1973

Studies on the first component of the bovine complement system.

Anthony Shing-Duen. Pang
University of Windsor

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

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

ABSTRACT

Bovine $\overline{C1}$ 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, diisopropylfluorophosphate and 3-(3,4-dichlorophenoxyacetamido)-N-(3,chloro-2-fluorosulphonylbenzyl) pyridinium bromide, which inhibited $\overline{C1}$ esterase activity also inhibited the ability of $\overline{C1}$ to form $EAC^{Bov}\overline{142}$ from the $EAC^{Bov}42$ intermediate. It was suggested that the esterase active site might be involved in the active $\overline{C142}$ enzyme or part of the $\overline{C1}$ molecule might be involved in somehow maintaining in active conformation of the lytic intermediate.

From the studies of the uptake of the active $\overline{C1}$ prior treatment of cells with C1-DFP and the uptake of C1-^{3}H-DFP suggested that this molecule was not taken up by the $EAC^{Bov}42$ complex. It was postulated that the $EAC^{Bov}\overline{142}$ complex was compact structure in which $\overline{C1}$ 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

敬獻双親

兒端

TABLES OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

E	Erythrocyte (sheep cell)
A	Rabbit antibody to sheep erythrocytes (antisheep haemolysin)
C	Complement
C1, C2 ...9	Complement components
EAC1 - n	Intermediate complexes formed by the reaction of the first n complement components
$\overline{Cn}$	Complement component that has aquired enzymatic or other biological activity
EDTA	Trisodium Ethylene Diamine Tetraacetate
C-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 x 10^{-3}M $MgCl_2$ and 1.5 x 10^{-4}M $CaCl_2$
GBS-Sucrose	Sucrose-Gelatin-Barbital Saline
GBS-Sucrose++	GBS-Sucrose containing 1 x 10^{-3}M $MgCl_2$ and 1.5 x 10^{-4}M $CaCl_2$
EDTA-GBS	GBS containing 0.01M EDTA
DFP	Diisopropylfluorophosphate
^{3}H-DFP	Diisopropyl-^{3}H-fluorophosphate
C1-DFP	$\overline{C1}$ treated with DFP
C1-^{3}H-DFP	$\overline{C1}$ treated with ^{3}H-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-phenylalanyl Chloromethane
MeOEtOH	Ethylene glycol monomethyl ether (methyl cellusolve)

N-Z-L-Tyr-p-Np	N-Carbobenoxy-L-tyrosine-p-nitrophenyl ester
RPM	Revolutions per minute
O.D.	Optical Density
Ci	Curie
d.p.m.	Disintegrations per minute

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 enchancement 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 complemtnt (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 permamently 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 C1q, C1r and C1s and they

TABLE I

Properties of Human Complement Protein (1)

Complement Component	Serum Conc. µg/ml	Sed. Coeff. (S)	App. Mol. Weight	Electrophoretic Mobility
C1q	190	11.1	400,000	γ_2
C1r	---	7.0	168,000	β
C1s	22	4.0	80,000	α_2
C2	20-40	5.5	117,000	β_2
C3	1,200	9.5	185,000	β_1
C4	430	10.0	240,000	β_1
C5	75	8.7	180,000	β_1
C6	?10 ?c50	5-6	95,000 (140,000)	β_2
C7		5-6	110,000 (140,000)	β_2
C8	10	8.0	(150,000)	γ_1
C9	10	4.5	79,000	α

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 $\overline{C1}$.

Erythrocytes sensitized with anti-erythrocyte antibodies may he 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 C1q, C1r and C1s, in the form of the C1 complex. The uptake requires calcium ions. Then the proesterase C1s is activated, presumably by some mechanism involving C1q and C1r (10). Activated C1 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, $\overline{C1}$ esterase is not involved in the actual process of cell lysis. The natural substrate of $\overline{C1}$ esterase is not the cell membrane but apparantly C4 and C2. The next event in the complement reaction therefore seems to be the sequential uptake by the complex, EA$\overline{C1}$, of C4 and C2. Following the formation of the complex EA$\overline{C1}42$, activation of C3 and its subsequent binding to the cell membrane occurs.

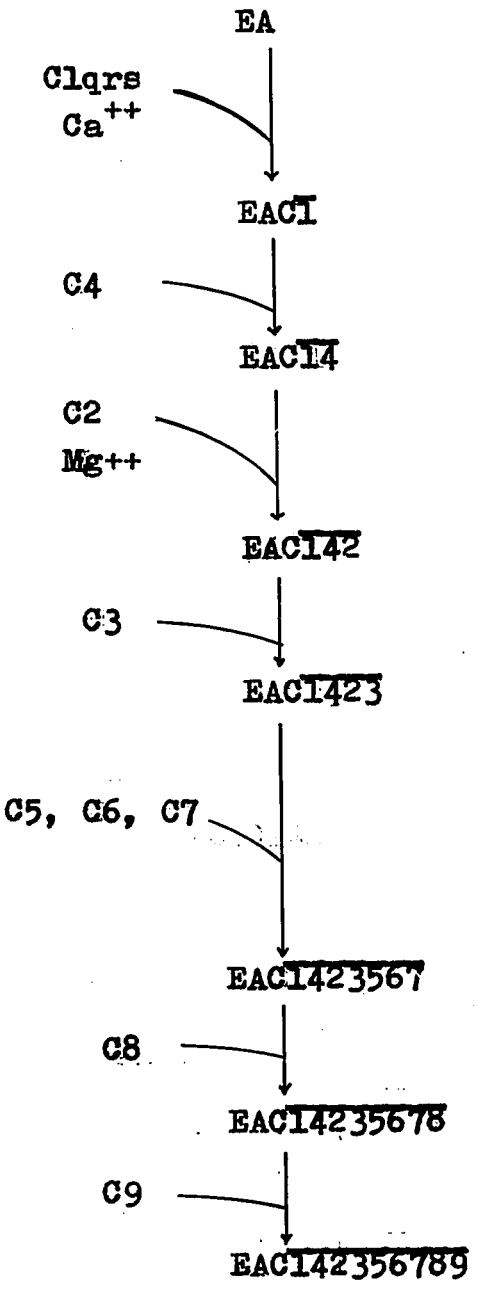
The subsquent event of immune lysis are less well understood. The bound C3 is thought to modify the EAC42 enzyme so that it initiates the activation of the remaining five components with the assembly on the membrane of a C56789 complex. At this stage lesions occur in the cell membrane, perhaps as the 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) repoarted 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 1×10^{-3}M Mg^{++} and 3×10^{-4}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 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 EAC$\overline{142}$ (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 C1 activity and contained no C4 or C2 activity, the identity of the decayed lytic intermediates appeared to be EAC42.

The first component of complement (C1) is an euglobulin. Studies on the effect of the divalent cation chelator trisodium ethylenediaminetetraacetate (EDTA) on C1 led to the recognition that human C1 is composed of at least three fragments, C1q, C1r and C1s, 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 C1 for immunoglobulins resides in the subunit C1q (19). Following binding of the complex to EA, a proenzyme (C1s) is activated through internal mechanism involving C1r (20); activated $\overline{C1}$ triggers the complement chain reaction. It may be postulated that C1r is able to act on C1s through a distortion or spatial rearrangement of the C1 complex

resulting from conbination with antibody through Clq. Human and guinea pig C1 is a proesterase which becomes an esterase following its activation to $\overline{C1}$ (21). The proesterase and esterase activity has been identified with the C1s (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, $\overline{C1}$ 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 $\overline{C1}$ was required by $EAC\overline{142}$ for it to function as a lytic intermediate (17). Therefore it is of interest to investigate the role of $\overline{C1}$ in activity of the $EAC^{Bov}\overline{142}$ 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 $\overline{C1}$ but not with C1 and blocks the esterase activity of both $\overline{C1}$ and the 4S fragment (C1s) derived from $\overline{C1}$ (27). That serum protein is called C1-esterase inhibitor with a sedimentation constant of 3S. It also inhibits the haemolytic activity of $\overline{C1}$. A relative high molecular weight substance, carrageenan, inhibits the haemolytic activity of C1 or $\overline{C1}$ (28) by preventing attachment of the complement to antigen-antibody complexes. But it does not inhibit the esterase activity of partially purified $\overline{C1}$. These show that the site on $\overline{C1}$ that binds to antigen-antibody complexes is distinct from the site possessing enzymatic activity (26).

Human and guinea pig $\overline{C1}$ 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 $C\bar{1}s$ and $C\bar{1}$ 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 $C\bar{1}$ and $C\bar{1}s$.

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 $EAC\bar{1}\bar{4}\bar{2}$ 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 $EAC\bar{1}\bar{4}\bar{2}$ can be lysed by treatment with purified $C\bar{2}$, followed by C-EDTA. This means that the decayed cells are in the state $EAC\bar{1}\bar{4}$ (35). On the contrary, $EAC^{Bov}\bar{1}\bar{4}\bar{2}$ 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 $EAC^{Bov}\bar{1}\bar{4}\bar{2}$ 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 $C\bar{1}$ (17). This provides a convenient assay for $C\bar{1}$. The $EAC^{Bov}\bar{1}\bar{4}\bar{2}$ 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 1×10^{-3} M

Mg^{++} and 1.5×10^{-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$\overline{142}$ 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.

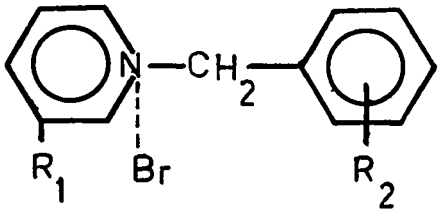
This manuscript presents an investigation of some of the characteristics of bovine C1. A method is presented for its functional purification. Its role in the $EAC^{Bov}\overline{142}$ complex has been investigated by subjecting the protein to various inhibitors and examining their effects on the enzymatic activity of C1 and its capacity to form the active intermediate $EAC^{Bov}\overline{142}$ 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 $\overline{C1}$, 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

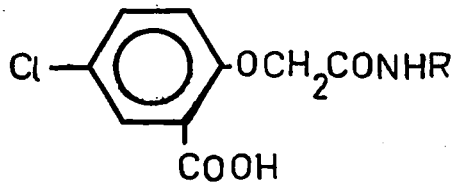
Proteolytic Enzyme active-site-directed Irreversible Inhibitors

Type I



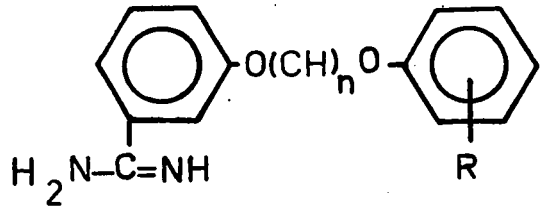
Examples		
	R_1	R_2
	$3,4\text{-}Cl_2C_6H_3OCH_2CONH$	$4\text{-}SO_2F$
	$3,4\text{-}Cl_2C_6H_3OCH_2CONH$	$2\text{-}SO_2F$

Type II



R
$m\text{-}CH_2C_6H_4NHCONHC_6H_3\text{-}3\text{-}Cl\text{-}4\text{-}SO_2F$

Type III



n	R
3	$m\text{-}NHCONHC_6H_4NO_2\text{-}p$
3	$m\text{-}NHCONHC_6H_4SO_2F\text{-}p$

