyme/ml in the control tubes containing
no inhibitor but only saline.
<table>
<thead>
<tr>
<th>Final Conc. of DFP mM</th>
<th>Time of Incubation (min.)</th>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15</td>
<td>30</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unit Enzyme %</td>
<td>Unit Enzyme %</td>
<td>Unit Enzyme %</td>
<td></td>
</tr>
<tr>
<td></td>
<td>per ml Inhib. x 10^-5</td>
<td>per ml Inhib. x 10^-5</td>
<td>per ml Inhib. x 10^-5</td>
<td></td>
</tr>
<tr>
<td>Bovine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>2.60 38.0 1.92</td>
<td>55.0 1.2 71.8</td>
<td></td>
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</tr>
<tr>
<td>0.5</td>
<td>0.097 76.8 0.86</td>
<td>79.8 0.44 89.7</td>
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<td></td>
</tr>
<tr>
<td>1.0</td>
<td>0.082 80.5 0.67</td>
<td>84.2 0.22 94.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>4.20 0 4.26</td>
<td>0 4.26 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>12.1 20.0 11.0</td>
<td>27.6 9.0 40.5</td>
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</tr>
<tr>
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<td>68.1 0.22 85.5</td>
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<td></td>
</tr>
<tr>
<td>1.0</td>
<td>0.57 61.1 0.17</td>
<td>88.4 0.10 93.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>15.2 0 15.2</td>
<td>0 15.1 0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
THE EFFECT OF TLCK, TPCK AND PMSF ON THE ENZYMATIC ACTIVITY OF GI

Legend

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Solvents</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMSF</td>
<td>Isopropyl Alcohol</td>
</tr>
<tr>
<td>TPCK</td>
<td>Methyl Alcohol</td>
</tr>
<tr>
<td>TLCK</td>
<td>Isopropyl Alcohol</td>
</tr>
</tbody>
</table>

One unit of enzyme is defined as the amount of protein which releases $1 \times 10^{-6}$ mM of p-nitrophenol in 5 min. at $25^\circ$C from $3 \times 10^{-5}$ M N-Z-L-Tyr-p-Np at pH 8.05.

Inhibition is expressed as a fractional percentage or the units of enzyme/ml observed after inhibition over the units of enzyme/ml in the control tubes containing no inhibition but only saline.
TABLE IV

THE EFFECT OF TLCK, TPCK AND PMSF ON THE ENZYMATIC ACTIVITY OF CT

<table>
<thead>
<tr>
<th>Solvent/Inhibitor 5 x 10^{-4}M</th>
<th>Time of Incubation (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Unit Enzyme per ml x 10^{-5}</td>
</tr>
<tr>
<td>Bovine</td>
<td></td>
</tr>
<tr>
<td>TLCK</td>
<td>5.70</td>
</tr>
<tr>
<td>(CH_3)_2CH_2OH</td>
<td>6.00</td>
</tr>
<tr>
<td>TPCK</td>
<td>6.70</td>
</tr>
<tr>
<td>PMSF</td>
<td>3.49</td>
</tr>
<tr>
<td>MeOH</td>
<td>6.36</td>
</tr>
<tr>
<td>Saline</td>
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<td>Human</td>
<td></td>
</tr>
<tr>
<td>TLCK</td>
<td>5.67</td>
</tr>
<tr>
<td>(CH_3)_2CH_2OH</td>
<td>5.67</td>
</tr>
<tr>
<td>TPCK</td>
<td>5.85</td>
</tr>
<tr>
<td>PMSF</td>
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</tr>
<tr>
<td>MeOH</td>
<td>5.67</td>
</tr>
<tr>
<td>Saline</td>
<td>5.85</td>
</tr>
</tbody>
</table>
of $5 \times 10^{-4}$M was observed after 60 minutes at $37^\circ$C. Similarly it was seen that neither TPCK nor TLCK at final concentrations of $5 \times 10^{-4}$M were able to inhibit $5.85 \times 10^{5}$ enzyme units/ml of human CI.

Though the two histidine inhibitors have no effect, it is not possible to conclude that histidine is non-essential in either bovine or human CI for its action as an esterase without actual demonstration of histidine alteration.

Since DFP and PMSF inhibit the esterase activity of human and bovine CI, it is possible to question whether these inhibitors have an effect on the capacity of CI to form a lytic intermediate with EAC$_{Bov}^{42}$ cells.

Tables V and VI shows the effect of DFP, PMSF, TLCK and TPCK on the capacity of bovine CI to form EAC$_{Bov}^{142}$ from EAC$_{Bov}^{42}$ cells. Both DFP and PMSF are effective inhibitors. At a final concentration of $1 \times 10^{-3}$M PMSF and after 30 minutes of incubation at $37^\circ$C it inhibits completely the haemolytic activity of 4475 CH$_{50}$ units/ml of bovine CI. On the other hand DFP at a final concentration of 0.5 mM and after 15 minutes of incubation at $37^\circ$C it inhibits almost completely the haemolytic activity if 4160 CH$_{50}$ units/ml of bovine CI. Therefore DFP and PMSF inactivated CI is not capable of forming active EAC$_{Bov}^{142}$ complex. Neither TLCK nor TPCK at a final concentration of $1 \times 10^{-3}$M were effective in inhibiting the haemolytic activity of CI. The per cent of inhibition by TPCK at $1 \times 10^{-3}$M after 60 minutes incubation at $37^\circ$C is 43.6.

