BIOCHEMICAL STUDIES ON THIRD COMPONENT OF OVINE COMPLEMENT

THESIS

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BY

Shakeel A. Khan

TO

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..... TO MY FATHER,

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C E R T I F I C A T E

Certified that the research work embodied in this thesis entitled "Biochemical studies on third component of ovine complement" submitted by Dr. Shakeel A. Khan for the award of Master's Degree of Indian Veterinary Research Institute, is the original work carried out by the candidate himself under my supervision and guidance.

It is further certified that Dr. Shakeel A. Khan has worked for more than 24 months in the Institute and has put in more than 150 days' attendance under me from the date of registration for the Master's Degree of Deemed University, as required under the relevant ordinance.

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C E R T I F I C A T E

Certified that the thesis entitled "**Biochemical studies on third component of ovine complement**" submitted by **Dr. Shakeel A. Khan** in partial fulfilment of **M.V.Sc. Degree** of Indian Veterinary Research Institute, embodies the original work done by the candidate. The candidate has carried out his work sincerely and methodically.

We have carefully gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented by him for the award of **M.V.Sc. Degree** of this Institute.

It is further certified that the candidate has completed all the prescribed requirements governing the award of **M.V.Sc. Degree** of Indian Veterinary Research Institute.

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[SHAKEEL A. KHAN]

C O N T E N T S

ABBREVIATION

A°	:	Angstrom
ACP	:	Alternate complement pathway
CVF	:	Cobra venom factor
C_3/I	:	Third component of complement/Inulin
Conc.	:	Concentration
cm	:	Centimeter
CM	:	Carboxy methyle
°C	:	Centrigrade (celsius) degree
D.W.	:	Distilled water
DEAE	:	Diethyle amino ethyle
EAC_1	:	Erythrocyte antibody complement
ELISA	:	Enzyme linked immuno sorbent assay
Fig.	:	Figure
hr	:	Hour
kD	:	Kilo Dalton
Log	:	Logarithm
M	:	Molar
m	:	meter
M.W.	:	Molecular weight
mA	:	Milli ampere
min	:	Minute
ml	:	Milliliter
mM	:	Milli Molar
/ug	:	Microgram
/ul.	:	Microlitre
nm	:	Nanometer
sec	:	Seconds
R_m	:	Relative mobility
%	:	Percentage
TEMED	:	N, N, N', N' - Tetramethylethylenediamine
V_e	:	Elution volume
V_o	:	Void volume

INTRODUCTION

A. INTRODUCTION

Vertebrates possess in their blood several activation systems that have been recognised and studied. These activation systems viz. blood clotting, clot-lysis, the renin-angiotesin and kinin systems and complement, are found to be essential for the survival of the animal and all have a common feature; dependence on the sequential activation or cascade type of reactions.

Complement is the most complex of these systems, and its existence was known with the observation of **Pfeiffer** in 1984 that, fresh immune serum obtained from guinea pigs immunized with cholera causing organism, lysed the very organisms. After extensive research in this exciting biological phenomenon, it has firmly been established as a system responsible for the destruction and elimination of foreign materials from body notably bacteria and viruses, which usually occur after the interaction of antibodies with the foreign organisms or substances and complement is thus the effector mechanism (Porter & Reid, 1978). Receptors for activated complements proteins, such as the third component C_3 are present on lymphocytes, macrophages and some other cells of immune system.

Not surprisingly, therefore, interest in the complement system has increased greatly, and the progress in isolating and characterising the various components has led to attempts to determine their structure and the biochemical mechanisms of the activation. Complement system comprises of a complex group of some thirty plasma proteins, recognised to date. These proteins of complement as a rule are designated as C_1, C_2, C_3 and so on for first, second and third components of complement system respectively apart from complement factors like B, P, I, etc.

These proteins of complement system have their distinct biochemical and immunological identity. They are capable of interacting with one another, with antibody and with cell membrane receptors once the system get activated. Complement system on activation follows a cascade type sequential biochemical phenomenon. For activation, this system follows two parallel but exclusively independent pathways, viz. classical pathway of complement activation and the alternate pathway of complement activation. A terminal and biologically crucial sequence of reactions adopted by C_5 to C_9 is common to both pathways. Terminal pathway gives rise to the generation of membrane attack complex (MAC) that eventually targets the activator for lysis.

The essential difference between these two pathways lies in the agents that trigger them. Classical pathway for complement activation may get activated by antigen antibody complexes or aggregated immunoglobulins (IgG and IgM), as well as by various non immunological agents viz. DNA, C-reactive protein, some trypsin like enzymes etc. and for this pathway C_1, C_2, C_3 and C_4 actively participate. On the other hand alternate pathway proceeds in a different manner, as it does not require the preformed antibodies. An essential requirment for activation is the presence of C_{3b}, which is continuously generated in small amounts in the circulation and most of the newly generated C_{3b} remains in fluid phase though some bind to various cellular surfaces. In either case, C_{3b} is rapidly inactivated by various control proteins like factor I and H which cleave it.