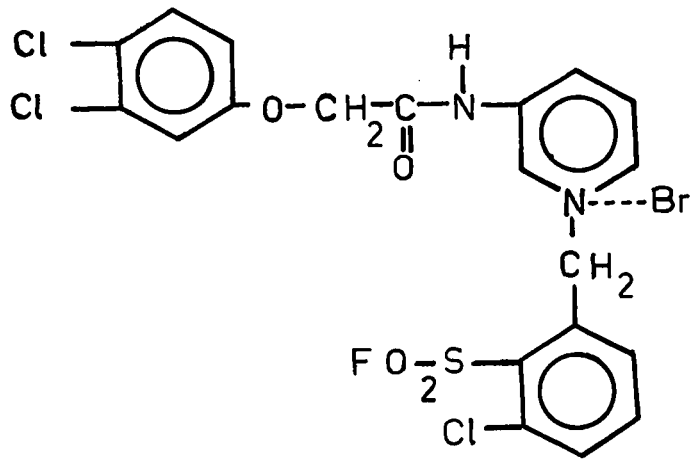
not surprising that other inhibitors of trypsin and chymotrypsin inhibit $C\bar{1}$ and other components of complement since some are 'tryptic' or 'chymotryptic' or both in character (29). Baker showed that there were two types of chymotryptic inhibitor (Type I and Type II) and one type of tryptic inhibitor (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 $C\bar{1}$ 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 $C\bar{1}$. 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 $C\bar{1}$ 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 avtivities of bovine $C\bar{1}$. 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 C1 (38). It is hoped that the studies with PABPB on bovine $C\overline{1}$ will give helpful information concerning the function of $C\overline{1}$, as an esterase and its capacity to form $EAC\overline{1}42$ 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 Difico 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 $\overline{C1}$ was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.

The inhibitor, 3-(3,4-dichlorophenoxyacetamido)-N-(3-chloro-2-fluorosulphonylbenzyl) pyridinium bromide (PABPB) 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. Phenylmethyl-sulphony fluoride (PMSF) and 5M diisopropylfluorophosphate (DFP) in isopropyl alcohol were purchased from Pierce Chemical,

Rockford, Illinois.

Diisopropyl-1-^{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 μ = 0.15, containing 0.1% gelatin, 1 x 10^{-3}M $MgCl_2$ and 1.5 x 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 $\overline{CI}$, $EAC^{Bov}\overline{142}$ and $EAC^{Bov}42$ the diluent used was GBS-Sucrose++ at pH 7.35 and ionic strength μ = 0.075. This was prepared by mixing equal quanities of GBS++ and 9% sucrose and adjusting the final concentration of gelatin to 0.1% and divalent cation concentration to 1 x 10^{-3}M $MgCl_2$ and 1.5 x 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

μ = 0.094 containing 0.1% gelatin, 1 x 10^{-3}M $MgCl_2$ and 3 x 10^{-4}M $CaCl_2$. It was prepared by mixing 3 volumes of GBS++ with 2 volumes of GBS-Sucrose++ (μ = 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 $\pm$ 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-phenyloxazolyl))-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 Unlix II Scintillator Counter (Nuclear-Chicago Corp.). The effeciency 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 C1

Bovine C1 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 2°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 C1. The remainder was stored at -20°C where it remained stable with

respect to its capacity to form $EAC^{Bov}\overline{142}$ cells for about 3 months. The pH of the serum was adjusted to 7.5 by dropwise addition 0.1M K_2HPO_4 while stirring and then dialysed in $1^7/_8$ Visking tubing overnight against distilled water brought up to pH 7.5 by addition of 0.1M K_2HPO_4. For every liter of dialysing fluid, 1 ml of 0.15M $CaCl_2$ was added. The resulting precipitate was centrifuged at 12,000 RPM (173,000 g) for 20 minutes. The supernatent was a R1 (i.e. it contains all the other complement components except C1) and the precipitate (containing C1) 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 1×10^{-3}M $MgCl_2$ and 1.5×10^{-4}M $CaCl_2$. 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 1×10^{-3}M $MgCl_2$ and 1.5×10^{-4}M $CaCl_2$ for 4 - 6 hours. The precipitated C1 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 1×10^{-3}M $MgCl_2$ and 1.5×10^{-4}M $CaCl_2$. The resulting solution was centrifuged to clear. The preparation was stored at $-20^{\circ}C$.

Further purification of bovine C1 was achieved by molecular sieve chromatography at $2^{\circ}C$ 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 CI, 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 $C\bar{1}$ 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% $EAC^{Bov}42$ 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 $C\bar{1}$, was determined by measuring the optical density at 412nm of the haemoglobin released into the fluid phase. Fractions containing $C\bar{1}$ haemolytic activity were pooled. The C1 was precipitated by dialysing GBS++ diluted 1/5 (μ = 0.03) overnight at 2°C. The $C\bar{1}$ was harvested by centrifugation at 8,000 RPM for 20 minutes and washed twice with the diluted GBS++ (μ = 0.03). The $C\bar{1}$ was then dissolved in 2 ml of GBS++ to which was added 0.15M NaCl and stored at -20°C.

B. Preparation of EA, $EAC^{Bov}\bar{1}42$ and $EAC^{Bov}42$ 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_2CO_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_2CO_3 resulted in an O.D. of 0.504 at 541nm, therefore the number of cells was calculated to be 7.2×10^8.

b. Guinea Pig EA

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

2. $EAC^{Bov}\overline{142}$

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 $EAC^{Bov}\overline{142}$ 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% $EAC^{Bov}\overline{142}$ in GBS-Sucrose and stirred at 37°C for 15 minutes. The mixture was centrifuged, the supernatent discarded and the cells resuspended to 0.33% in a buffer containing one volume of GBS-Sucrose and two volumes of EDTA-GBS. Following a further 15 minutes at 37°C the cells were washed three times with GBS-Sucrose++ and finally suspended to appropiate dilution in GBS-Sucrose++. The $EAC^{Bov}42$ 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 $\overline{CI}$ (diluted 1/50 with GBS-Sucrose++) for 20 minutes (see haemolytic assay for $\overline{CI}$). The cells were stable after a week at 2°C. The term stable refers to their capacity to form a lytic intermediate with $\overline{CI}$.

C. Haemolytic Assays

1. Method For Determining CH_{50} 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_{50} 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 sensitized 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

lysis 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 centrigugation. 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 μ = 0.094 containing 0.1% gelatin, 1 x 10^{-3}M $MgCl_2$ and 3 x 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 $C\bar{1}$

The diluent used was GBS-Sucrose++ at pH 7.35 and ionic strength μ 0.075. Bovine $EAC\bar{1}42$ cells were used for the assays of bovine and human $C\bar{1}$. The assay was performed in tubes 1 x 7.5 cm. Serial dilutions of $C\bar{1}$ 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 $\overline{C1}$ 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 1×10^{-3}M. The assay system consisted of 3 ml of buffer, 50 ul of $\overline{C1}$ 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 1×10^{-6}mM of p-nitrophenol in 5 minutes at 25°C from 3×10^{-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 1.66×10^{4} (43).

E. Inhibitor Studies on Whole Complement, $\overline{C1}$ and Intermediate Complexes

1. Whole Complement

PABPB : Inhibition of guinea pig and human whole complement was performed as described by Baker and Erickson (40) using sensitised sheep erythrocytes as target cells except that the diluent used was GBS++ pH 7.35 and ionic strength μ = o.15 containing 0.1% gelatin, 1×10^{-3}M $MgCl_2$ and 1.5×10^{-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 μ = 0.094 containing 0.1% gelatin,

1 x 10^{-3}M $MgCl_2$ and 3 x 10^{-4}M $CaCl_2$ was used as diluent and the target cells were guinea pig erythrocytes sensitized with bovine natural antibody. In nine 1 x 7.5 cm centrifuge tubes were placed 0.25 ml of 2.5% EA cells. To each tube was added 50 ul of MeOEtOH plus or minus inhibitor. The final concentration of inhibitor in tubes 2, 5 and 8 was 1 mM while in tubes 3, 6 and 9 was 0.5 mM. To tubes 1, 2 and 3 was added 0.2 ml of 1:50 guinea pig complement; to tubes 4, 5 and 6 was added 0.2 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°C for 15 minutes, then lysis was stopped by addition of 1 ml EDTA-GBS. The cells were then centrifuged and the degree of haemolysis determined by measuring the O.D. at 541nm in a 1 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 $\overline{C1}$ from Bovine and Human Species

a. PABPB : The NaCl concentration in the stock solution of $\overline{C1}$ was lowered to 0.15M by dilution with equal volume of 0.01M phosphate buffer at pH 7.3 containing 1 x 10^{-3}M $MgCl_2$ and 1.5 x 10^{-4}M $CaCl_2$. An aliquot of 0.9 ml of the $\overline{C1}$ enzyme was treated with 0.1 ml of various concentrations of PABPB (2.5, 1.25, 0.25, 0.125 and 0.0625 mM) in MeOEtOH at 37°C for 30, 60 and 90 minutes periods. Controls were performed using 0.9 ml of $\overline{C1}$ enzyme and 0.1 ml of either MeOEtOH or phosphate buffered saline at pH 7.3 and the ionic strength μ = 0.15. The $\overline{C1}$ enzyme activity in 50 µl aliquots of each sample was determined spectrophotometrically using N-Z-L-Tyr-p-Np as substrate.

Haemolytic assays employed bovine $EAC^{Bov}42$ cells and GBS-Sucrose++ (μ = 0.075) as the diluent. For each interval of incubation, several dilutions of $C\bar{1}$ were in 0.2 ml aliquot and to each dilution was added to 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 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 supernatent was read at 541nm in a 1 cm glass cuvette. The amount of $C\bar{1}$ required for 50% haemolysis was expressed as CH_{50} units.

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

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 5×10^{-3}M using GBS-Sucrose++ and methyl alcohol respectively as solvents. Only one concentration of inhibitor, namely: 1×10^{-3}M was used in the studies of their effects on active $C\bar{1}$ in haemolytic assays, while in esterase assays the final concentration of the inhibitors was 5×10^{-4}M. The methods employed were similar to those described in the PABPB studies.

3. Effect of Inhibitors in $EAC^{Bov}\overline{142}$ and $EAC^{Bov}42$ Intermediates

a. $EAC^{Bov}\overline{142}$: Stock solutions of the inhibitors, 1×10^{-2}M DFP in GBS-Sucrose++, 0.1M PMSF in isopropyl

alcohol, 5 x 10^{-3}M TLCK in GBS-Sucrose++ and 5 x 10^{-3}M TPCK in methanol were diluted to either or both 10^{-3} or 10^{-4}M in GBS-Sucrose++. $EAC^{Bov}\overline{142}$ 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 curvette. Controls using solvents minus the inhibitors were performed at the same time.

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

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

Bovine $\overline{CI}$ was diluted 1:20 with GBS-Sucrose++. Equal volumes of 1% $EAC^{Bov}42$ cells and diluted bovine $\overline{CI}$ 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 CI was incubated at the same time. Graph of O.D. against time of incubation were plotted.