However there is significant inhibition of the CI by methyl alcohol solvent used for TPCK i.e. 20% inhibition during 60 minutes at $37^\circ$C. Therefore it seems that the observed inhibition is partly due to the solvent. On the other hand the compound which does not appear to inhibit the esterase function may inhibit the haemolytic function.
THE EFFECT OF DFP ON THE HAEMOLYTIC ACTIVITY OF BOVINE AND HUMAN C1

Legend

Inhibition of C1 by DFP is expressed as a fractional percentage of the \( CH_{50} \) units/ml observed after inhibition over the \( CH_{50} \) units/ml in the saline control.

Controls, which were used for correcting for non-specific lysis, were performed at the same time using saline.
### TABLE V

**THE EFFECT OF DFP ON THE HAEMOLYTIC ACTIVITY OF BOVINE AND HUMAN CT**

<table>
<thead>
<tr>
<th>Final DFP Conc. (mM)</th>
<th>Time of Incubation (min.)</th>
<th>15</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CH&lt;sub&gt;50&lt;/sub&gt;</td>
<td>%</td>
<td>CH&lt;sub&gt;50&lt;/sub&gt;</td>
<td>%</td>
</tr>
<tr>
<td>Bovine</td>
<td>Inhib.</td>
<td></td>
<td>Inhib.</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>1820 56.4</td>
<td></td>
<td>955 83.1</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>10 ~100</td>
<td></td>
<td>0 100</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>0 100</td>
<td></td>
<td>0 100</td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>4160 0</td>
<td></td>
<td>5620 0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Human</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>---</td>
<td></td>
<td>4050 19</td>
<td></td>
<td>1350 73.0</td>
</tr>
<tr>
<td>0.5</td>
<td>---</td>
<td></td>
<td>850 83</td>
<td></td>
<td>0 100</td>
</tr>
<tr>
<td>Saline</td>
<td>---</td>
<td></td>
<td>5000 0</td>
<td></td>
<td>5000 0</td>
</tr>
</tbody>
</table>
THE EFFECT OF TLCK, TPCK AND PMSF ON THE
HAEMOLYTIC ACTIVITY ON BOVINE C1

Legend

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Solvents</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMSF</td>
<td>Isopropyl Alcohol</td>
</tr>
<tr>
<td>TPCK</td>
<td>Methyl Alcohol</td>
</tr>
<tr>
<td>TLCK</td>
<td>Saline</td>
</tr>
</tbody>
</table>

Inhibition of C1 by inhibitors is expressed as a fractional percentage of the CH50 units/ml observed after inhibition over the CH50 units/ml in the saline control.

Controls, which were used for correcting for non-specific lysis, were performed at the same time using saline.
TABLE VI

THE EFFECT OF TLCK, TPCK AND PMSF ON THE HAEMOLYTIC ACTIVITY OF BOVINE C1

<table>
<thead>
<tr>
<th>Solvent/Inhibitor</th>
<th>Time of Incubation (min)</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CH(_{50}) % Inhibition</td>
<td>CH(_{50}) % Inhibition</td>
<td></td>
</tr>
<tr>
<td>TLCK 1 x 10(^{-3})M</td>
<td>4475</td>
<td>0.0</td>
<td>4475</td>
</tr>
<tr>
<td>TPCK 1 x 10(^{-3})M</td>
<td>2330</td>
<td>20.0</td>
<td>1025</td>
</tr>
<tr>
<td>PMSF 1 x 10(^{-3})M</td>
<td>0</td>
<td>100.0</td>
<td>---</td>
</tr>
<tr>
<td>(CH(_3))(_2)CH(_2)OH</td>
<td>4475</td>
<td>0.0</td>
<td>4475</td>
</tr>
<tr>
<td>MeOH 1 x 10(^{-3})M</td>
<td>3300</td>
<td>13.5</td>
<td>2330</td>
</tr>
<tr>
<td>Saline 1 x 10(^{-3})M</td>
<td>4475</td>
<td>0.0</td>
<td>4475</td>
</tr>
</tbody>
</table>
of Cl by acting somewhere else on the molecule.

Since inactivation of the Cl esterase site makes it incapable of forming an active EAC\textsuperscript{Bov142} complex, it might be concluded that either directly or indirectly the esterase site or its intermediate vicinity are important in the function of Cl in EAC\textsuperscript{Bov142}. Inactivation of haemolysis appears greater than the inactivation of the esterase. Since the current composition of human Cl has been suggested to be composed of Clq : Clr : Cls in the molecular ratio of 1 : 2 : 4 (51). The multiple Cls units in the molecule make it possible that perhaps only partial inactivation of the total number of esterase active sites per molecule is capable of preventing the Cl from forming an active lytic intermediate.

E. **Effect of Inhibitors on EAC\textsuperscript{Bov42} cells**

When the inactivated Cl is tested for its ability to reform a lytic intermediate the relatively large excess of inhibitor is not removed from the system during the incubation of the Cl with EAC\textsuperscript{Bov42}. It is possible that the inhibitor may have an effect on bound C4 and/or C2. A 1% suspension of EAC\textsuperscript{Bov42} cells was made in GBS-Sucrose++ containing DFP at final concentrations of $1 \times 10^{-3}$M or $1 \times 10^{-4}$M. After various periods of time at 37°C the cells were washed well with GBS-Sucrose++ to remove the inhibitor. The cells were then used for titration of active Cl in the usual manner. The results in Table VII shows that DFP over the concentration range used to inhibit Cl was not capable of significantly affecting bound C4 or C2 by impairing the capacity of EAC\textsuperscript{Bov42} to form EAC\textsuperscript{Bov142}.
EFFECT OF DFP ON EAC\textsuperscript{Bov42} CELLS