This steady state continuous turnover of C_3 to C_{3b} coupled with rapid inactivation of newly formed C_{3b} is vastly dependent and modified by particulate activators of alternate pathway of complement (APC), e.g. insoluble polysaccharides and certain cell membranes mostly having a heterologous antigenic character. A part of the C_{3b} that is continuously generated gets deposited on the surface of activators as also on the non activator surfaces but C_{3b} deposited on the activator surface in contrast to non activator surface is protected from destruction by factor I and II.

The surface bound protected C_{3b} interacts with factors B and D to give rise to C_{3bBb}, an enzyme called alternate pathway C_3 convertase, which is able to cleave large amount of native C_3. This amplification of C_{3b} formation is followed by getting more alternate pathway C_3-convertase to deposit an activator surface, finally the C_{3b} generated binds in close proximity to C_{3bBP} by virtue of which a new enzyme like substance takes shape on activator surface, that is able to cleave C_5, which then proceeds to the formation of MAC in a sequential order viz. terminal pathway of complement.

From this simplified overview of complement system activity it is quite discernible to realise the importance of this system in the protection of a body from infectious agents, especially via alternate pathway where the system is able to defend even when the body has no antibodies against the foreign agents. It will be over simplification to lessen the importance of C_3 molecule which stands pivotal in both pathways of complement activation due to its multifunctional role and versatility. C_3 is one of the thirty complement proteins recognised to date and has been the focus of intensive studies due to its important role in complement activation pathways.

In human, this 185 kD protein is the most abundant among the complement proteins in serum (1-2 $mg.ml^{-1}$). It is comprised of two polypeptide chains, a 110 kD x Chain and a 75 kD B chain linked together by one disulfide bond and other non covalent forces (**Lambris** 1988). C_3 is synthesized as a single chain precursor and after post synthetic modifications the native two chain molecule is generated. The synthesis of C_3 along with other complement proteins in tissues other than liver plays a vital role in local inflammatory process (**Lambris** 1988).

It is quite fascinating to realise that such a complex, interacting and interdependent series of plasma proteins collectively termed as complement system has been functionally conserved throughout evolution, as confirmed by the interchangeability of various components of complement system between various diverse branches of vertebrates (**Horton** and **Lackie** 1989).

REVIEW OF LITERATURE

B. REVIEW OF LITERATURE

The third component of complement (C_3) plays a central role in complement pathways, and its relative abundance in blood, (80-130mg/100 ml of human serum) which can not be overlooked, proved instrumental in its sustained and extensive research since its first isolation in 1960 by **Muller-Eberhard** and **Nilsson.** The third component of complement system forms a vital link in complement activation both by classical as well as alternate pathway. Due to its high profile role, its versatility and multifunctional position it was but natural for investigators to get inquisitive about its biochemical and biological parameters.

A wide range of work relating to C_3 : genetics, biochemistry, its association with other molecules and receptors on other cells, has been dealt with detail, by a wide spectrum of researchers. Till date, C_3 has been isolated and characterized from a number of species, human (**Tack** and **Prahl** 1976; **Hammer et al.**, 1981), Rabbits (**Giclas et al.**, 1981), Cats (**Helen et al.**, 1980), Japanese quails (**Kai et al.**, 1983), Rats (**Daha et al.**, 1979), Porcine (**Paques**, 1980), Bovine (**Menger** and **Aston** 1985; **Phipps** and **Aston** 1988) and mouse (**Pepys et al.**, 1977).

Serum contains C_3 in its native conformation, as well as C_3 fragments viz. C_{3b}, C_{3c}, etc. (**Parkes et al.**, 1981). A lot of literature is available for isolation and purification of native C_3, but purification of C_3 fragments has attracted little attention, though this can be conveniently acheived by using aged serum, in which C_3 undergoes *transformation to* C_{3c} *(Harrison and Lachmann 1980).*

An effort has been made here to provide a brief overview of the literature and work done regarding various aspects of C_3 molecule under relevant headings.

B.1 C_3 - ISOLATION AND PURIFICATION

C_3 was first recognised and isolated in 1960 (**Muller Eberhard**); but the methods employed were not sensitive enough to make it possible for the workers to get C_3 with a higher percentage of recovery. Reports of better recovery in purification of human C_3 (**Harrison** and **Lachmann** 1979; **Molenaar** et al., 1973; **Kunkel** et al., 1980) are now available.

The third component of guinea pig complement system was purified and characterized in a more effective manner by **Shin** et al. (1968). C_3 was isolated from the pseudoglobulin fraction of guinea pig serum by DEAE and CM-cellulose chromatography and by Pevikon block electrophoresis. It was reported that the final purified C_3 contained no impurity detectable by any of the fractionation procedures like hydroxyapatite chromatography, IEP or disc electrophoresis. As reported by the workers, the final purified C_3 did not contain any of the other components of complement system.

Molenaar et al. (1973) developed a new innovative procedure for human C_3 isolation using affinity chromatography. This was a new preparative method for isolation of a highly purified C_3 from human serum, using a reversed application of affinity chromatography. Antisera against contamination usually present in crude C_3 preparations was developed and these antibodies were irreversibly coupled to sepharose-4B and used in a column form. When crude C_3 preparations were applied to these columns, the impurities were bound and thus eliminated. The resulting C_3 preparation was pure and homogeneous.