G. Effect of Active CI on $EAC^{Bov}42$ previously treated with DFP-inactivated C1

Bovine CI (0.9 ml 2,000 CH_{50} units/ml) was incubated with DFP (0.1 ml of 2 $x10^{-3}$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% $EAC^{Bov}42$ 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 o.2 ml of active bovine CI (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 412nm.

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

A control experiment was performed using bovine CI in the absence of inhibitor.

H. Stability of $EAC^{Bov}\overline{142}$ 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, 1×10^{-3}M $MgCl_2$ and 1.5×10^{-4}M $CaCl_2$ from GBS++ and 9% sucrose containing 0.1% gelatin, 1×10^{-3}M $MgCl_2$ and 1.5×10^{-4} $CaCl_2$.

μ Ionic Strength	Volumes of 9% Sucrose++	Volumes of GBS++
0.150	----	10
0.135	1	9
0.120	2	8
0.105	3	7
0.090	4	6
0.075	5	5
0.060	6	4

$EAC^{Bov}\overline{142}$ 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 C-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 412nm.

I. Stability of $EAC^{Bov}\overline{142}$ in EDTA-GBS-Sucrose and EDTA-GBS

Different concentrations of EDTA in GBS and GBS-Sucrose solutions were prepared. The intermediate, $EAC^{Bov}\overline{142}$, 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-H^3-DFP by EA, $EAC^{Bov}\overline{142}$ and $EAC^{Bov}42$ cells

A reaction mixture containing 0.675 ml of bovine $\overline{CI}$ (14,400 CH_{50} units), 0.675 ml of GBS++, 0.075 ml of H^3-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 $\overline{CI}$ was completely inactivated with respect to its capacity to form $EAC^{Bov}\overline{142}$ from $EAC^{Bov}42$. 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-H^3-DFP was diluted with an equal volume of ice cold 9% sucrose solution containing 1 x 10^{-3}M $MgCl_2$ and 1.5 x 10^{-4}M $CaCl_2$. In three 1 x 7.5 cm centrifuged tubes were placed 0.4 ml of Cl-H^3-DFP. To tube 1 was added 0.4 ml 5% EA cells; to tube 2 was added 0.4 ml 5% $EAC^{Bov}42$; and to tube 3 was added 0.4 ml 5% $EAC^{Bov}\overline{142}$ cells. Two control tubes were performed: to tubes 4 and 5 were added 0.4 ml GBS-Sucrose++ and then equal volume of Cl-H^3-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% $EAC^{Bov}42$ and 5% $EAC^{Bov}\overline{142}$ 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 PPO and 60 mgm/liter of POPOP with methyl cellusolve (MeOEtOH) in the proportion 1:0.7 by volume (58). Five counts of each sample were made. The counting effiniency of rach 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 $\overline{C1}$

Tamura and Nelson (44) found that at pH 7.5 and low ionic strength (μ = 0.03 - 0.04) guinea pig, human and canine C1 could be selectively precipitated from all the other serum complement components and the C1 inactivator. Thus C1 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 C1. 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 C1 thus obtained was capable of substituting for purified human $\overline{C1}$ in its capacity to form $EAC^{Bov}\overline{142}$ from EAC^{Bov}_{42}. Further neither the bovine C1 nor the R1 were capable of forming $EAC^{Bov}\overline{142}$ cells from EA cells. A typical preparation of C1 (50 ml) obtained by this method had an optical density at 280nm of 5.522 and containing 5,000 CH_{50} units/ml and 5.64×10^5 esterase units/ml.

As observed by other workers (44) during the preparation of C1, by precipitation at low ionic strength, it becomes fully activated. C1 may be converted to $\overline{C1}$ by warning it at 37°C for 15 minutes (59). Such treatment of the bovine C1 did not increase either its haemolytic or esterase activity. It had also been observed that though some human C1 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 $\overline{Cls}$.

Rabbit and guinea pig C1 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 C1 thus obtained was completely free of all other complement components and contained 1 - 2 times as much C1 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 C1 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 C1.

Five ml of a sample of twice-precipitated bovine $\overline{C1}$ which has activity of 908 CH_{50} unit/ml/O.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 alexinated cells.

The $\overline{C1}$ 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 $\overline{C1}$ was precipitated by dialysis at low ionic strength ($\mu = 0.03$) and pH 7.35. The $\overline{C1}$

PURIFICATION OF BOVINE $\overline{CI}$ BY SEPHADEX G-200

Legend

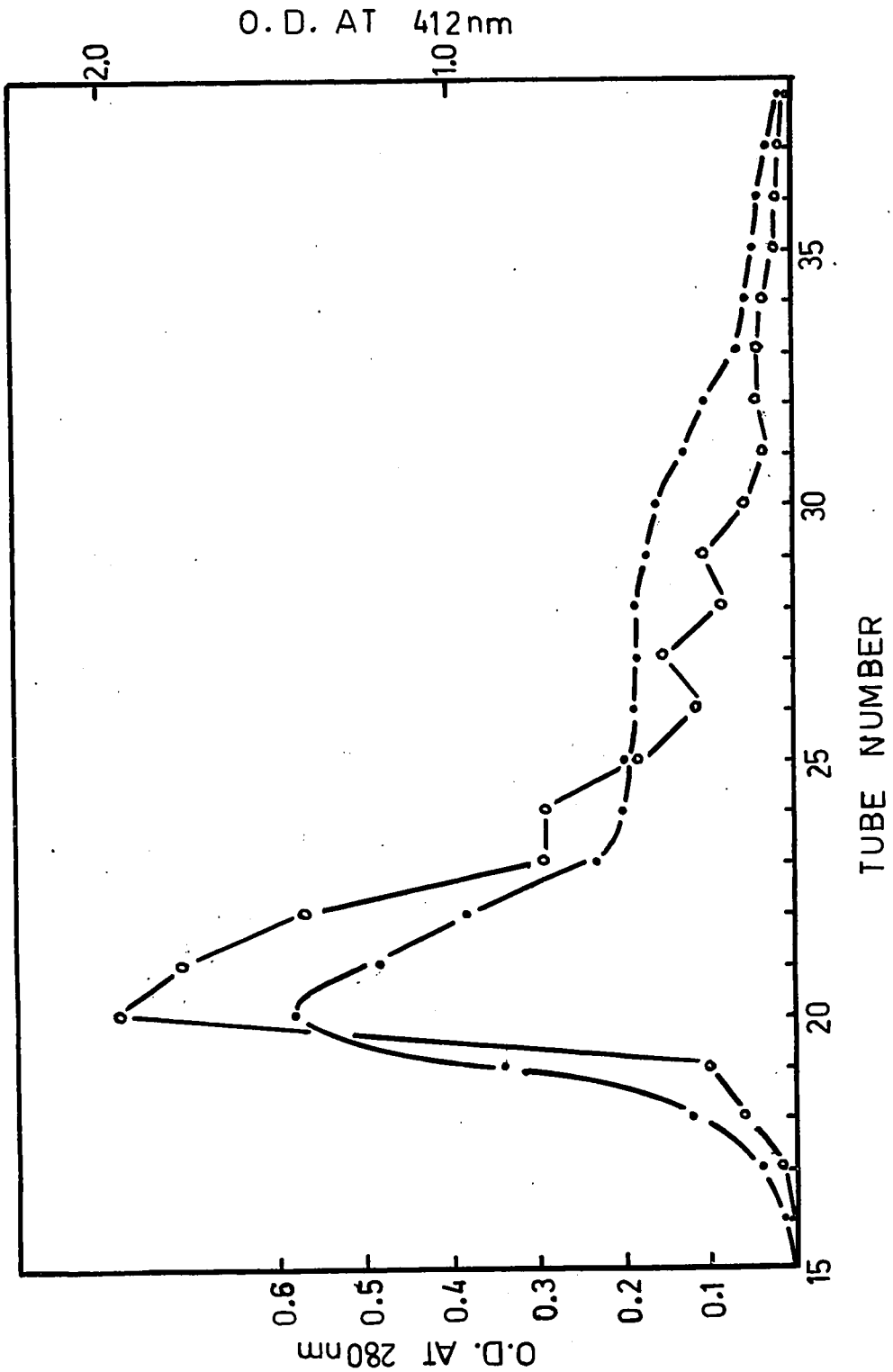
. . . . . Protein content of each fraction measured in terms of O.D. at 280nm

o o o o o Bovine $\overline{CI}$ 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 1×10^{-3}M $MgCl_2$ and 1.5×10^{-4}M $CaCl_2$. The resulting solution was diluted to 1/15 with GBS-Sucrose++ (i.e. the final dilution was 1/30). The haemolytic function of $\overline{CI}$ was assayed by its capacity to convert EAC^{Bov}_{42} cells to the lytic intermediate $EAC^{Bov}_{\overline{142}}$ which could then be lysed by C-EDTA.

FIGURE IV

CHROMATOGRAPHY OF BOVINE CI ON SEPHADEX G-200



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 $\overline{CI}$ could be dissolved. Titration of $\overline{CI}$ 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/O.D. unit at 280nm the chromatographed $\overline{CI}$ had 1310 CH_{50} units/ml/O.D. unit at 280nm. This represents a 1.45 fold purification. It is difficult to estimate the yield with respect to whole serum because Cl can not accurately be titrated in whole serum. However the $\overline{CI}$ 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 $\overline{CI}$ could be achieved either by sucrose gradiant or zonal centrifugation procedure (53) or by affinity chromatography on γ-globulin linked to Sepharose (54).

B. <u>The Stability of $EAC^{Bov}\overline{142}$ in varying Ionic Strength Buffers</u>

The specific interaction between $\overline{CI}$ and the antibody portion of immune complexes is more stable at low ionic strength than at physicological ionic strength (55). Fong <u>et al</u> found that $EAC^{Bov}\overline{142}$ 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). Im this laboratory it was not possible corroborate this. The lytic intermediate, $EAC^{Bov}\overline{142}$ 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^{Bov}142 IN VARIOUS IONIC STRENGTH BUFFERS

Ionic Strength	O.D. at 412nm after 30 min. Incubation at 37°C				
	Time of Incubation in Minutes				
	5	10	15	30	60
0.150	2.580	2.550	2.571	2.553	2.583
0.135	2.579	2.538	2.540	2.548	2.586
0.120	2.544	2.585	2.571	2.671	2.587
0.105	2.548	2.532	2.534	2.564	2.541
0.090	2.532	2.534	2.556	2.535	2.540
0.075	2.500	2.500	2.500	2.490	2.509
0.060	2.550	2.560	2.553	2.584	2.570

Table II shows that $EAC^{Bov}\overline{142}$ 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 $EAC^{Bov}\overline{142}$ cells in various concentrations of EDTA in either GBS-Sucrose or GBS

Becker (48) has shown that guinea pig C1 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 $EAC^{Bov}\overline{142}$ 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 $EAC^{Bov}\overline{142}$ 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 $EAC^{Bov}\overline{142}$ cells and this decay was greater in the higher ionic strength buffer. The decay of $EAC^{Bov}\overline{142}$ 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 $EAC^{Bov}\overline{142}$ at $\mu = 0.15$ and 0.01M EDTA resulted in the detachment of C1 from the complex. Since C1 is required for the action of $EAC^{Bov}\overline{142}$ as a lytic intermediate (16,17) then it is highly