Legend

EAC\textsuperscript{Bov42} cells were incubated with DFP at concentration $10^{-4}$M and $10^{-3}$M at 37°C for an hour. The cells were washed twice with GBS-Sucrose+++ and were incubated with different dilutions of bovine CI at 37°C for 20 minutes.
### TABLE VII

**EFFECT OF DFP ON EAC\textsuperscript{BoV}42 CELLS**

<table>
<thead>
<tr>
<th>DFP Conc.</th>
<th>1/3</th>
<th>1/9</th>
<th>1/27</th>
<th>1/81</th>
<th>1/243</th>
<th>1/729</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x 10\textsuperscript{-4}M</td>
<td>2.235</td>
<td>2.564</td>
<td>2.630</td>
<td>2.604</td>
<td>2.622</td>
<td>2.389</td>
</tr>
<tr>
<td>1 x 10\textsuperscript{-3}M</td>
<td>2.540</td>
<td>2.589</td>
<td>2.589</td>
<td>2.506</td>
<td>2.590</td>
<td>2.375</td>
</tr>
</tbody>
</table>

O.D. at 412nm after 30 min. at 37°C
F. Effect of Inhibitors on EAC$^{Bov \text{142}}$ cells

It has been shown that several inhibitors inactivate the esterase activity of bovine and human C\text{I} and that such inactivation prevents the C\text{I} from forming a lytic intermediate with EAC$^{Bov \text{42}}$. Various inhibitors were used to see if they could abolish the activity of C\text{I} when it was bound in an essential way in the EAC$^{Bov \text{142}}$ complex. TLCK and TPCK, although they do not inhibit the esterase and haemolytic activities of free C\text{I}, were tried since the bound C\text{I} may be in a different conformation from the unbound form and have an exposed essential histidine susceptible to attack.

The results in Table VIII show that none of the inhibitors at the concentrations used had any apparent effect on the capacity of the lytic intermediate to be completely lysed by C-EDTA. The EAC$^{Bov \text{142}}$ cells were incubated in the inhibitors at concentrations which were effective on free C\text{I} for various periods of time then the inhibitor was removed by washing and the cells treated with C-EDTA.

The results for PABPB will be discussed later.

From the above results it may be concluded that:

a. either the active site of C\text{I} is not involved in the activity of EAC$^{Bov \text{142}}$ complex

b. or, if it is involved it is masked by the interaction between C\text{I} and C\text{4} and/or C\text{2}.  

EFFECT OF DFP, PMSF, TLCK AND TPCK ON EAC\textsuperscript{Bov}142 CELLS

**Legend**

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Solvents</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFP</td>
<td>GBS-Sucrose++</td>
</tr>
<tr>
<td>PMSF</td>
<td>Isopropyl Alcohol</td>
</tr>
<tr>
<td>TLCK</td>
<td>GBS-Sucrose++</td>
</tr>
<tr>
<td>TPCK</td>
<td>Methyl Alcohol</td>
</tr>
</tbody>
</table>

O.D. values were corrected for any small amount of lysis occurring in the absence of G-EDTA.
### Table VIII

**EFFECT OF DFP, PMSF, TLCK AND TPCK ON EAG\textsuperscript{BovT42} CELLS**

<table>
<thead>
<tr>
<th>Inhibitors or Solvents</th>
<th>0.D. at 412nm after 30 min at 37°C</th>
<th>Time of Incubation (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>DFP ((1 \times 10^{-4} \text{M}))</td>
<td>2.515</td>
<td>2.517</td>
</tr>
<tr>
<td>DFP ((1 \times 10^{-3} \text{M}))</td>
<td>2.503</td>
<td>2.525</td>
</tr>
<tr>
<td>PMSF ((1 \times 10^{-4} \text{M}))</td>
<td>2.502</td>
<td>2.490</td>
</tr>
<tr>
<td>PMSF ((1 \times 10^{-3} \text{M}))</td>
<td>2.492</td>
<td>2.500</td>
</tr>
<tr>
<td>TLCK ((1 \times 10^{-3} \text{M}))</td>
<td>2.495</td>
<td>2.483</td>
</tr>
<tr>
<td>TPCK ((1 \times 10^{-3} \text{M}))</td>
<td>2.512</td>
<td>2.523</td>
</tr>
<tr>
<td>((\text{CH}_3)_2\text{CH}_2\text{OH})</td>
<td>2.455</td>
<td>2.480</td>
</tr>
<tr>
<td>\text{CH}_3\text{OH})</td>
<td>2.475</td>
<td>2.482</td>
</tr>
<tr>
<td>GBS-Sucrose++</td>
<td>2.552</td>
<td>2.533</td>
</tr>
</tbody>
</table>
G. Uptake by EAC_{Bov}^{42} of Bovine C\text{I} and Bovine C\text{I} inactivated by DFP

Figure VII shows the uptake of bovine C\text{I}
(2,000 CH\text{50} units/ml) diluted 1/20 in GBS-Sucrose++ by an equal volume of 1% in GBS-Sucrose++ at 37°C over a period of 60 minutes. Initial uptake is quite fast and after 20 to 30 minutes the uptake is sufficient to form enough EAC_{Bov}^{42} sites on the alexinated cells to cause their lysis in C-EDTA.