Tack and **Prahl** (1976) purified and characterized C_3 of human complement from plasma. It is noteworthy that 33% of C_3 was recovered

in the final purified product. The third component of human complement was purified from fresh plasma employing an intial fractionation with polyethyleneglycol, followed by sequential depletion of plasminogen by affinity adsorbents. Chromatography on DEAE-cellulose, gel filtration on agarose, and batch-adsorption/desorption on hydroxyapatite. As reported by these workers the homogeneity was ascertained by immunological methods and PAGE. A partial specific volume of 0.736±0.003 ml gm^{-1} was determined for C_3 by the mechanical oscillator technique.

For human C_3 an improved method was reported by Harrison et al. (1979). The basic mode of precipitation followed by chromatography was used and improved upon.

Gyongyossy and Assimeh (1977) described a simple method for isolation and purification of the third component of mouse complement to homogeneity, satisfying physico-chemical and immunochemical criteria of purity and retaining biological activity. As reported by the workers, the third component of mouse complement was purified from pseudoglobulin by two chromatographic steps, involving sequential cation and anion-exchange chromatography.

Rat, being primarily a species used as a model for immunological studies, an insight into C_3 activity was an obvious choice Daha et al. (1978) following the same procedure as previously used, isolated C_3 by initial precipitation by PEG in presence of benzamidine, followed by chromatography of the precipitate on cellulose, hydroxyapatite and QAE-A50 sephadex and gel filtration on sephadex G-200 SF. C_3 was claimed to be isolated in a native form, as assessed by its functional activity and immunoelectrophoretic mobility. The recovery of C_3 was found to be between 18-26%, and was homogeneous on SDS-PAGE and finally gel filtration on sepharose was employed.

Kunkel et al. (1980) reported purification of C_3 and C_5 from human complement employing the hydrophobic chromatography technique.

They reported the application of both hydrophobic and salt mediated hydrophobic chromatography, thus purifying the 3rd and 5th component of human complement. Serum was initially adsorbed on to the hydroxyapatite resin (omega amino hexyl agarose); this first step yield was stated to be greater than 95% of the starting C_3 and C_5. Subsequent fractionation of C_3/C_5 pool on hydroxyapatite was used to separate C_5 from C_3. Salt mediated hydrophobic chromatography and either gel filtration (C_3) or calcium phosphate (C_5) yielded samples of C_3 and C_5 to homogeneity, as assessed by SDS-PAGE. Qualitative and radial immunodiffusion and hemolytic assay were used to measure recoveries at each step in purification scheme. The final recovery of C_3 was reported to be 34% of the initial C_3.

The porcine species gained the distinction of being the first among the species of veterinary importance to be probed for C_3 studies. Purification and partial characterization of porcine third component of complement was reported by Paques (1980). The purification was carried out at 4°C and interestingly no protease inhibitors were used. A general procedure of PEG precipitation, ion exchange chromatography on DEAE-Sephadex followed by Sepharose 6B-chromatography and use of hydroxyapatite column were adopted.

The method for isolation and purification of C_{3b} by affinity chromatography using factor H-Sepharose was used by Scott et al. (1982). It may be noted that C_{3b} a cleavage product of native C_3 has great affinity for factor H of plasma. The workers reported C_{3b} purification using rabbit, bovine and human serum. Initially, the serum was subjected to PEG precipitation, DEAE-Sepharose ion exchange chromatography and use of an affinity chromatography human factor H-Sepharose column. The reported yield was as high as 35-40% and the whole process was complete in three days. It is established that C_{3b} binding to factor H-Sepharose was equimolar and had a sharp optimum pH (7.6) and quite sensitive to changes in ionic strength. An added advantage of this method was the ability of the affinity column for repeated use.

C_3 of Japanese quails (Kai et al., 1983) was attempted by using rabbit antiserum prepared against quail serum-treated zymosan (zx) as an initial reagent. Purification protocol was reported as salt precipitation followed by sephadex gel filtration and DEAE-Sephadex column chromatography.

Cannine C_3 was isolated and purified (Johnson et al., 1985) from plasma of dog by initial precipitation with 4% (w/v) polythyleneglycol-4000, followed by depletion of plasminogen using speharose-4B-Lysine column and as reported the effluent was again fractionated with PEG 4000 at 10% w/v. The precipitate was used in DEAE-Sephacel column, and positive for C_3 fractions were passed on to CM-Sepharose-Cl-6B column to yield the final purified product.

Bovine C_3 was purified and studied in detail by Menger & Aston (1985). Not much change was exercised in the purification protocol. Using bovine serum in presence of protease-inhibitors, PEG precipitation by PEG-6000 followed by ion exchange chromatography on DEAE and CM-Sephadex, native C_3 was obtained after gel filtration on Sephacryl-S-200, reportedly purified to homogeneity.

Reports are also available for isolation and purification of C_3 and C_5 component from rabbit (Giclas et al., 1981). Procedures used do not differ much from those already reviewed.