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

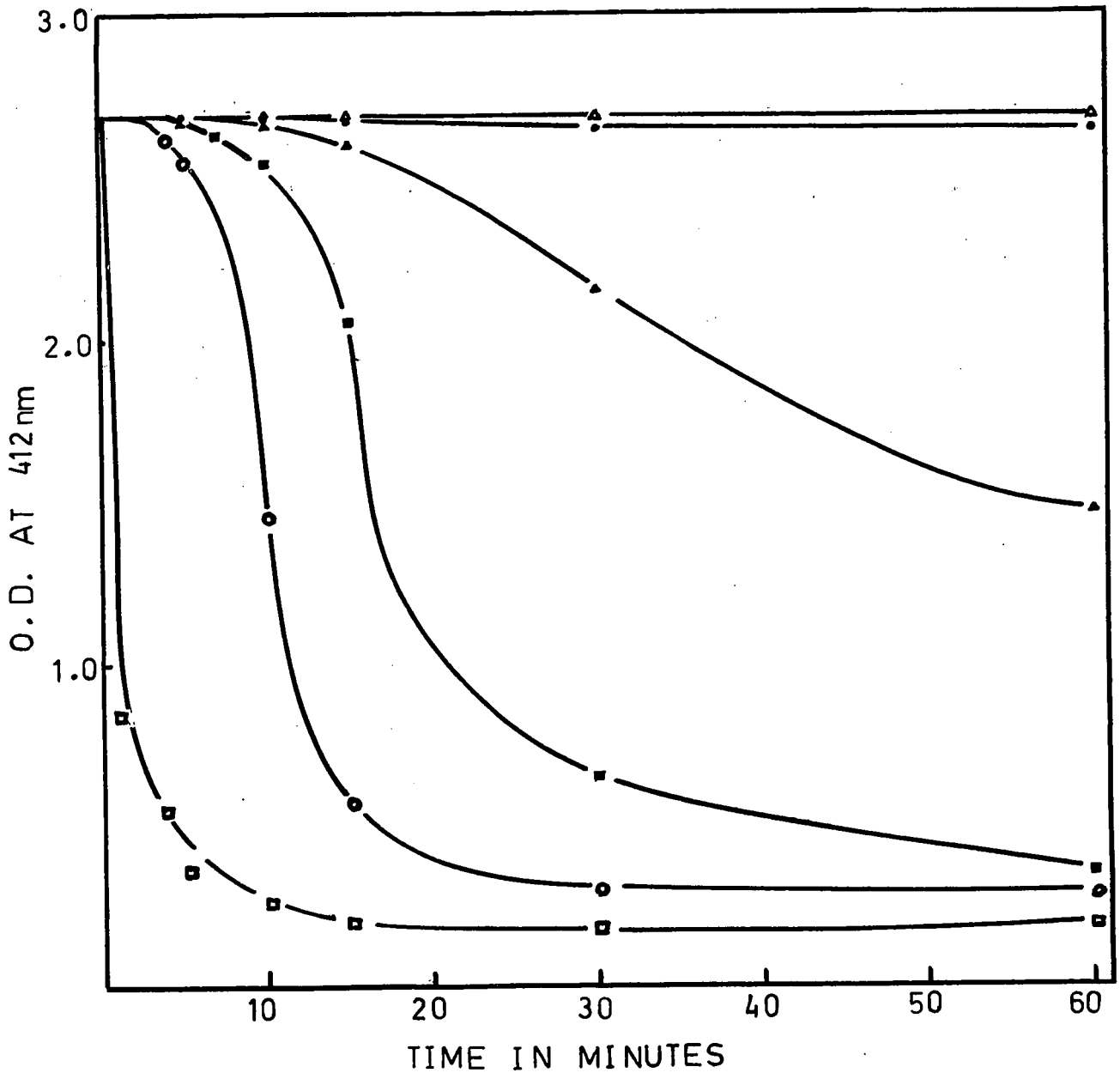
Legend

One per cent $EAC^{Bov}\overline{142}$ cells were incubated with different concentrations of EDTA in GBS-Sucrose at 37°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 C-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.

▲ ▲ ▲ ▲	GBS-Sucrose
• • • •	0.001M EDTA-GBS-Sucrose
▲ ▲ ▲ ▲	0.0025M EDTA-GBS-Sucrose
■ ■ ■ ■	0.005M EDTA-GBS-Sucrose
o o o o	0.010M EDTA-GBS-Sucrose
□ □ □ □	0.050M EDTA-GBS-Sucrose

FIGURE V

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



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

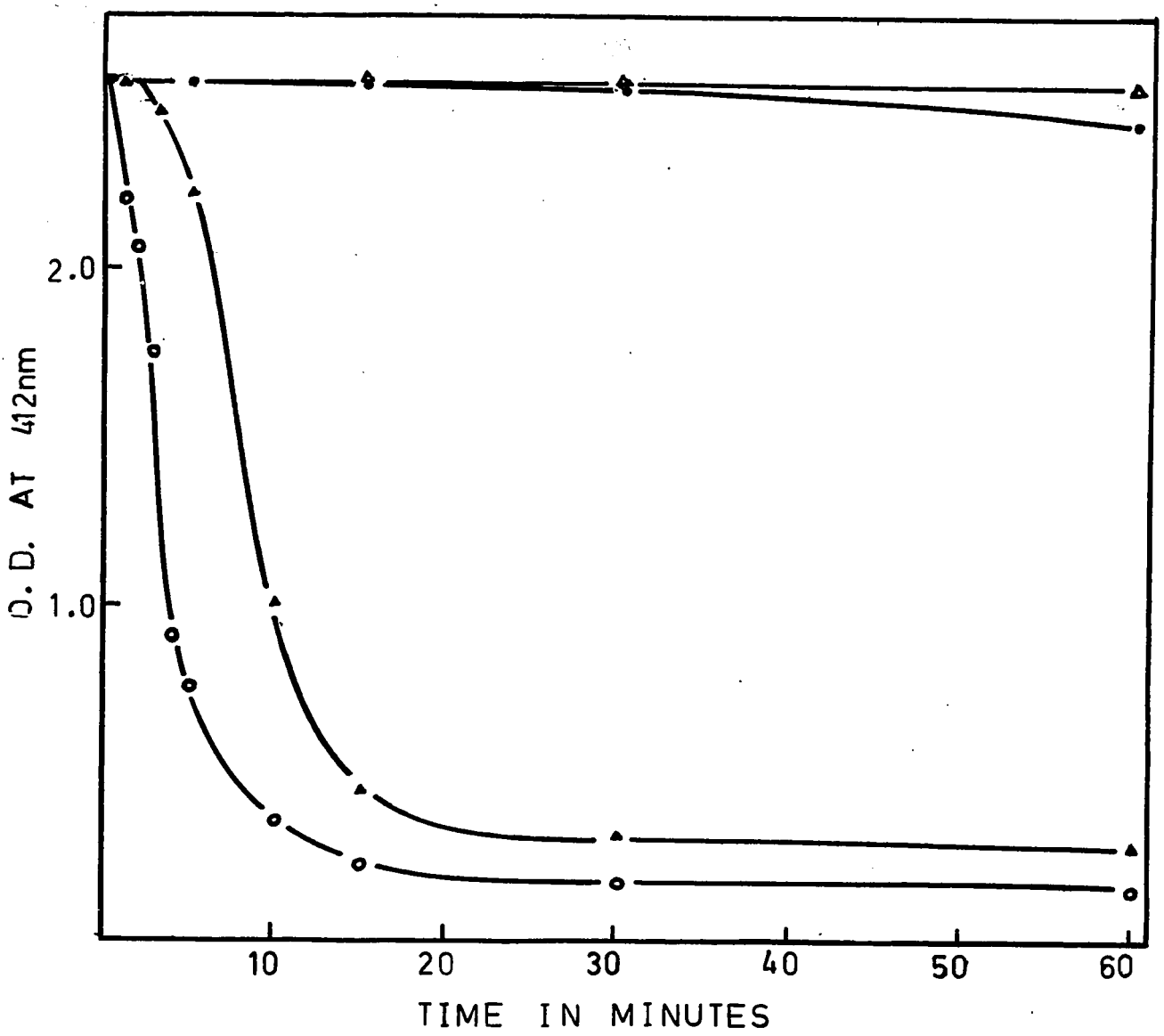
Legend

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

△ △ △	GBS
• • •	0.001M EDTA-GBS
▲ ▲ ▲	0.0025M EDTA-GBS
o o o	0.005M EDTA-GBS

FIGURE VI

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



probable that the $\overline{C1}$ interacts in some way with C4 and /or C2. In buffers of ionic strength, $\mu = 0.075$, which favour the formation of $EAC^{Bov}\overline{142}$ there is possibly a unique and rather firm association between the antibody molecule, C1, C4 and /or C2/. On raising the ionic strength to $\mu = 0.15$ then perhaps the association of $\overline{C1}$ with C4 and /or C2 is sufficient to maintain an active complex even though the association between $\overline{C1}$ and the antibody is weakened. Treatment with EDTA in sufficient concentration to affect the integrity of C1 probably affects the interaction of C1 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 C1 in the $EAC^{Bov}\overline{142}$ complex allowing its rapid detachment and decay of the intermediate.

D. Effect of Inhibitors on the Esterase and Haemolytic Functions of Bovine $\overline{C1}$

Since $\overline{C1}$ is required by $EAC^{Bov}\overline{142}$ complex for its action on later components, it is possible to ask the following questions.

1. Is the active site of $\overline{C1}$ important for its function in the $EAC^{Bov}\overline{142}$ complex ?
2. Does $\overline{C1}$ 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 $\overline{C1}$ have esterase enzyme activities and both tryptic and chymotryptic substrate specificities can be demonstrated (25). It is expected that bovine $\overline{C1}$ would have similar properties.

Bovine $\overline{C1}$ is capable of hydrolysing N-Z-L-Tyr-p-Np in a manner similar to that described by Bing (25) for human $\overline{C1}$ 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 1×10^{-3}M DFP is quite potent and after 60 minutes at 37°C it inhibits almost completely the esterase activity of 4.26×10^5 units of enzyme/ml of bovine CI and of 1.515×10^6 units of enzyme/ml of human CI. The inactivation of human and guinea lig 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) specificially inactivates trypsin and TPCK (tosyl-phenyl-alanine-chloromethyl ketone) is specific for chymotrypsin. The effect of these inhibitors on the esterase action of bovine and human CI is seem in Table III.