It is possible that inactivation of the esterase activity of C\text{I} by DFP prevents the molecule from being taken up by the EAC_{Bov}^{42} cells. Bovine C\text{I} was partially and completely inactivated with respect to its haemolytic function by DFP. Treatment 2,000 CH\text{50} units of bovine C\text{I} with 2 \times 10^{-4} M DFP at 37°C for 15 minutes reduced its activity to 800 CH\text{50} units. When a similar sample of the C\text{I} was treated with 5 \times 10^{-4} M DFP at 37°C for 30 minutes its haemolytic function was completely abolished.

Both inactivated samples of the C\text{I} at a dilution of 1/15 in GBS-Sucrose++ were incubated separately with an equal volume of 1% EAC_{Bov}^{42} cells in GBS-Sucrose++ for various periods of time ranging from 2 to 60 minutes. After each time interval the cells were harvested by centrifugation, washed with GBS-Sucrose++ to remove any unbound C\text{I} and excess DFP and finally resuspended to 1% in GBS-Sucrose++. The cells were treated with an equal volume of active C\text{I} (2,000 CH\text{50} units/ml) diluted to 1/20 with GBS-Sucrose++ and after 20 minutes at 37°C (condition of maximum effective uptake of C\text{I}). C-EDTA was added and the resulting lysis determined spectrophotometrically.

Table IX shows the uptake by EAC_{Bov}^{42} of the partially inactivated C\text{I} as measured by the capacity to form a lytic intermediate which lysed in the absence of C-EDTA. The pattern of uptake though much reduced is
BOVINE C\text{I} (2000 CH\textsubscript{50} UNITS/ml) UPTAKE
BY EAC\textsuperscript{Bov} 42 CELLS

Legend

Bovine C\text{I} (2000 CH\textsubscript{50} units/ml) was diluted 1:20 with GBS-Sucrose++. Equal volumes of 1% EAC\textsuperscript{Bov} 42 cells and diluted C\text{I} were incubated at 37\degree C for 2, 5, 7, 10, 15, 20, 30 and 60 minutes. Then it was followed by normal procedures for haemolytic assay.

O.D. values were corrected for any small amount of lysis occurring in the absence of bovine C\text{I}. 
FIGURE VII

BOVINE Cl (2000 CH$_{50}$ UNITS/ml) UPTAKE BY EAC$^{Bov}$ 42 CELLS
EFFECT OF TREATING EAC$^{Bov}_{42}$ FIRST WITH Cl–DFP AND THEN WITH Cl–

**Legend**

A. Uptake of partially DFP (at a final concentration of $2 \times 10^{-4} M$, incubated for 15 minutes at $37^\circ C$) inactivated bovine Cl (1/15 dilution with GBS–Sucrose++).

B. The cells from (A) for each interval of time were washed twice with ice cold GBS–Sucrose++ and incubated with active bovine Cl (1/120 dilution) for 20 minutes at $37^\circ C$. Then to each tube 0.2 ml C–EDTA was added followed by the normal procedure for haemolytic assays.

C. As in (A) but the final concentration of DFP was $5 \times 10^{-4} M$ and incubated at $37^\circ C$ for 30 minutes.

D. As in (B) using cells from (C).
### Table IX

**Effect of Treating EAC\textsuperscript{Bov}42 First with Cl–DFP and Then with Cl**

<table>
<thead>
<tr>
<th>Time of Incubation of EAC42 cells with Cl–DFP (in minutes)</th>
<th>2</th>
<th>5</th>
<th>7</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.296</td>
<td>0.515</td>
<td>0.569</td>
<td>0.602</td>
<td>1.057</td>
<td>1.429</td>
<td>1.313</td>
<td>1.200</td>
</tr>
<tr>
<td>B</td>
<td>2.254</td>
<td>2.376</td>
<td>2.198</td>
<td>2.101</td>
<td>2.236</td>
<td>2.300</td>
<td>2.407</td>
<td>2.400</td>
</tr>
<tr>
<td>C</td>
<td>0.271</td>
<td>0.261</td>
<td>0.323</td>
<td>0.290</td>
<td>0.305</td>
<td>0.329</td>
<td>0.341</td>
<td>0.431</td>
</tr>
<tr>
<td>D</td>
<td>2.433</td>
<td>2.451</td>
<td>2.475</td>
<td>2.447</td>
<td>2.464</td>
<td>2.538</td>
<td>2.447</td>
<td>2.487</td>
</tr>
</tbody>
</table>

O.D. at 412nm after Incubation with C–EDTA for 30 minutes at 37°C
similar to that observed for active CI (Fig. VII).

The treatment of EAG\textsuperscript{Bov 42} cells with either partially or totally inactive CI does not appear to inhibit the capacity of them to interact with active CI and form a lytic intermediate (Table IX). It appears from this that the DFP-inactivated CI is not taken up by the cells alternatively it may be taken up weakly and is capable of exchanging with the active CI so that a functional lytic intermediate may form.

Another alternative is that there are many more EAC42 sites on the EA than could be blocked by all the offered inactivated CI and on offering active CI the unblocked sites take it up to form functional lytic intermediate. Which, if any of these hypotheses is correct must await further investigation.

These hypotheses are explained diagrammatically in Figure VIII.

H. Uptake of CI-\textsuperscript{3H}-DFP by EA, EAG\textsuperscript{Bov 142} and EAG\textsuperscript{Bov 42} cells

Bovine CI inactivated with tritium labelled DFP has been used to investigate the uptake of CI-DFP. CI (14400 CH\textsubscript{50} units/ml) was treated with 1.05 x 10\textsuperscript{-3} M DFP in a final volume of 1.5 ml. The specific activity of the DFP was 7.15 pCi/mM. The CI was completely inactivated with respected to its capacity to form a lytic intermediate with EAC42 cells. The activity of the CI-\textsuperscript{3H}-DFP after dialysis to remove all the excess inhibitor was found to be 4.32 x 10\textsuperscript{-4} d.p.m.