C_3 has been isolated and partially purified by various workers while adopting procedures by which entire complement protein series could be worked on. Vroon et al. (1970) gave a detailed scheme for separation of nine components (C_1 to C_9) and two inactivators of C_1 and C_3. From a single pool of human serum workers adopted salt precipitation at different pH levels, chromatography on DEAE or CM-cellulose and finally gel filtration. Sargent et al. (1976) claimed to functionally purify the first seven components of cannine complement by using euglobulin portion of dog serum by ammonium sulfate precipita-

tion, ion exchange and exclusion chromatography, on DEAE-cellulose, CM-cellulose, hydroxyapatite and sephadex G-200, respectively.

Hammer et al. (1981) reported large scale isolation of functionally active components of human complement system. A large volume of fresh EDTA plasma was used as the starting material. Use of inhibitors was reported in usual procedures of ion exchange and gel filtration chromatography, PEG-precipitation followed by plasminogen-depletion.

Phipps et al. (1988) in a simple procedure isolated all components of bovine ACP-C_3-convertase, including C_3 component.

B.2 BIOCHEMICAL CHARACTERS

There appears to be a general agreement regarding the molecular weight and a well established two chain structure of C_3 molecule. The available literature narrows down the molecular weight (M.W.) range of C_3 molecules in various species between 170,000 to 200,000 daltons.

Molecular weight of gunlea pig C_3 was reported as 180,000 (**Shin et al.** 1968) while that of human as 187,650 ± 5650 (**Tack et al.**, 1976) as assessed by low speed sedimentation equilibrium method. Further characterizing the same authors reported a molar-extinction-coefficient of $1.82 \times 10^5/\text{mole}^{-1}\ \text{cm}^{-1}$ at 280nm as calculated from boundary spreading experiments in ultra-centrifuge and an assumed negative index increament. The M.W. of 210,000 was reported for mouse C_3, a β-glycoprotein by **Gyongyossy et al.** (1977) having two non-identical disulfide linked polypeptide chains of M.W. 130,000 and 75,000 dalton, respectively.

A detailed report of the M.W. of human C_3 and its fragments was made by **Molenaar et al.** (1975). The M.W. of native C_3 was 190,000 as

assesed by SDS-PAGE. The number of polypeptide chains ascribed to C_3 were two with M.W. 115,000 and 75,000 dalton respectively. Comparatively, C_{3b} had a M.W. of 179,000 with 4 polypeptide chains of the m.w. of 82,000, 63,000, 24,000 and 11,000 daltons as observed by SDS-PAGE. **Bokisch et al.** (1975) in contrast, reported for human C_3 a M.W. of 195,000. For C_{3b} a M.W. of 181,000 dalton was assigned. C_3 dissociated on reduction into two chains of 120,000 and 75,000, respectively. Identical treatment of C_{3b} resulted in dissociation of only two chains (111,000 and 75,000 dalton).

Rat C_3 has a m.w. of 187,000 (**Daha et al.**, 1979) with two nonidentical disulphide linked polypeptide chains of 123,000 and 76,000 dalton; porcine C_3 comparatively has a m.w. of 190,000 dalton (**Paques**, 1980) with two disulfide linked polypeptide chains, alpha and beta of M.W. 16000 and 74000 dalton respectively.

In Japanese quails the C_3 equivalent of mammals has a M.W. of 184,000 dalton (**Kai et al.**, 1983). Dog C_3 has a M.W. of 179,000±7000 **Johnson et al.**, 1985) and composed of two disulphide linked chains of 114,000±6000 and 65000±3500 dalton. Bovine C_3 molecule has a reported M.W. of 183,000 (**Menger and Aston, 1985**) with two disulfide linked chains of 114,000 and 69,000 dalton. Characterization of the third component of cannine and feline component was reported by **Gorman et al.** (1981). Cannine and feline C_3 were shown to have a M.W. of 198,000 and 197,000 dalton respectively, each comprised of two polypeptide chains, alpha and beta (cannine alpha-126,000 dalton, beta 72,000 dalton : feline alpha-125,000 dalton beta-72,000 dalton).

Amino acid composition of the C_3 molecule always posed a challenge to analytical biochemists. In this regard, preliminary work was done in human C_3 utilising automated Edman degradation method (Tack **et al.**, 1976) revealing no unusual or distinctive characteristics. A double N terminal sequence, Ser-val, Pro-Glx, Met-Leu, Tyr-Thr, Ser-Glx, Ile-Lys, Gly-Arg, Thr-Met, Pro-Asx, in agreement with the two chain structure observed on SDS-PAGE was found indicating that

both chains were available for degradation. Serine was postulated as initiating the sequence in the both chains and a Alanine as the ultimate carboxyterminal amino acid. Aminoacid composition of C_3 and its fragments C_{3b}, C_{3c}, C_{3d} on a comparative presentation (**Molenaar et al.**, 1975) revealed that in comparison to C_3, the concentration of basic residues was diminished to a greater extent than that of the amino-acid residues in C_{3b}. This can be imagined from the release of C_{3a} (**Budzko et al.**, 1971), the amount of lysine and half-cystine were relatively low in C_{3c}; in C_{3d} the amount of aliphatic aminoacid and phenylalanine were relatively low, whereas, the amount of lysine, glutamic-acid, glycine and alanine were relatively high (**Molenaar et al.**, 1975).