At a final concentration of 5×10^{-4}M TLCK is observed to inhibit the bovine CI enzyme (6.84×10^5 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 x 10^{-6} mM of p-nitrophenol in 5 min. at 25°C from 3 x 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 III

THE EFFECT OF DFP ON THE ENZYMATIC ACTIVITY OF BOVINE AND HUMAN CI

	Final Concn. of DFP mM	Time of Incubation (min.) 15 Unit Enzyme per ml x 10^{-5}	15 % Inhib.	30 Unit Enzyme per ml x 10^{-5}	30 % Inhib.	60 Unit Enzyme per ml x 10^{-5}	60 % Inhib.
Bovine	0.1	2.60	38.0	1.92	55.0	1.2	71.8
	0.5	0.097	76.8	0.86	79.8	0.44	89.7
	1.0	0.082	80.5	0.67	84.2	0.22	94.7
	Saline	4.20	0	4.26	0	4.26	0
Human	0.1	12.1	20.0	11.0	27.6	9.0	40.5
	0.5	0.87	42.5	0.48	68.1	0.22	85.5
	1.0	0.57	61.1	0.17	88.4	0.10	93.4
	Saline	15.2	0	15.2	0	15.1	0

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

Legend

Inhibitors	Solvents
PMSF	Isopropyl Alcohol
TPCK	Methyl Alcohol
TLCK	Isopropyl Alcohol

One unit of enzyme is defined as the amount of protein which releases 1 x 10^{-6}mM of p-nitrophenol in 5 min. at 25°C from 3 x 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

Solvent/ Inhibitor 5×10^{-4}M	Time of Incubation (min.) 30		60	
	Unit Enzyme per ml $\times 10^{-5}$	% Inhibition	Unit Enzyme per ml $\times 10^{-5}$	% Inhibition
Bovine				
TLCK	5.70	16.6	6.45	14.0
$(CH_3)_2CH_2OH$	6.00	12.5	6.83	8.6
TPCK	6.70	2.0	7.38	3.0
PMSF	3.49	51.0	3.56	52.5
MeOH	6.36	7.0	7.30	2.5
Saline	6.84	0.0	7.50	0.0
Human				
TLCK	5.67	3.0	5.22	15.0
$(CH_3)_2CH_2OH$	5.67	3.0	5.40	12.0
TPCK	5.85	0	6.12	0
PMSF	2.92	50.0	3.06	50.0
MeOH	5.67	3.0	5.97	2.5
Saline	5.85	0	6.12	0

of 5 x 10^{-4}M was observed after 60 minutes at 37°C. Similarly it was seen that neither TPCK nore TLCK at final concentrations of 5 x 10^{-4}M were able to inhibit 5.85 x 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 $EAC^{Boc}\overline{I42}$ from $EAC^{Bov}42$ cells. Both DFP and PMSF are effective inhibitors. At a final concentration of 1 x 10^{-3}M PMSF and after 30 minutes of incubation at 37°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°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 $EAC^{Bov}\overline{I42}$ complex. Neither TLCK nor TPCK at a final concentration of 1 x 10^{-3}M were effective in inhibiting the haemolytic activity of CI. The per cent of inhibition by TPCK at 1 x 10^{-3}M after 60 minutes incubation at 37°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°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 $\overline{C1}$

Legend

Inhibition of $\overline{C1}$ 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 $\overline{C1}$

Final DFP Concn. mM	Time of Incubation (min.) 15		30		60	
	CH_{50}	% Inhib.	CH_{50}	% Inhib.	CH_{50}	% Inhib.
Bovine						
0.1	1820	56.4	955	83.1	10	~100
0.5	10	~100	0	100	0	100
1.0	0	100	0	100	0	100
Saline	4160	0	5620	0	5620	0
Human						
0.1	---	---	4050	19	1350	73.0
0.5	---	---	850	83	0	100
Saline	---	---	5000	0	5000	0

THE EFFECT OF TLCK, TPCK AND PMSF ON THE HAEMOLYTIC ACTIVITY ON BOVINE $\overline{C1}$

Legend

Inhibitors	Solvents
PMSF	Isopropyl Alcohol
TPCK	Methyl Alcohol
TLCK	Saline

Inhibition of $\overline{C1}$ by inhibitors 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 VI

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

Solvent/ Inhibitor 1×10^{-3}M	Time of Incubation (min)			
	30		60	
	CH_{50}	% Inhibition	CH_{50}	% Inhibition
TLCK	4475	0.0	4475	0.0
TPCK	2330	20.0	1025	43.6
PMSF	0	100.0	---	---
$(CH_3)_2CH_2OH$	4475	0.0	4475	0.0
MeOH	3300	13.5	2330	20.0
Saline	4475	0.0	4475	0.0

of $\overline{C1}$ by acting somewhere else on the molecule.

Since inactivation of the $\overline{C1}$ esterase site makes it incapable of forming an active $EAC^{Bov}\overline{142}$ complex, it might be concluded that either directly or indirectly the esterase site or its intermediate vicinity are important in the function of $\overline{C1}$ in $EAC^{Bov}\overline{142}$. Inactivation of haemolysis appears greater than the inactivation of the esterase. Since the current composition of human C1 has been suggested to be composed of C1q : C1r : C1s in the molecular ratio of 1 : 2 : 4 (51). The mutiple C1s 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 C1 from forming an active lytic intermediate.

E. Effect of Inhibitors on $EAC^{Bov}42$ cells

When the inactivated C1 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 C1 with $EAC^{Bov}42$. It is possible that the inhibitor may have an effect on bound C4 and/or C2. A 1% suspension of $EAC^{Bov}42$ cells was made in GBS-Sucrose++ containing DFP at final concentrations of 1×10^{-3}M or 1×10^{-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 $\overline{C1}$ in the usual manner. The results in Table VII shows that DFP over the concentration range used to inhibit $\overline{C1}$ was not capable of significantly affecting bound C4 or C2 by impairing the capacity of $EAC^{Bov}42$ to form $EAC^{Bov}\overline{142}$.

EFFECT OF DFP ON $EAC^{Bov}42$ CELLS

Legend

$EAC^{Bov}42$ 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 $EAC^{Bov}42$ CELLS

DFP Concn.	O.D. at 412nm after 30 min. at 37°C					
	Dilution of Bovine $\overline{CI}$					
	1/3	1/9	1/27	1/81	1/243	1/729
-----	2.166	2.619	2.609	2.613	2.601	2.413
1×10^{-4}M	2.235	2.564	2.630	2.604	2.622	2.389
1×10^{-3}M	2.540	2.589	2.589	2.506	2.590	2.375

F. Effect of Inhibitors on $EAC^{Bov}\overline{142}$ cells

It has been shown that several inhibitors inactivate the esterase activity of bovine and human $\overline{C1}$ and that such inactivation prevents the $\overline{C1}$ from forming a lytic intermediate with $EAC^{Bov}42$. Various inhibitors were used to see if they could abolish the activity of $\overline{C1}$ when it was bound in an essential way in the $EAC^{Bov}\overline{142}$ complex. TLCK and TPCK, although they do not inhibit the esterase and haemolytic aetivities of free $\overline{C1}$, were tried since the bound $\overline{C1}$ may be in a different conformation from the unbound form and have an exposed essential histidine susceptable 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 $EAC^{Bov}\overline{142}$ cells were incubated in the inhibitors at concentrations which were effective on free $\overline{C1}$ 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 $\overline{C1}$ is not involved in the activity of $EAC^{Bov}\overline{142}$ complex

b. or, if it is involved it is masked by the interaction between $\overline{C1}$ and C4 and/or C2.

EFFECT OF DFP, PMSF, TLCK AND TPCK ON $EAC^{Bov}1,4,2$ CELLS

Legend

Inhibitors	Solvents
DFP	GBS-Sucrose++
PMSF	Isopropyl Alcohol
TLCK	GBS-Sucrose++
TPCK	Methyl Alcohol

O.D. values were corrected for any small amount of lysis occurring in the absence of C-EDTA.

TABLE VIII

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

Inhibitors or Solvents	O.D. at 412nm after 30 min at 37°C				
	Time of Incubation (min.)				
	5	10	15	30	60
DFP (1×10^{-4}M)	2.515	2.517	2.530	2.500	2.470
DFP (1×10^{-3}M)	2.503	2.525	2.512	2.507	2.460
PMSF (1×10^{-4}M)	2.502	2.490	2.510	2.500	2.480
PMSF (1×10^{-3}M)	2.492	2.500	2.497	2.494	2.510
TLCK (1×10^{-3}M)	2.495	2.483	2.457	2.512	2.474
TPCK (1×10^{-3}M)	2.512	2.523	2.453	2.446	2.481
$(CH_3)_2CH_2OH$	2.455	2.480	2.447	2.464	2.455
CH_3OH	2.475	2.482	2.514	2.481	2.510
GBS-Sucrose++	2.552	2.533	2.504	2.491	2.491

G. Uptake by $EAC^{Bov}42$ of Bovine $\overline{CI}$ and Bovine CI inactivated by DFP

Figure VII shows the uptake of bovine $\overline{CI}$ (2,000 CH_{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 $EAC^{Bov}\overline{I42}$ sites on the alexinated cells to cause their lysis in C-EDTA.

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

Both inactivated samples of the C1 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 C1 and excess DFP and finally resuspended to 1% in GBS-Sucrose++. The cells were treated with an equal volume of active $\overline{CI}$ (2,000 CH_{50} units/ml) diluted to 1/20 with GBS-Sucrose++ and after 20 minutes at 37°C (condition of maximum effective uptake of $\overline{CI}$). C-EDTA was added and the resulting lysis determined spectrophotometrically.

Table IX shows the uptake by $EAC^{Bov}42$ of the partially inactivated C1 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 $\overline{CI}$ (2000 CH_{50} UNITS/ml) UPTAKE BY $EAC^{Bov}{}_{42}$ CELLS