Table X shows the relative uptake of CI-\textsuperscript{3H}-DFP by 1 x 10\textsuperscript{8} cells of EA, EAC42 and EAC142.

The EA intermediate was used to form the EACT42 from which the EAC42 cells were prepared. Therefore all three cell preparations should have the same average number of complement fixing sites. The erythrocyte
FIGURE VIII

PROPOSED HYPOTHESIS FOR THE UPTAKE OF Cl-DFP BY EAC\textsuperscript{Bov}42 CELLS

\[
\begin{align*}
x \text{EAC42} + y \text{Cl}^{DFP} & \rightarrow y \text{EAC1}^{DFP}42 + (x - y)\text{EAC42} \\
\text{EAC42} + \text{Cl}^{DFP} & \rightarrow \text{EAC142} \rightarrow \text{Lysis} + \text{Cl} + \text{C-EDTA} \\
\text{Lysis} & \rightarrow \text{EAC142} \rightarrow \text{C-EDTA} \\
\text{Lysis} & \rightarrow \text{C-EDTA} \\
\end{align*}
\]
### TABLE X

**THE UPTAKE OF $^3H$-DFP INACTIVATED Cl BY EA, EAC$^{Bov}42$, AND EAC$^{Bov}142$ CELLS**

Activity of Cl-$^3H$-DFP offered to cells = 8638 d.p.m. $^b$

<table>
<thead>
<tr>
<th>Cell Suspension</th>
<th>Actual Cell Conc. in 0.4 ml of 5% cells $^c$</th>
<th>Activity taken up by 0.4 ml of cells d.p.m.</th>
<th>Activity taken up by $1 \times 10^8$ cells d.p.m.</th>
<th>Relative uptake Activity $^e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>EA</td>
<td>$1.04 \times 10^8$</td>
<td>2827</td>
<td>2718</td>
<td>100%</td>
</tr>
<tr>
<td>EAC$^{42}$</td>
<td>$1.10 \times 10^8$</td>
<td>2683</td>
<td>2438</td>
<td>89.7%</td>
</tr>
<tr>
<td>EAC$^{142}$</td>
<td>$1.16 \times 10^8$</td>
<td>2461</td>
<td>2121</td>
<td>78.0%</td>
</tr>
</tbody>
</table>

$^a$ All activities are corrected for background counting and represent an average of 5 independent countings.

$^b$ The efficiency of counting was 13.5%.

$^c$ The cell concentration per ml was determined spectrophotometrically for 5 ml suspensions as described in Methods and Materials.

$^d$ The efficiency of counting was 13.5% in all instances.

$^e$ The relative uptake is calculated assuming that the uptake by EA is 100% i.e. it represents the possible maximum amount of Cl uptake.
surface represents a mosaic of antigenic determinants and therefore each sensitized cell probably has many Cl fixing sites. In a preparation of EAC_{Bov}^{142} from EA and bovine complement, under the prescribed, controlled conditions which prevent significant uptake of C3, there are most likely complexes representing each stage in the fixation of Cl, C4 and C2 (see Figure VIII).

Removal of Cl from the EAC_{Bov}^{142} cell preparation results in the general of potential sites for the uptake of Cl-^{3}H-DFP i.e. EA, EAC_{Bov}^{4} and EAC_{Bov}^{142} (Figure IX). Therefore the interpretation of the results is made difficult by the probable heterogeneity of the EAC_{Bov}^{142} and EAC_{Bov}^{42} preparations. The uptake of Cl-^{3}H-DFP by EAC_{Bov}^{142} is not surprising. They probably contain a relatively large number of EA sites relative to the other complexes because of the condition under which they were formed. Removal of Cl from the EAC_{Bov}^{142} results in the generation of potential sites for the fixation of Cl-^{3}H-DFP (see Figure IX) i.e. new sites and also EAC4 and EAC42 sites. It is observed that EAC_{Bov}^{42} takes up more radioactivity (about 10%) than EAC_{Bov}^{142} cells, whether this is due to the Cl-^{3}H-DFP being fixed to EA, EAC4 and EAC42 sites, is not determined. However the EAC_{Bov}^{42} preparation doesn't take up as much radioactivity as the EA preparation which suggests that the Cl-^{3}H-DFP is not able to occupy all the potential sites. It is attractive, in view of all previous evidence presented in this manuscript, to suggest that the Cl-^{3}H-DFP is not capable of binding with EAC_{Bov}^{142} and possibly EAC4 complexes. If this so then it appears that the esterase site of Cl is involved either directly or indirectly in its interaction with C4 and C2 in maintaining an active EAC_{Bov}^{142} complex. Further more this interaction is sensitive to the binding of a relatively small molecule such as DFP to the esterase site.
I. Effect of PABPB on whole Guinea Pig, Human and Bovine Complements

It is well understood that the guinea pig complement has a greater haemolytic activity than the human using sensitized sheep erythrocytes as target cells. Although bovine serum lacks haemolytic complement activity against sensitized sheep erythrocytes, the use of sensitized guinea pig cells described by Barta and Barta (15) makes it possible to assay its haemolytic activity. Table XI shows the CH$_{50}$ units of the three complement species.

Compounds derived by quaterization of N-(3-pyridylmethyl)-3,4-dichlorophenoxyacetamide with substituted fluorosulphonyl benzyl bromides form a family of proteolytic enzyme irreversible inhibitors which are in general good inactivators of chymotrypsin and both guinea pig whole complement and C1 (41). PABPB is chosen as a representative of this group of inhibitors. Though quite effective other compounds in this series are more potent inhibitors of guinea pig C1 (38). However PABPB was chosen because of its relative ease of synthesis.