The glycoprotein nature of human C_3 was reported (**Hirani et al.**, 1986) after a detailed study. A 2% of the total mass of human C_3 was attributed to carbohydrate content. C_3 was found to contain N-acetylglucosamine and mannose (in the molar ratio of 1:4), both chains being glycosylated and site of asparagine glycosylation at residue 67 (β chain) and 917 (α chain) was established. The carbohydrate moiety of α chain has been found to be the binding site for bovine plasma protein conglutinin.

Electron microscopy of native C_3 in guinea pig confirms a spherical shape of molecule (**Suzuki et al.**, 1972). In case of human, electron microscopy of C_3 and its fragments was reported by **Molenaar et al.** (1975) C_3 was seen as having spherical configuration in which no subunit like particles could be distinguished, having a maximum size of sphere as 23nm. In contrast to C_3, C_{3b} demonstrated a thread like structure consisting of molecules probably laid end to end.

Circular dichroism (CD) measurement for purified C_3 and its fragments C_{3b}, C_{3d} and C_{3c} (human) were done, (**Molenaar et al.**, 1975), CD measurements revealed that the conversion of C_3 to C_{3b} resulted in a change of secondary structure, and also confirmed structural changes during the conversion of C_{3b} into C_{3c} and C_{3d}. The same workers also reported the relative % of α-helix, β-helix and random

coils structure of C_3 and its fragments from CD curves. C_3 was assigned 10% α-helix, 35% β-helix, 55% random coil structure, while as C_{3b} and C_{3c} showed 1% α helix, 44 to 41% β-helix and 55 to 58% random coil structure.

Variants of human C_3 have been identified by Immuno-electric-focussing (I.E.F.) technique (**Vogel et al.**, 1984). The IEF pattern of human C_3 and Cobra venom factor (CVF) were found similar. Both proteins exhibit extensive heterogeneity and a similar banding pattern of three groups of variants, focussing between pH 5.5 and 6.5 with a characteristic gap at pH 6.0 (**Vogel et al.**, 1984). In contrast, murine C_3 focusses as a single C_3 band of PI 6.0-6.1 depending upon the strain of mouse (**Natsuume-Sakai et al.**, 1978). Bovine C_3 focusses as a single cluster of at least three discernible variants within a relatively narrow range of 6.55-6.85 (**Menger&Aston**, 1984).

B.3 C_3 MOLECULE - BIOLOGICAL ACTIVITY

The third component of complement plays a central role as the protein at which the classical and alternate pathways of activation converge (**Tack et al.**, 1976). C_3 interacts with C_3 convertases C_{42} via classical pathway of complement activation to give rise to C_5 convertases $C_{4,2,3}$ (**Muller-Edberhard et al.**, 1976), or with the proteins of the alternate pathway of complement activation giving rise to an alterate pathway C_3 convertase C_{3bBb} (**Sandberg et al.**, 1970; **Gotze and Muller-Eberhard**, 1971).

The activating enzyme, C_3 convertases cleaves the molecule into two fragments C_{3a} and C_{3b}, the activation peptide C_{3a}, constitutes one of the two known anaphylatoxins, (**Bokisch, et al.**, 1975) which answers for the involvement anaphylactic activity on C_3 activation (**Dasilva et al.**, 1967). In very low concentrations the peptide effects release of histamine and related products from mast cells and chemotatic migration of polymorphonuclear leukocytes (**Bokisch et al.**, 1969; **Ward et al.**, 1973).

Cleavage of C_3 between residues 77 and 78 (Arg-Ser) of alpha chain, either by classical (C_{42}) or the alternate (C_{3bBb}) pathway C_3 convertases, lead to the generation of C_{3b} and C_{3a} fragments (Lambris 1988). Only native haemolytically active C_3 molecules are cleaved by the classical pathway C_3 convertase C_{42} (Jonatova et al., 1980). The existence of C_{3b} receptors on monocytes, macrophages, polymorphonuclear leucocytes and B-cells has emphasized the role of complement in opsinization, immune adherence and the humoral immune response (Lay and Nussenzwerg 1968; Huber et al.; 1968). C_{3b} being the larger fragment, in its nascent state is capable of binding to the surface of cells and other particles (Bokish 1975) including bacteria and immune complexes and thus facilitate their ingestion by phagocytic cells (Gigli et al.,1968). The main role of C_{3b} is to modulate several complex enzymes of classical and alternate complement pathways (Muller-Eberhard 1972; Muller-Eberhard et al., 1975). The C_{3b} molecule transiently acquires the ability to be fixed covalently through an ester or amide bond to the hydroxyl or amino groups present on cell surfaces, complex carbohydrates or immune complexes (Lambris 1988). Covalent binding of C_{3b} is the function of its thioester group (Pangburn et al., 1980) and involves sulphur of cysteine 988 and carbonyl group of glutamic acid 990, both of which are located in the C_{3d} fragment of C_3 (Tack 1983). C_{3b} was shown to contain a sulphydryl group not detectable in native C_3 (Tack et al., 1980). Both the sulphydryl and reactive carbonyl groups are found in "d" (C_{3d}) domain of alpha chain, thus the two groups give rise to an internal thioester in native C_3 (Pangburn et al. 1980). In contrast to native C_3, C_{3b} expresses multiple binding sites for other complement components, including C_5, properdin (P) factors H, B and I, C_4 binding protein (C_{4bp}), CR1 (C_{3b} receptor), membrane cofactor protein (MCP) and decay-accelerating factor (DAF). Binding of these proteins to C_{3b} leads either to amplification of C_3 convertase (by B and P in presence of factor D) and initiation of MAC or to inactivation of C_{3b} (MCP, CRI, H, C_{4bp} by factor I) (Lambris 1988).