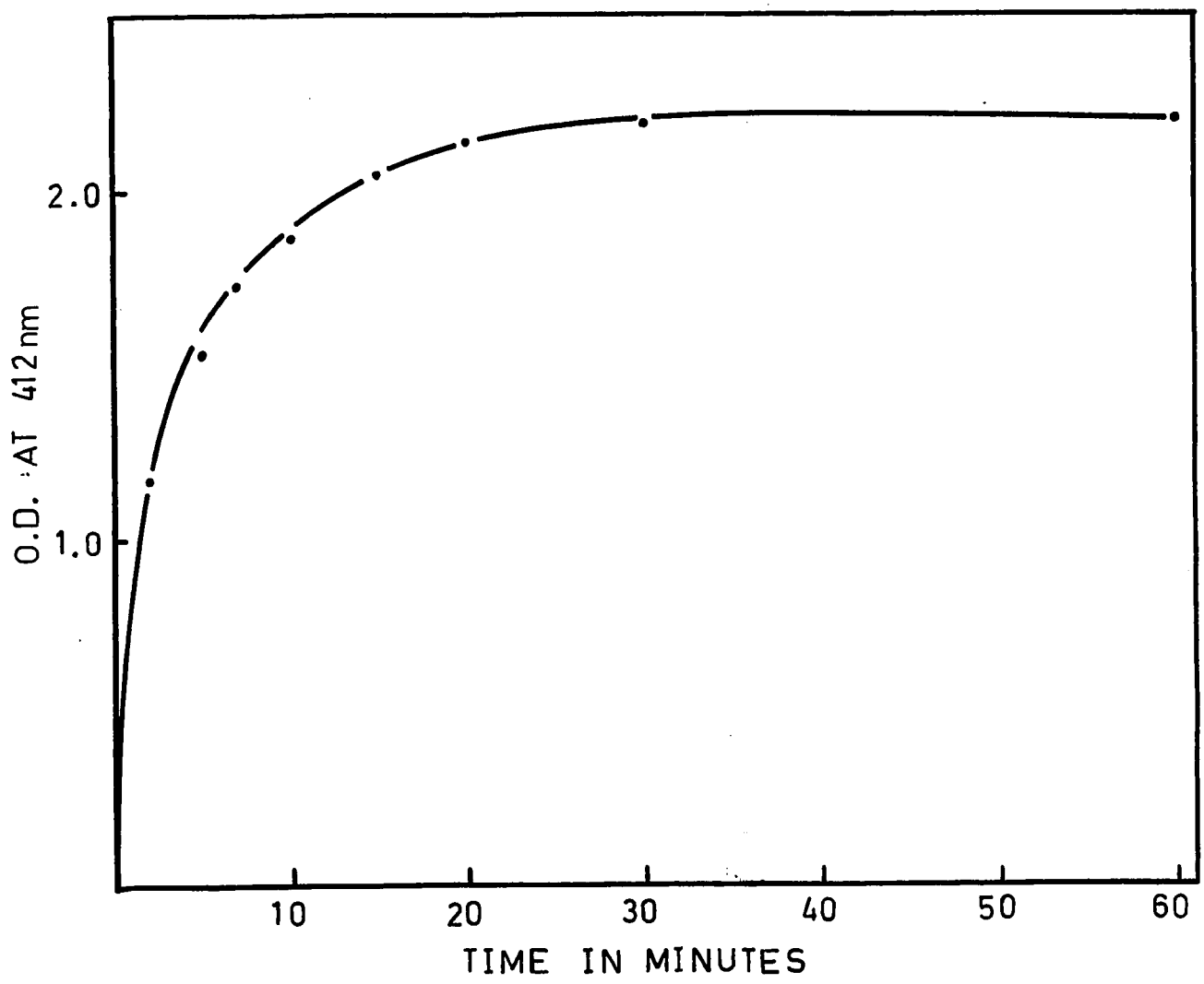
Legend

Bovine $\overline{CI}$ (2000 CH_{50} units/ml) was diluted 1:20 with GBS-Sucrose++. Equal volumes of 1% $EAC^{Bov}{}_{42}$ cells and diluted $\overline{CI}$ were incubated at $37^{o}C$ 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 $\overline{CI}$.

FIGURE VII

BOVINE $\overline{C1}$ (2000 CH_{50} UNITS/ml) UPTAKE BY $EAC^{Bov}42$ CELLS



EFFECT OF TREATING $EAC^{Bov}42$ FIRST WITH C1-DFP AND THEN WITH $\overline{CI}$

Legend

A. Uptake of partially DFP (at a final concentration of 2×10^{-4}M, incubated for 15 minutes at 37°C) inactivated bovine C1 (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 $\overline{CI}$ (/120 dilution) for 20 minutes at 37°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 5×10^{-4}M. and incubated at 37°C for 30 minutes.

D. As in (B) using cells from (C).

TBALE IX

EFFECT OF TREATING $EAC^{Bov}42$ FIRST WITH Cl-DFP AND THEN WITH CI

	O.D. at 412nm after Incubation with C-EDTA for 30 minutes at 37°C							
	Time of Incubation of EAC42 cells with Cl-DFP (in minutes)							
	2	5	7	10	15	20	30	60
A	0.296	0.515	0.569	0.602	1.057	1.429	1.313	1.200
B	2.254	2.376	2.198	2.101	2.236	2.300	2.407	2.400
C	0.271	0.261	0.323	0.290	0.305	0.329	0.341	0.431
D	2.433	2.451	2.475	2.447	2.464	2.538	2.447	2.487

similar to that observed for active $\overline{Cl}$ (Fig. VII).

The treatment of $EAC^{Bov}42$ cells with either partially or totally inactive Cl does not appear to inhibit the capacity of them to ineract with active $\overline{Cl}$ and form a lytic intermediate (Table IX). It appears from this that the DFP-inactivated Cl is not taken up by the cells alternatively it may be taken up weakly and is capable of exchanging with the active Cl 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 Cl and on offering active $\overline{Cl}$ 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 $\overline{Cl}$-^{3}H-DFP by EA, $EAC^{Bov}\overline{1}42$ and $EAC^{Bov}42$ cells

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

Table X shows the relative uptake of $\overline{Cl}$-^{3}H-DFP by 1×10^8 cells of EA, EAC42 and $EAC\overline{1}42$.

The EA intermediate was used to form the $EAC\overline{1}42$ 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 $EAC^{Bov}42$ CELLS

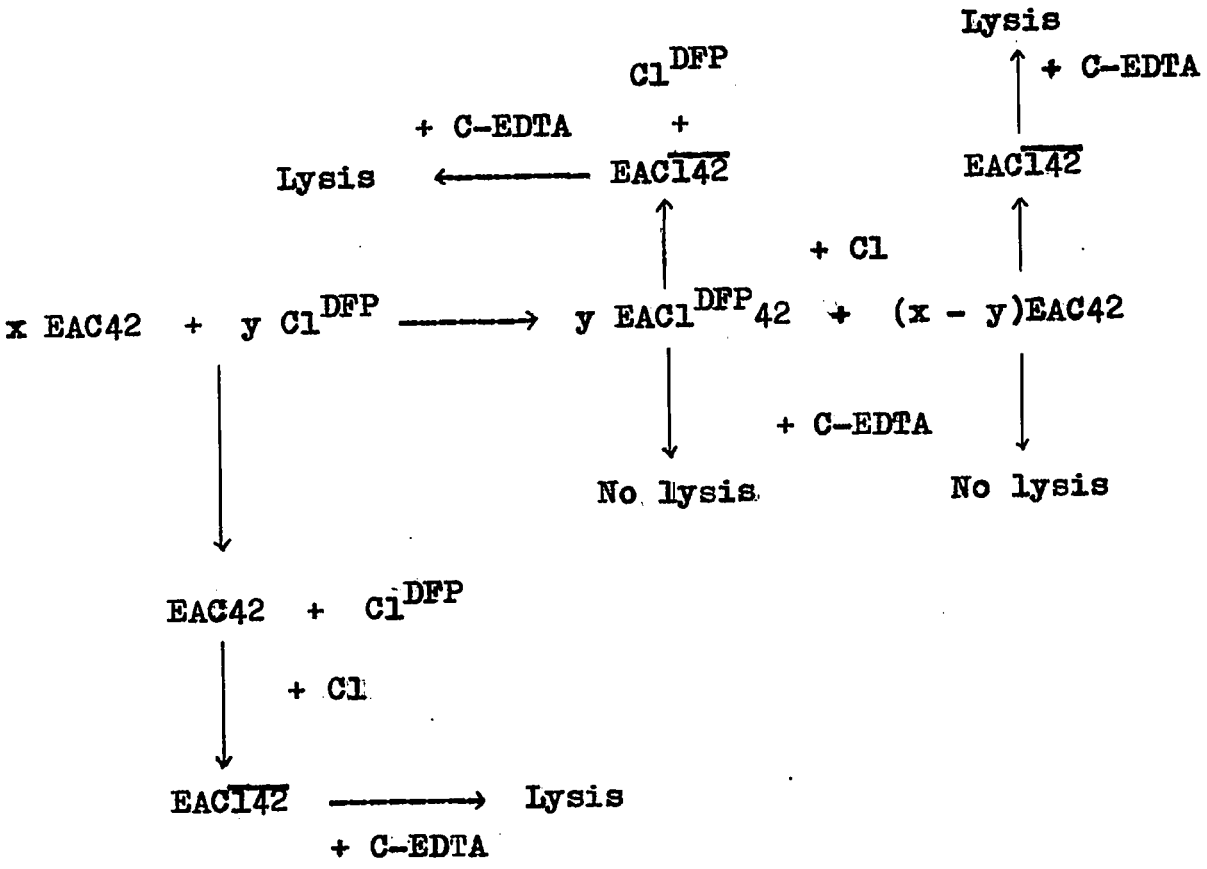


TABLE X

THE UPTAKE OF ^{3}H-DFP INACTIVATED C1 BY EA, $EAC^{Bov}42$, AND $EAC^{Bov}\overline{142}$ CELLS[a]

Activity of Cl-H^{3}-DFP offered to cells = 8638 d.p.m.[b]

Cell Suspension	Actual Cell Concn. in 0.4 ml of 5 % cells[c]	Activity taken up by 0.4 ml of cells[d] d.p.m.	Activity taken up by 1 x 10^{8} cells d.p.m.	Relative uptake Activity[e]
EA	1.04 x 10^{8}	2827	2718	100%
EAC42	1.10 x 10^{8}	2683	2438	89.7%
$EAC\overline{142}$	1.16 x 10^{8}	2461	2121	78.0%

a. All activities are corrected for back ground counting and represent an average of 5 independing 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.

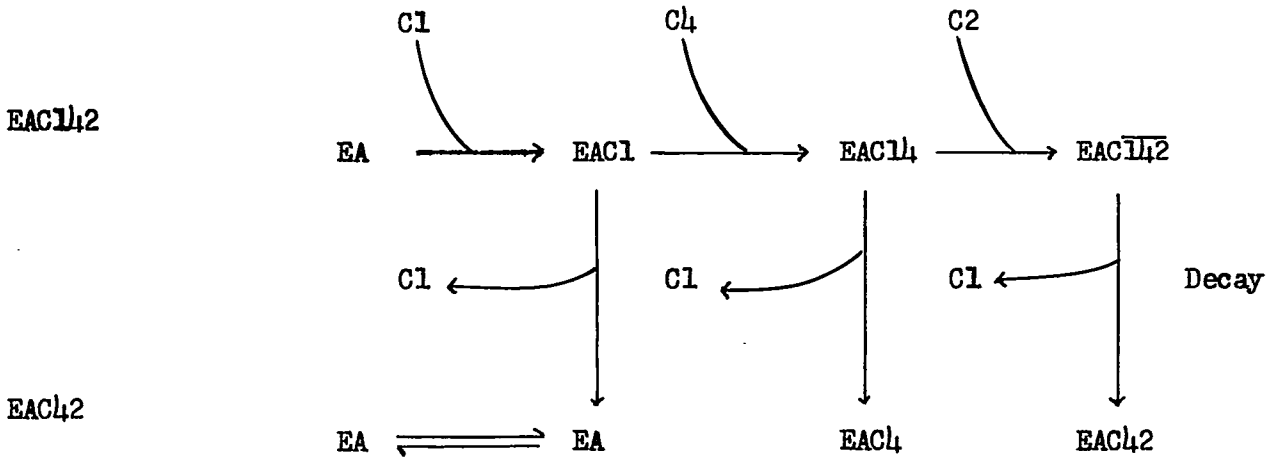
e. The relative uptake is calculated assuming that the uptake by EA is 100% i.e. it represents the possible maximum amount of C1 uptake.

surface represents a mosaic of antigenic determinants and therefore each sensitized cell probably has many C1 fixing sites. In a preparation of $EAC^{Bov}\overline{142}$ 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 C1, C4 and C2 (see Figure VIII).