Table XII shows the effect of two different concentrations of PABPB on the haemolytic activities of human, guinea pig and bovine complements. The results with guinea pig complement are comparable with those previously reported for the inhibitory effect of PABPB (38). Although direct comparisons between the complement from the three species is not possible because of using different target cells, pH, diluent etc. PABPB seems to be an effective inhibitor of each complement system. It appears that PABPB is a more effective inhibitor of guinea pig and human than of bovine complement.
HAEMOLYTIC TITRATION OF BOVINE, HUMAN AND GUINEA PIG COMPLEMENTS

Legend

One per cent sheep erythrocytes optimally sensitized with rabbit haemolysin were used as the target cells (EA) in the human and guinea pig systems and GBS++ (pH 7.35, ionic strength \( \mu = 0.15 \)) as diluent.

One per cent guinea pig erythrocytes optimally sensitized with bovine haemolysin were used as the target cells in the bovine system and GBS-Sucrose++ (pH 7.0, ionic strength \( \mu = 0.094 \)) as diluent.
**TABLE XI**

**HAEMOLYTIC TITRATION OF BOVINE, HUMAN AND GUINEA PIG COMPLEMENTS**

<table>
<thead>
<tr>
<th>Complement</th>
<th>Target Cells</th>
<th>CH$_{50}$ Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea Pig</td>
<td>Sensitized Sheep RBC</td>
<td>700</td>
</tr>
<tr>
<td>Human</td>
<td>Sensitized Sheep RBC</td>
<td>150</td>
</tr>
<tr>
<td>Bovine</td>
<td>Sensitized Guinea Pig RBC</td>
<td>400</td>
</tr>
</tbody>
</table>
THE INHIBITION OF WHOLE COMPLEMENT FROM GUINEA PIG, HUMAN AND BOVINE SPECIES BY PABPB

Legend

Sheep erythrocytes optimally sensitized with rabbit haemolysin were used as the target cells (EA) in the human and guinea pig complement systems and GBS++ as diluent (pH 7.35, ionic strength μ = 0.15).

Guinea pig erythrocytes optimally sensitized with bovine haemolysin were used as the target cells and GBS-Sucrose++ (pH 7.0, μ = 0.094) as diluent.

O.D. is optical density at 541nm in 1 cm glass cuvettes corrected for EA + MeOEtOH control, and in tubes 2, 3, 5, 6, 8 and 9 for slight haemolysis produced by PABPB acting in absence of complement.

Inhibition of complement by PABPB is expressed as a fractional percentage of the O.D. observed over the O.D. in standard tubes 1, 4 or 7.
<table>
<thead>
<tr>
<th></th>
<th>33</th>
<th>0</th>
<th>67</th>
<th>0</th>
<th>77</th>
<th>0</th>
<th>Per centage Inhibition</th>
</tr>
</thead>
</table>
| 0.56  | 1.0 | 0.05 | 0.0 | 0.05 | 0.0 | 0.0 | P的情况 | P.I.
| 0.05  | - | - | - | - | - | - | T: 10 bovine C Mt | T: 10 human C Mt | T: 50 guinea pig C Mt | 0.5% PABB in Melt | 1.0% PABB in Melt | Motion Mt | 2.5% E & Mt | Type number |
| 0.56  | 0.05 | 0.0 | 0.05 | 0.0 | 0.0 | 0.0 |
| 0.05  | - | - | - | - | - | - |
| 0.56  | 0.05 | 0.0 | 0.05 | 0.0 | 0.0 | 0.0 |
| 0.05  | - | - | - | - | - | - |

The inhibition of whole complement from guinea pig, human and bovine species by PABB

**Table XI**
J. **Effect of PABPB on Bovine C\textsuperscript{T}\**

It has been suggested that the major site of complement inactivation by PABPB and related compounds in the guinea pig system is the C\textsuperscript{I} molecule. Baker and Cory (38) incubated PABPB and related compounds with guinea pig C\textsuperscript{I} and then without removing the large excess of inhibitor, RI (all the other complement components except C\textsuperscript{I}) and the sensitized sheep red cells were added. The extent of inhibition was related to the amount of haemolysis. Since the inhibitor was not removed it is not possible to conclude that compound was affecting only the activity of C\textsuperscript{I}, it may also have affect the activity of the later acting components present in the RI.

The decay of the lytic EAC\textsuperscript{Bov\textsuperscript{42}} cells to nonlytic EAC\textsuperscript{Bov\textsuperscript{42}} cells provides a stable intermediate for C\textsuperscript{I} assays. Table XIII shows the effect of PABPB on the ability of bovine C\textsuperscript{I} to form an active EAC\textsuperscript{Bov\textsuperscript{42}} complex from EAC\textsuperscript{Bov\textsuperscript{42}} cells. PABPB at final concentrations ranging from 0.25 to 2.5 mM for 60 minutes at 37\degree C completely inhibit the haemolytic activity of 14400 CH\textsubscript{50} units/ml of bovine C\textsuperscript{I}. Only partial inhibition is observed after 30 minutes with the same inhibitor concentrations. Complete inhibition was not achieved by either 0.125 or 0.0675 mM PABPB even after 90 minutes at 37\degree C. It was found that integrity of the EAC\textsuperscript{Bov\textsuperscript{42}} cells is unaffected by 0.308 mM PABPB or lower concentrations. When the effect of inhibitor on the haemolytic bovine C\textsuperscript{I} is determined the C\textsuperscript{I} and inhibitor are serially diluted so that the inhibitor concentration in contact with the EAC\textsuperscript{Bov\textsuperscript{42}} cells is made less than 0.30 mM. This indicates that the inhibitor in this experiment has little or no effect on the cell bound C4 and C2.