The inactivation of C_{3b} proceeds in three steps and requires one of the several cofactor molecules (MCP, CRI, CR2, H, C_4bp). These,

cause a conformational change in C_{3b} thus making factor I to bind and exert its inactivation action (**Harrison et al.**, 1980; **Ross et al.**, 1982; **Medof et al.**, 1982). It has been suggested that C_{3b} (alpha chain) is cleaved at three points by factor I. (**Lambris** 1988), first and second cleavage of alpha chain give rise to a 2KD residue C_{3f} and yields C_{3b} to iC_{3b} (**Harrison et al.**, 1980; **Medof et al.**, 1982). It is still debatable whether I effects a third cleavage and thus generating C_{3dg} with concommitant liberation of C_{3c} (**Lambris** 1988). Further proteolysis of C_{3dg} by trypsin, elastase, plasmin generates C_{3d} (**Ross et al.**, 1982). The C_3 fragments generated during activation process have the inherent capacity to bind specifically to several cell surface complement receptors called CR1, CR2, CR3, CR4, CR5 and C_{3a} receptors which lead to various biological responses (**Lambris** 1988).

Complement receptors (CR1, CR2, CR3, CR4 and C_{3aR}) present on cell surfaces on interaction with various fragments of C_3 give rise to a wide spectrum of biological activity (**Lambris** 1988). CR1 is found mainly on RBC, Langerhans cell, neutrophills, monocytes, eosinophills, B and some T cells (**Ross** 1985). On interaction with C_3 fragments specifically, activity like inhibition of complement activation by increasing the decay/dissociation of C_3 convertases of both activation pathways, or behaving as a co-factor in I-mediated inactivation of C_{3b}; phagocytosis of C_{3b}-coated particles is enhanced (**Lambris** 1988). Receptor activities for cell bound degradation products of third component, C_{3b} and C_{3d} were demonstrated on the cells from nine cultured human lymphoid cell lines and spleen lymphocytes of eight strains of mice (**Manfred et al.**, 1974). Some cultured human lymphocytes were found to display receptor preference for both C_{3b} and C_{3d} while others showed preference for only C_{3d}. But murine cell receptors were found to interact only with murine C_{3d} and to a lesser extent to C_{3d} human.

CR2 receptor has got specificity for C_{3d}, iC_{3b} and C_{3dg} fragments of C_3. CR2 is mostly present on B. lymphocytes, follicular dendritic cells and some B and T cells lines (**Ross et al.**, 1985), CR2 also serves as a receptor for Epstein-Barr-Virus (EBV). The involve-

ment of CR2 in the modulation of immune response is well established, though the mechanism of this is unclear (**Lambris** 1988). Complement receptor type three (CR3) found on cell surfaces of monocytes, macrophages, granulocytes and large granulocytes binds iC_{3b} fragments of C_3 (**Ross** et al., 1985). CR3 functions in the phagocytosis of iC_{3b} coated particles and plays and role as an adhesive molecule in cellular interactions. CR4, a leukocyte adhesion receptor molecule is expressed on myeloid cells and some activated lymphocytes. It binds iC_{3b} in a diavalent cation-dependent manner (**Micklem** et al., 1985; **Myones** et al., 1988).

Complement receptor five CR5 found on neutrophills and platelets binds C_{3dg} and C_{3d} fragments of C_3 and this interaction is independent of divalent cations (**Micklem** et al., 1985). C_{3a} receptor (C_{3aR}) is highly specific for C_{3a}, present mainly on mast cells, neutrophills, basophills, macrophages, eosinophills and T. lymphocytes (**Lambris** 1988). The interaction with C_{3a} leads to release of vasoactive amines from mast cells, eosinophills, and thromboxane-A from macrophages (**Hugli** 1984).

Membrane cofactor protein (MCP) is mainly found on neutrophills, monocytes, platelets and reticulocytes (**Lambris** 1988) MCP is specific for C_{3b} and functions as a surface cofactor for inactivation of C_{3b}, by factor I. (Seya et al., 1986). On the other hand DAF, in addition to cells that express MCP, is also found on erythrocytes and shows preference for C_3 convertases of classical as well as alternate pathways (**Nicholson-Wellar** et al., 1982), its function is to accelerate the decay of these C_3 convertases.