Removal of C1 from the $EAC^{Bov}\overline{142}$ cell preparation results in the general of potential sites for the uptake of C1-^{3}H-DFP i.e. EA, $EAC^{Bov}4$ and $EAC^{Bov}42$ (Figure IX). Therefore the interpretation of the results is made difficult by the probable heterogeneity of the $EAC^{Bov}\overline{142}$ and $EAC^{Bov}42$ preparations. The uptake of C1-^{3}H-DFP by $EAC^{Bov}\overline{142}$ 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 C1 from the $EAC^{Bov}\overline{142}$ results in the generation of potential sites for the fixation of C1-^{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 $EAC^{Bov}\overline{142}$ cells, Whether this is due to the C1-^{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 C1-^{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 C1-^{3}H-DFP is not capable of binding with $EAC^{Bov}42$ and possibly EAC4 complexes. If this so then it appears that the esterase site of C1 is involved either directly or indirectly in its interaction with C4 and C2 in maintaining an active $EAC^{Bov}\overline{142}$ complex. Further more this interaction is sensitive to the binding of a relatively small molecule such as DFP to the esterase site.

FIGURE IX

HETEROGENEITY OF $EAC^{Bov}\overline{1,4,2}$ AND $EAC^{Bov}4,2$ CELL INTERMEDIATES



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 $\overline{C1}$ (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 $\overline{C1}$ (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

Complement	Target Cells	CH_{50} Unit
Guinea Pig	Sensitized Sheep RBC	700
Human	Sensitized Sheep RBC	150
Bovine	Sensitized Guinea Pig RBC	400

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 tagret 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 XII

THE INHIBITION OF WHOLE COMPLEMENT FROM GUINEA PIG, HUMAN AND BOVINE SPECIES BY PABPB

	Guinea Pig C			Human C			Bovine C		
Tube number	1	2	3	4	5	6	7	8	9
2.5% EA ml	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
MeOEtOH ml	0.05	-	-	0.05	-	-	0.05	-	-
1.0 mM PABPB in MeOEtOH ml	-	0.05	-	-	0.05	-	-	0.05	-
0.5 mM PABPB in MeOEtOH ml	-	-	0.05	-	-	0.05	-	-	0.05
1 : 50 Guinea Pig C ml	0.20	0.20	0.20	-	-	-	-	-	-
1 : 10 Human C ml	-	-	-	0.20	0.20	0.20	-	-	-
1 : 10 Bovine C ml	-	-	-	-	-	-	0.20	0.20	0.20
Final Concn. PABPB mM	0	0.10	0.05	0	0.10	0.05	0	0.10	0.05
O. D.	0.304	0.071	0.147	0.291	0.096	0.177	0.536	0.325	0.522
Percentage Inhibition	0	77	52	0	67	39	0	39	3

J. Effect of PABPB on Bovine $\overline{CI}$

It has been suggested that the major site of complement inactivation by PABPB and related compounds in the guinea pig system is the $\overline{CI}$ molecule. Baker and Cory (38) incubated PABPB and related compounds with guinea pig $\overline{CI}$ and then without removing the large excess of inhibitor, $\overline{R1}$ (all the other complement components except C1) 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 $\overline{CI}$, it may also have affect the activity of the later acting components present in the $\overline{R1}$.

The decay of the lytic $EAC^{Bov}\overline{142}$ cells to nonlytic $EAC^{Bov}42$ cells provides a stable intermediate for $\overline{CI}$ assays. Table XIII shows the effect of PABPB on the ability of bovine $\overline{CI}$ to form an active $EAC^{Bov}\overline{142}$ complex from $EAC^{Bov}42$ cells. PABPB at final concentrations ranging from 0.25 to 2.5 mM for 60 minutes at 37°C completely inhibit the haemolytic activity of 14400 CH_{50} units/ml of bovine $\overline{CI}$. 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°C. It was found that integrity of the $EAC^{Bov}42$ cells is unaffected by 0.308 mM PABPB or lower concentrations. When the effect of inhibitor on the haemolytic bovine $\overline{CI}$ is determined the $\overline{CI}$ and inhibitor are serially diluted so that the inhibitor concentration in contact with the $EAC^{Bov}42$ 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 $\overline{CI}$ TO FORM THE LYTIC INTERMEDIATE $EAC\overline{142}$ FROM EAC^{Bov}_{42}

Legend

Inhibition of $\overline{CI}$ 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 XIII

INHIBITION BY PABPB OF THE CAPACITY OF C1 TO FORM THE LYTIC INTERMEDIATE $EAC\overline{14}2$ FROM EAC42

Final PABPB Concentration mM	Time of Incubation: 30 min CH_{50} Unit/ml	30 min Percentage Inhibition	60 min CH_{50} Unit/ml	60 min Percentage Inhibition	90 min CH_{50} Unit/ml	90 min Percentage Inhibition
2.5	2750	81	0	100	0	100
1.25	7800	46	0	100	0	100
0.25	13155	9	155	99	0	100
0.125	13155	9	2660	82	1575	88
0.0625	12700	12	9200	35	4150	69
Saline	14400	0	14400	0	13600	0

adding C-EDTA and the inhibitor had no effect on the $EAC^{Bov}42$ cells, the effect of PABPB is presumed to be associated with the capacity of $\overline{CI}$ to react with the $EAC^{Bov}42$ to form the lytic intermediate. This result supports the hypothesis that a major site of complement inactivation by PABPB is the $\overline{CI}$ molecule (38).

Table XIV and Table XV show the effect of PABPB on the enzymatic activity of bovine and human $\overline{CI}$ with respect to the esterolytsis of N-Z-L-Tyr-p-Np. The results show that the enzymatic activities of both human and bovine $\overline{CI}$ (1.815×10^6 enzyme units/ml and 5.64×10^5 enzyme units/ml respectively) are not completely destroyed by 0.0625 to 2.5 mM final concentrations of PABPB even after 90 minutes at $37^{\circ}C$. Higher concentrations of inhibitor were not used because they resulted in extensive precipitation of the $\overline{CI}$. It seems that the esterase inhibition on human $\overline{CI}$ is greater than on bovine $\overline{CI}$. This result correlates with the greater inhibition in whole human complement than in bovine complement (Table XII). Like the DFP inhibitor, for the same amount of $\overline{CI}$ the PABPB inhibits the capacity to form $EAC^{Bov}\overline{142}$ 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 $\overline{CI}$ at sites other than those which can lead to complete enzymatic interaction and in doing so prevents $\overline{CI}$ from combining effectively with $EAC^{Bov}42$ to form a reactive $EAC^{Bov}\overline{142}$ 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 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

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 XIV

THE EFFECT OF PABPB ON THE ENZYMATIC ACTIVITY OF BOVINE C1

Final PABPB Concentration mM	Time of Incubation: 30 min — Unit Enzyme per ml x 10^{-5}	30 min — Percentage Inhibition	60 min — Unit Enzyme per ml x 10^{-5}	60 min — Percentage Inhibition	90 min — Unit Enzyme per ml x 10^{-5}	90 min — Percentage Inhibition
2.5	7.35	—	6.0	13.0	4.88	12.0
1.25	4.55	19.0	3.05	47.0	2.60	53.0
0.25	3.48	37.5	2.70	53.0	2.27	59.0
0.125	5.08	9.4	4.20	27.0	3.59	35.3
0.0625	5.39	4.0	5.40	6.0	4.94	11.0
MeOEtOH	5.62	—	5.75	—	5.64	—
Saline	5.62	—	5.75	—	5.64	—

TABLE XV

THE EFFECT OF PABPB ON THE ENZYMATIC ACTIVITY OF HUMAN C1

Final PABPB Concentration mM	Time of Incubation: 30 min		60 min		90 min	
	Unit Enzyme per ml x 10^{-5}	Percentage Inhibition	Unit Enzyme per ml x 10^{-5}	Percentage Inhibition	Unit Enzyme per ml x 10^{-5}	Percentage Inhibition
2.5	6.4	58.5	6.6	61.8	6.9	62.0
1.25	6.6	58.0	4.4	74.3	4.2	77.0
0.25	11.0	29.0	10.3	40.0	9.1	50.0
0.125	13.7	11.4	13.3	23.0	11.5	36.5
0.0625	14.4	7.2	15.4	11.7	15.4	15.0
MeOEtOH	15.0	4.3	16.4	5.8	17.9	1.5
Saline	15.5	—	17.2	—	18.2	—

and human $\overline{CI}$ more inhibition is obtained with 0.25 mM PABPB in bovine $\overline{CI}$ and 1.25 mM human $\overline{CI}$ than with higher concentrations of 1.25 and 2.5 mM respectively. This could result from multiple binding site on the $\overline{CI}$ 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 $\overline{CI}$ help to explain the inhibition of $\overline{CI}$ interaction with $EAC^{Bov}42$ 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, $EAC^{Bov}\overline{142}$, which may be lysed with C-EDTA. Removal of $\overline{C1}$ from $EAC^{Bov}\overline{142}$ 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 $\overline{C1}$ 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 affininity chromatography (53,54).

The assay procedures for bovine $\overline{C1}$ used here are based upon its esterase and haemolytic functions. The enzymatic activity is measured by the hydrolysis of N-Z-L-Tyr-p-Np in five minutes as described for human $\overline{C1}$ and $\overline{C1}$s by Bing (25). The decay of $EAC^{Bov}\overline{142}$ to $EAC^{Bov}42$ provides a stable intermediate for both human and bovine $\overline{C1}$ assays. Hence its haemolytic activity can be estimated by its capacity to reform lytic $EAC\overline{142}$ from non-lytic EAC42 cells. The effect of the inhibitors have been observed on these two activities.

The effect of various inhibitors, namely: DFP, PMSF and PABPB, on bovine $\overline{C1}$ esterase activity is similar to

the human $\overline{C1}$. The two histidine inhibitors, TLCK and TPCK, have no effect on bovine and human $\overline{C1}$. Though these two inhibitors have no effect, it is not possible to conclude that histidine is non-essential in either bovine and human $\overline{C1}$ 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 $EAC^{Bov}\overline{142}$ from $EAC^{Bov}42$. This is rather surprising because one expects that after activation of C4 and C2 the $\overline{C1}$ enzymatic activity is no longer required. Hence the involvement of the $\overline{C1}$ esterase active site may be direct i.e. it may be involved in the active $\overline{C142}$ enzyme or the $\overline{C1}$ 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 $\overline{C1}$ results in the loss of functional activity of $EAC^{Bov}\overline{142}$ cells.

The results on the study of the uptake of active $\overline{C1}$ prior treatment of cells with C1-DFP and the uptake of $C1-^{3}H$-DFP suggest that this molecule may not be taken by the $EAC^{Bov}42$ complex. From the results on the stability of $EAC^{Bov}\overline{142}$ in different ionic strength buffer, with or without EDTA, it may be suggested that $EAC^{Bov}\overline{142}$ has a compact structure in which $\overline{C1}$ forms a close association with the antibody, C4 and/or C2. Therefore it is possible that the $EAC^{Bov}\overline{142}$ complex is a compact structure such that even slight modification of $\overline{C1}$ by DFP is sufficient to prevent $\overline{C1}$ from reforming the lytic $EAC^{Bov}\overline{142}$ from $EAC^{Bov}42$ 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 $\overline{C1}$ component. Hence other inhibitors may be of greater use. Recently Bing reported (56) that m-(o-(2-chloro-5-fluorosulphonylphenylureido) phenoxybutoxy)-benzamidine (MCFPB) irreversibly inactivates purified human $\overline{C1}$ and dialysis of $\overline{C1}$ 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 $\overline{C1}$s subunit of the $\overline{C1}$ 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 $\overline{C1}$ with respect to esterase and haemolytic activities are comparable to those with DFP. The inhibitiom of $\overline{C1}$ interaction with EAC42 probably is enchanced by interaction of PABPB at sites on the $\overline{C1}$ molecule other tham the active site.