Since excess inhibitor was removed before
INHIBITION BY PABPB OF THE CAPACITY OF CI TO FORM THE
LYTIC INTERMEDIATE EAC142 FROM EAC1ROV 42

Legend

Inhibition of CI by PABPB is expressed as a
fractional percentage of the CH$_{50}$ units/ml observed over
the CH$_{50}$ units/ml in the saline control.
<table>
<thead>
<tr>
<th>Time of Inhibition</th>
<th>Unit/m²</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>90 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Inhibition by PABA of the Capacity of G1 to Form the Intermediate Amino Acid from Eucalts**

**Table XIII**
adding C-EDTA and the inhibitor had no effect on the EAC\textsuperscript{Bov42} cells, the effect of PABPB is presumed to be associated with the capacity of C\text{I} to react with the EAC\textsuperscript{Bov42} to form the lytic intermediate. This result supports the hypothesis that a major site of complement inactivation by PABPB is the C\text{I} molecule (38).

Table XIV and Table XV show the effect of PABPB on the enzymatic activity of bovine and human C\text{I} with respect to the esterolytisis of N-Z-L-Tyr-p-Np. The results show that the enzymatic activities of both human and bovine C\text{I} (1.815 \times 10\textsuperscript{6} enzyme units/ml and 5.64 \times 10\textsuperscript{5} enzyme units/ml respectively) are not completely destroyed by 0.0625 to 2.5 mM final concentrations of PABPB even after 90 minutes at 37\textdegree C. Higher concentrations of inhibitor were not used because they resulted in extensive precipitation of the C\text{I}. It seems that the esterase inhibition on human C\text{I} is greater than on bovine C\text{I}. This result correlates with the greater inhibition in whole human complement than in bovine complement (Table XIII). Like the DFP inhibitor, for the same amount of C\text{I} the PABPB inhibits the capacity to form EAC\textsuperscript{Bov42} more effectively than the inhibition of enzymatic activity. Besides the explanation for this phenomenon given in the DFP section, this could also suggest that the inhibitor may be interacting with C\text{I} at sites other than those which can lead to complete enzymatic interaction and in doing so prevents C\text{I} from combining effectively with EAC\textsuperscript{Bov42} to form a reactive EAC\textsuperscript{Bov42} complex.

The proposed mechanism of action of PABPB and related compounds is by exoalkylation (57). Bridging with some nucleophilic group on the enzyme surface outside the active site promotes irreversible interaction by stabilizing the interaction of the sulphonyl fluoride group with the active site of the enzyme. With both bovine
THE EFFECT OF PABPB ON THE ENZYMATIC ACTIVITY OF BOVINE AND HUMAN CT

Legend

One unit of enzyme is defined as the amount of protein which releases $1 \times 10^{-6}$ mM of p-nitrophenol in 5 minutes at $25^\circ C$ from $3 \times 10^{-5}$ M N-Z-L-Tyr-p-Np at pH 8.05.

Inhibition is expressed as a fractional percentage of the units of enzyme/ml observed after inhibition over the units of enzyme/ml in the control tubes containing either MeOEtOH or Saline.
<table>
<thead>
<tr>
<th>Time of Incubation</th>
<th>Concentration</th>
<th>Nitrogenase Activity of Bovine C1</th>
</tr>
</thead>
<tbody>
<tr>
<td>90 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time of Incubation</td>
<td>Percentage Inhibition</td>
<td>Concentration (mm)</td>
</tr>
<tr>
<td>-------------------</td>
<td>-----------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>90 min</td>
<td>60 min</td>
<td>30 min</td>
</tr>
<tr>
<td>≈ 10%</td>
<td>≈ 10%</td>
<td>≈ 10%</td>
</tr>
<tr>
<td></td>
<td>l ≈ 10%</td>
<td></td>
</tr>
</tbody>
</table>

The Effect of Papb on the Enzyme Activity of Human CA
and human CT more inhibition is obtained with 0.25 mM PABPB in bovine CT and 1.25 mM human CT than with higher concentrations of 1.25 and 2.5 mM respectively. This could result from multiple binding sites on the CT molecule for PABPB. Preferential binding at one or more sites may inhibit the binding of PABPB at the active site; this effect could be enhanced at higher concentrations of the inhibitor but become less apparent at lower concentrations. The possibility of multiple binding sites on CT help to explain the inhibition of CT interaction with EAC^Bov42 without complete interaction of its enzymatic nature.
CHAPTER IV

CONCLUSION

Although bovine serum has a potent bactericidal complement system, it is non-haemolytic in standard haemolytic complement assays. But under controlled conditions the early acting components may be assembled onto sensitized sheep erythrocytes to give a stable intermediate, EAC\textsuperscript{Bov-142}, which may be lysed with C-EDTA. Removal of CI from EAC\textsuperscript{Bov-142} renders it no longer reactive with the C-EDTA, but it is not true in the guinea pig system. These unique characters of the early acting components of bovine complement leads to the studies of bovine complement.

In order to study the role of bovine CI in the system it is necessary to obtain it in a relatively pure form. This is achieved by using the method described by Nelson (44). Further purification has been tried using Sephadex G-200 and there was a 1.4 fold increase in haemolytic activity. It is expected that further purification can be achieved by using Bio-gel, zonal ultracentrifugation or affinity chromatography (53,54).