Besides cell membrane receptors, a host of serum proteins interact with C_3 and its fragments eliciting a wide range of activity. The effects of factor H on complement activation, via alternate pathway are exerted by binding to C_{3b} and acting as an antagonist of factor B binding to C_{3b}, as a decay-accelerating factor for C_3/C_5 convertases,

and as a cofactor for factor I mediated cleavage of C_{3b} (Pangburn 1986). Initial C_3 convertases of alternate pathway, as is well known, cleave C_3 to C_{3b} which in presence of factor B and D can form C_{3bBb} whose formation by factor B and D is Mg^{+2} dependent (Lambris 1988). C_{3b} after modifications, which is decided by the type of factors C_{3b} interacts in a particular micro-environment. For inactivation it is cleaved by factor I, a serine protease (Pangburn et al 1983). Another plasma glycoprotein Properdin (P) exerts influence on complement activity by stablising alternate pathway C_3 convertase after binding to the latter (Pangburn 1986). The site of (P) binding in C_3 lies within residues 1402-1435 of C_3 (Daoudaki et al., 1987). C_5 convertase comprising of C_{3bBBb}, having two C_3b molecules with other factors; (Lambris 1988) on intraction with C_5, cleaves it and gives rise to C_{5a} an anaphylatoxin and initiation of membrane attack complex MAC (Pangburn 1986).

Complement activity via the C_3 shunt (alternate pathway activation) can be triggered by various agents as reviewed but requires magnesium and not calcium ions (Fine et al., 1972) and proceeds in vitro in the presence of 10 mM ethyleneglycotetraacetic acid (EGTA). The complement activity via classical pathway requires calcium and thus gets inhibited by EGTA.

A detailed study of trypsin cleavage of C_3 was reported by Minta et al. (1977). Trypsin was shown to cleave C_3 in a stepwise manner, initially cleaving alpha chain and generating C_{3a} and C_{3b}, further cleavage of alpha chain of C_{3b} converted it to C_{3d} and C_{3c}. Cleavage of beta chain by trypsin occured at C_{3c} stage with a release of a small polypeptide chain MW 12,000 dalton from C_{3c} to give rise to C_{3c}.

Some non complement enzymes can also make C_4 and C_3 bind and elicit activity on a RBC surface, (Kirschfink et al., 1988). Trypsin, chymotrypsin, plasmin, elastase, thrombin, kallikrein, and enzymes from bacillus-subtilis, staphylococcus-aureus, were found capable of binding C_{3b} and C_{4b} to sheep RBC. Both classical pathway as well as

alternate pathway of complement activation could be thus elicited by most enzymes except plasmin and thrombin.

Interaction of B-determinant of C_3 with amidino compounds as reported by Asgar et al. (1976) revealed that amidino compounds after interaction with B-determinant render C_3 incapable of reacting with anti-B-determinant antiserum; these amidino compounds also inhibit plasmin, trypsin and human plasma kallikreins. Thus there seems to be some similarity in B- determinant of C_3 with these enzymes.

C_3 is also activated by gamma (γ)-I-immune aggregates from guineapig serum, when mixed appropriatly with other serum cofactors and Mg^{+2}, (Konig et al., 1973). It has been shown that zymosan, (Thompson et al., 1970), Inulin (Gotze et al., 1971), Endotoxin (Marcus et al., 1971) and guineapig gamma-I-immune aggregates (antigen-gamma-I-antibody complexes) are able to activate the complement system at C_3 directly or via alternate pathway of C_3 activation.

The esterase-like activity of covalently bound C_3 was reported by Venkatesh et al. (1988), further clarifying that a acyl-ester bond between the C_3 and a variety of molecules is hydrolysed spontaneously at neutral pH. They suggested that a functional group other than SH acts as a "catylytic" group in this intramolecular hydrolytic reaction. Complete inhibition of the esterase like activity is observed with stoichiometric amounts of mercurric chloride and palladium chloride.

B.4 C_3 ASSAY

The identification of C_3 during purification steps has been achieved by development of hemolytic assays (Barta and Hubbert 1978). These assays were performed by preparing various cellular intermediates like EAC_1 guineapig, C_4 human, C_2 guineapig and using functionally purified C_3 to C_9 components of complement (Shin and Mayer 1968; Tack and Prahl 1976; Daha et al., 1979). For recovery of C_3

hemolytic activity, quantitated by using $2x10^7$ $EAC_{1\ 4}$ cells and incubating at 37°C for 1 hr; the hemolytic unit CH_{50} was taken as the reciprocal of dilutions of C_3 that would lyse $1x10^7$ $EAC_{1\ 4}$ cells (Mayer 1961). Methods for C_3-assay by classical pathway of activation were less sensitive and tedious due to their inherent complexity. So, a simple rapid and insensitive to C_3 degradation products assay for C_3, that utilizes the alternate pathway of complement activation is now established (Jessen et al., 1983) called as alternate pathway C_3 assay ($AP-C_3$). This uses rabbit RBC as complement activator and methylamine treated plasma, depleted of C_3 and C_4, as complement source, $AP-C_3$ assay was optimised at 2mM Mg^{+2} 5mM EGTA and at $5x10^7$ RBC cell/ml.