APPENDIX

I. Method for Determining CH_{50} Unit

This is an arbitary unit, since its magnitude depends on the concentration of red cells, the fragibity 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)

$$X = K \left(\frac{Y}{1 - Y} \right)^{1/n}$$ has been employed.

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

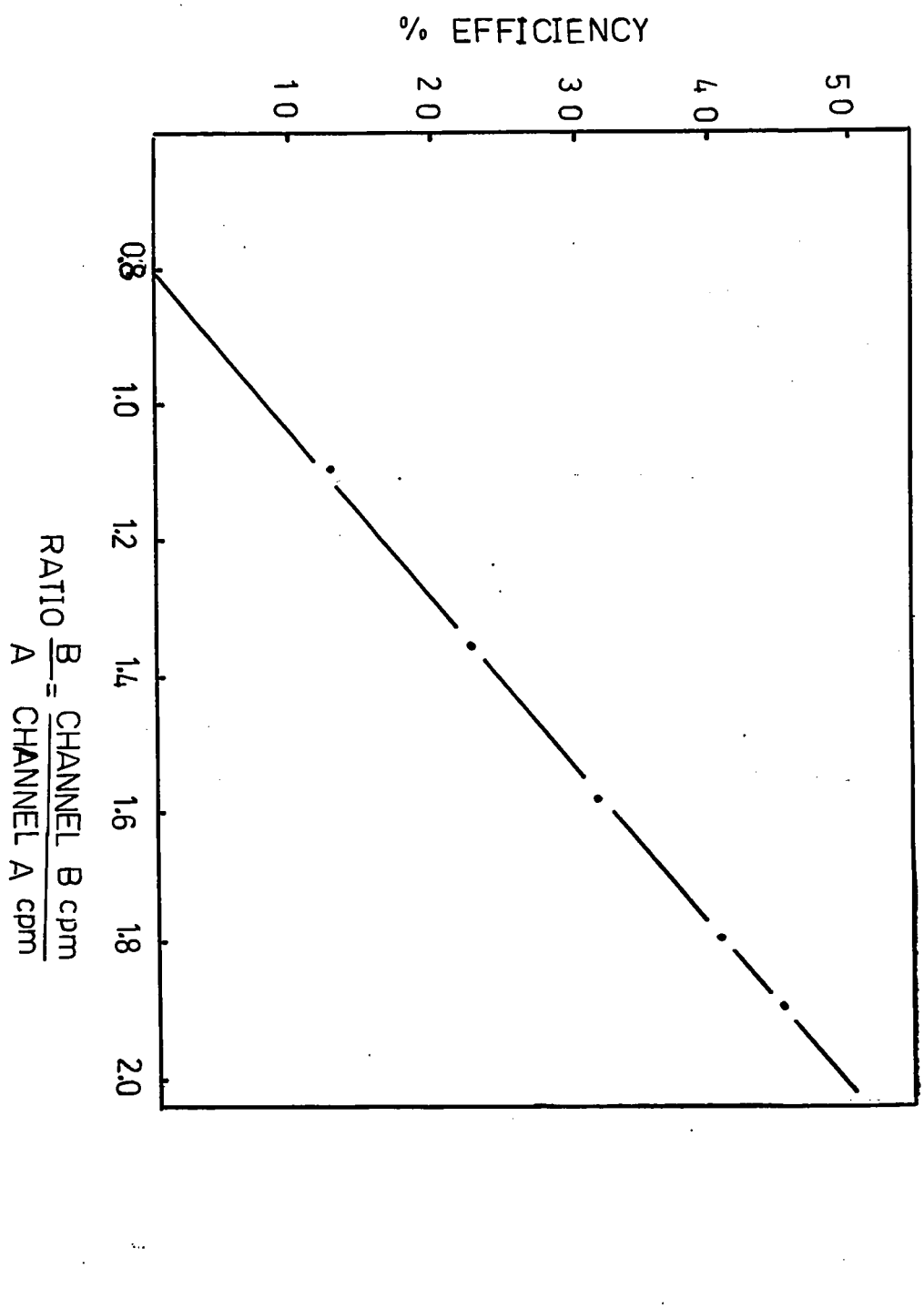
Y = degree of lysis (i.e. 100Y = % haemolysis)

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 $\pm$ 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.

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

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



BARIUM QUENCH CURVE

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 CCl_4 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.

BIBLIOGRAPHY

1. Muller-Eberhard, H.J., Annual Review Biochem., 38, 389(1969)
2. Rapp, H.J. and Borsos, T., J. Immunol., 91,826(1963)
3. Muir, R., J. Pathol. Bacteriol., 16,523(1911)
4. Shiegley,E.W. and Irwin, M.R., J. Immunol., 32, 281(1937)
5. Gigli, I. and Austin, K.F., Annual Review Micro., 25, 1573(1971)
6. Borsos, T. and Rapp, H.J., Immunochem., 3, 496(1966)
7. Grameritzki, M., Biochem. Z., 38, 501(1912)
8. Coulter, C.B., J. Gen. Physiol., 3, 771(1921)
9. Bulletin of the W.H.O., 39, 935(1968)
10. Lepow I.H., Naff, G.B. and Pensky, J., Ciba Found. Symp. Complement, 74,1965.
11. Borsos, T., and Mayer, M.M., "Immunopathology", (Grabar, P., and Miescher, P.A., eds.) Vol.II Basel, Benno Schwabe and Co., Pubs., 1961
12. Stolfi, R.L., J. Immunol., 100,46(1968)
13. Rice, C.E., Adv. Vet. Sci., 12,106(1968)
14. Muschel, L.H., Ann. N.Y. Acad. Sci., 88, 1265(1960)
15. Barta, O. and Barta, V., J. Immunol. Method, 1,363(1972)
16. Fong, J.S.C., Muschel,L.H. and Good, R.A., J. Immunol., 107, 28(1971)
17. Fong, J.S.C., Muschel, L.H. and Good, R.A., J. Immunol., 107, 34(1971)
18. Lepow, I.H., Naff, G.B., Todd, E.N., Pensky, J. and Hinz, C.F., J. Expt. Med., 117, 983(1963)

19. Muller-Eberhard, H.J. and Calcott, M.A., Immunochem., 3, 500(1966)

20. Naff, G.B. and Ratnoff, O.D., J. Expt. Med., 128, 571 (1968)

21. Lepow, I.H., Ratnoff, O.D., Roslin, F.S. and Pillemar, L., Proc. Soc. Expt. Biol. Med., 92, 32(1956)

22. Lepow, I.H., Annual N.Y. Acad. Sci., 103, 829(1963)

23. Becker, E.L., J. Immuno., 77, 469(1956)

24. Ratnoff, O.D. and Lepow, I.H., J. Expt. Med., 106, 327(1957)

25. Bing, D.H., Biochem., 8, 4503(1969)

26. Becker, E.L., J. Immunol., 84, 299(1960)

27. Levy, R.L. and Lepow, I.H., Proce. Soc. Expt. Biol. Med., 101, 608(1959)

28. Davis, G.E., Immunol., 6, 561(1963)

29. Muller-Eberhard, H.J., Adv. Immunol., 8, 1(1968)

30. Levine, L., Biochim et Biophys Acta, 18, 283(1955)

31. Ball, A.K. and Jansen, E.F., Advances Enzymol., 13, 321(1952)

32. Becker, E.L., J. Immunol., 77, 462(1956)

33. Jansen, E.F., Nutting, H.D.F. and Ball, A.K., J. Biol. Chem., 179, 189(1946)

34. Mikulaszek, E., Bull. Acad Sci. Pol., 11, 101(1954)

35. Mayer, M.M., in: Experimental Immunochemistry, 2nd ed., eds. Kabat, E.A. and Mayer, M.M.(Charles Thomas, Springfield, Illinois) pp. 133-240 (1961)

36. Rapp, H.J. and Borsos, T., in : Molecular Basis of Complement Action, 1st ed., (Appleton-Century-Crofts, Educational Division, Meredith Corp., N.Y.)(1970)

37. Baker, B.R., J. Med. Chem., 10, 1123(1967)

38. Baker, B.R. and Cory, M., J. Med. Chem., 14, 119(1971)

39. Mares-Guia, M. and Shaw, E., J. Biol. Chem., 240, 1579(1964)

40. Baker, B.R., J. Med. Chem., 10, 1123(1967)

41. Baker, B.R. and Hurlbat, J.A., J. Med. Chem., 12, 902(1969)

42. von Krogh, M., J Infect. Dis., 19, 452(1916)

43. Chase, T. and Shaw, E., Biochem. Biophys. Res. Commun. 29, 508(1967)

44. Tamura, T. and Nelson, R.A., J. Immunol., 101, 1333(1968)

45. Nelson, R.A., in: The Inflammatory Process (Academic Press, N.Y.) (1965)

46. Borsos, T. and Rapp, H.J., J. Immunol., 99, 263(1967)

47. Rommel, F.A. and Stolfi, R., Immunol., 15, 469(1968)

48. Becker, E.L., J. Immunol., 82, 43(1959)

49. Cooper, N.R., in : Progress in Immunology, (Amos, B., ed.) Academic Press, New York, N.Y.(1971)

50. Shaw,E., Mares-Guia, M. and Cohen, W., Biochem., 4, 2219(1965)

51. Muller-Eberhard, H.J., in: Progress in Immunology, (Amos, B., ed.) Academic Press, New York, N.Y. (1971) p.553

52. Linscott, W.D., Immunochem., 5, 311(1968)

53. Colten, H.R., Bond, H.E., Borsos, T. and Rapp, H.J., J. Immunol., 103, 862(1969)

54. Bing, D.H., J. Immunol., 107, 1243(1971)

55. Borsos, T. and Rapp, H.J., J. Immunol., 95, 559(1965)

56. Bing, D.H., Biochem., 23, 4266(1972)

57. Baker, B.R., in : Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymatic Active-Site, (J. Wiley and Sons, Inc., New York, N.Y.) (1967)

58. Warner, A.H. and McClean, D.K., Developmental Biology, 18, 278(1968)

59. Lepow, I.H., Ratnoff, O.D. and Levy, L.R., J. Expt. Med., 107, 451(1958)

VITA AUCTORIS

1946 : Born in Cantom, Kwantung, China, March 28, 1946.

1964 : Awarded the Hong Kong English School Certificate.

1966 : Awarded the Chinese University of Hong Kong Matriculation

1968 : Awarded the World Universities Service Scholarship

1970 : Graduated with a B.Sc. (Hon.) Degree from United College, the Chinese University of Hong Kong.

1971 : Accepted into the Faculty of Graduate Studies at the University of Windsor, Windsor, Ontario, Canada.