The assay procedures for bovine CI used here are based upon its esterase and haemolytic functions. The enzymatic activity is measured by the hydrolysis of N-Z-L-Tyr-D-Np in five minutes as described for human CI and CIs by Bing (25). The decay of EAC\textsuperscript{Bov-142} to EAC\textsuperscript{Bov-42} provides a stable intermediate for both human and bovine CI assays. Hence its haemolytic activity can be estimated by its capacity to reform lytic EAC\textsuperscript{142} from non-lytic EAC\textsuperscript{42} cells. The effect of the inhibitors have been observed on these two activities.

The effect of various inhibitors, namely: DFP, PMSF and PARPB, on bovine CI esterase activity is similar to
the human CI. The two histidine inhibitors, TLCK and TPCK, have no effect on bovine and human CI. Though these two inhibitors have no effect, it is not possible to conclude that histidine is non-essential in either bovine and human CI for its action as an esterase without actual demonstration of histidine alteration.

It has been found that the inhibitors which prevent esterase activity also inhibit the formation of EAC<sup>Bov</sup> 142 from EAC<sup>Bov</sup> 42. This is rather surprising because one expects that after activation of C4 and C2 the CI enzymatic activity is no longer required. Hence the involvement of the CI esterase active site may be direct i.e. it may be involved in the active CI enzyme or the CI esterase site involvement may be indirect i.e. that part of the molecule may be involved in somehow maintaining in active configuration of the C42 complex. In either case the removal of CI results in the loss of functional activity of EAC<sup>Bov</sup> 142 cells.

The results on the study of the uptake of active CI prior treatment of cells with CI-DFP and the uptake of CI-<sup>3</sup>H-DFP suggest that this molecule may not be taken by the EAC<sup>Bov</sup> 42 complex. From the results on the stability of EAC<sup>Bov</sup> 142 in different ionic strength buffer, with or without EDTA, it may be suggested that EAC<sup>Bov</sup> 142 has a compact structure in which CI forms a close association with the antibody, C4 and/or C2. Therefore it is possible that the EAC<sup>Bov</sup> 142 complex is a compact structure such that even slight modification of CI by DFP is sufficient to prevent CI from reforming the lytic EAC<sup>Bov</sup> 142 from EAC<sup>Bov</sup> 42 cells.

It would be useful to have a reagent which could selectively inhibit complement action especially its involvement in certain disease states. In a series of papers, Baker and co-workers reported the synthesis and screening of a large series of potential specific,
active-site-directed irreversible inhibitors of the first component of guinea pig complement (38). It has been found in this manuscript that PABPB is a relatively poor inhibitor of bovine whole complement and the CI component. Hence other inhibitors may be of greater use. Recently Bing reported (56) that m-(α-(2-chloro-5-fluorosulphonylphenylureido) phenoxybutoxy)-benzamidine (MCFPB) irreversibly inactivates purified human CI and dialysis of CI treated with MCFPB fails to restore activity. However, MCFPB is apparently more active than DFP. The site of action of the MCFPB molecule is almost certainly the esteratic site on C1s subunit of the CI complex. Therefore the reagent MCFPB should prove very useful in future studies in the area of structure-function relationship of the first component of complement.

The results of the inhibitor, PABPB, on bovine CI with respect to esterase and haemolytic activities are comparable to those with DFP. The inhibition of CI interaction with EAC42 probably is enchanced by interaction of PABPB at sites on the CI molecule other than the active site.
APPENDIX
I. Method for Determining CH₂₀₀ Unit

This is an arbitrary unit, since its magnitude depends on the concentration of red cells, the fragility of the cells, the quality of antibody used for sensitization, the nature of the antibody, the ionic strength of the reaction system, the concentration of Ca⁺⁺ and Mg⁺⁺, pH, reaction time and temperature (35).

For mathematical description of the sigmoidal response curve of the haemolytic reaction, the equation of von Krogh (42)

\[ \frac{Y}{X} = K \left( \frac{1}{1 - Y} \right)^{1/n} \]

has been employed.

\( X \) = the amount of complement (expressed in ml of complement)

\( Y \) = degree of lysis (i.e. 100Y = % haemolysis)

The magnitude of the exponent, \( 1/n \), which determines the shape of the sigmoidal curve, depends on experiment conditions, but usually, a value of 0.2 ± 10%.

The constant \( K \) is the 50% unit of complement, since at this point \( Y = 0.5 \) and the term \( Y/(1 - Y) \) = unity.

Therefore \( X = K \).

Logarithmic transformation of the von Krogh equation furnishes a function which is convenient for evaluation of experimental results.

\[ \log X = \log K + \frac{1}{n} \log \left( \frac{Y}{1 - Y} \right) \]

If \( \log X \) is plotted against \( \log \left( \frac{Y}{1 - Y} \right) \), it gives a straight line of intercept \( \log K \) and slope \( 1/n \).
II.

BARIUM QUENCH CURVE

Channel A  Adjusted for balance point for least quench tritium standard

Channel B  Adjusted for balance point for Barium-133 external standard, using the same tritium sample

Use Nuclear-Chicago Model 180050 liquid scintillation tritium quenched standard set i.e. accurately assayed sealed samples of tritium labelled toluene in scintillation fluid with varying amounts of reagent grade CCl₄ quenching agent.

Activity of each sample in March 1968 is 455,000 d.p.m.
Activity of each sample in August 1972 is 353,100 d.p.m.
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