Immunochemical techniques for C_3 assay have been used to monitor C_3 presence during purification procedures, where a monospecific antisera against C_3 is raised in a distant heterologous species (Henson 1977; Kai et al., 1983; Gorman et al., 1981). Inulin, (Harrison and Lachmann 1980) or zymosan, are mostly used by workers as an activator surface for ACP and subsequent deposition of C_3; Mg^{+2} and EGTA, are integral components of this whole procedure; EGTA for its preferential chelation of Ca^{++} rather than Mg^{+2}, thus permitting only ACP activation (Fine et al., 1972) and C_3 deposition on activator. A simple method for Human C_3 and C_4 antisera raising in rabbits was reported (Camoen's et al., 1987), anti-C_3 was prepared by injecting rabbits with elutes of human RBC coated with C_3 obtained by inulin method. Fresh RBC, serum and 5% inulin were incubated at 37°C, treated cells washed and elution carried out by heating the treated cells in saline.

B.5 C_3 - DEFICIENCY AND DISEASE

A significant corelation appears to exist between the C_3 level and disease status in a body. In a study for circulating C_3 in equine (Perryman et al., 1971) the C_3 level of horses, experimentally given equine infectious anemia virus, was lowered. Doposition of C_3 in renal glomerulii of felines having feline infectious peritonitis was established

(Helen et al., 1980). In the affected cats localisation coincided with deposition of IgG also. C_3 deficiency can lead to increased susceptibility to infections that lead to purulent lesions (Lambris 1988).

B.6 C_3 - GENERATION

C_3 synthesis in body is usaully carried out by liver, monocytes, astrocytes, B-lymphocytes. Several tumor cell lines of different origins can also sythesize C_3 (Colten 1986; Barnum 1989). Genetic studies indicate the presence of twenty two variants of C_3, most common being C_{3F} and C_{3S} (Colten 1986). Murine C_3 gene located on chromosome number 17 is located at some distance from major histocompatibility complex (MHC), while in human, C_3 gene is on chromosome 19 (Lambris 1988). Extensive work on genetic polymophism of murine C_3 was demonstrated by use of Immuno-electric focussing (IEF) and immunofixation (Natsuume-Sakai et al., 1978) and established that PI variation of mouse C_3 is inherited as an autosomal Co-dominant trait controlled by a single locus.

B.7 C_3 - EVOLUTION

Rabbit, mice, chicken, quail, xenopus rainbow trout and cobra have a similar molecule, of a comparable molecular mass and chain structure that functions like human C_3 (Lambris 1988).

Evidence of mutual interchangeability of complement components C_1, C_2, C_3, C_4 among species like horse, sheep, cow, etc. has been established (Rice et al., 1952). A comparative study of human C_3 and cobra venom factor (CVF) revealed similiarity in amino acid composition, far and near UV-spectra, CD- spectra etc. CVF was suggested to be a modified cobra C_3 having structural and biological activity resembling that of human C_{3b} (Alper et al., 1976) and has the capacity to cleave human C_3 without being recognised by human factor I.

B.8 C_3 AND IMMUNE RESPONSE

Complement component C_3 is involved in a successful induction of normal antibody response, as established from the studies on comple-

ment deficient guinea pigs (**Bottger et al.**, 1987), the fact that no antibody response is elicited because of C_3 depletion, was established. The same authors also viewed that, C_3 may have a secondary function being directly or indirectly involved in the suppression of polyclonal activation of lymphocytes. There is a considerable evidence regarding the *induction and maintenance of memory cells of B. cell lineage during* an immune response to an antigen (**Klaus et al.**, 1986), C_3 split products when presented to immune response cells in a specific manner, act as B. cell growth factors. Participation of C_{3a} in immune response has been established by C_{3a}-mediated inhibition of certain lymphocyte functions including polyclonal expansion and antibody production induced by Concavalin A and Pokeweed mitogen (**Hugli** 1984). Antibody response to antigen was found normal in C_3 deficient patients but had an impaired switch from IgM to IgG, (**Ochs et al.**, 1986), that were immunised with limited dose of T dependent antigen. Recently, C_3 has been shown to play a role in B-cell proliferation (**Kramer et al.**, 1990) crosslinked C_{3b} and C_{3d} stimulate B-cell growth in the G1 phase of the cell cycle, while soluble C_3, C_{3d}, and a peptide containing the complement receptor CR2 binding site are all inhibitory.

MATERIALS AND METHODS

C. MATERIALS AND METHODS

C.1 ANIMALS

C.1.1 Laboratory Animals

Healthy adult male rabbits obtained from Laboratory of Animal Research Centre (LAR), Indian Veterinary Research Institute (I.V.R.I.), Izatnagar, U.P. were maintained in the Physiology and Climatology animal shed and fed ad libitum.

C.1.2 Large Animals

Healthy adult sheep (male and female) were procured from sheep and goat farm, I.V.R.I. and housed in Physiology and Climatology large animal shed, fed and maintained in appropriate conditions.

C.2 CHEMICALS

The following chemicals used in the present study were procured as indicated against them.

Bisacrylamide, beta-Mercapto-ethanol, Phenylmethylsulfonyl-flouride (PMSF), C. amino-n-caproic-acid, Ethyleneglycol tetraacetic acid (EGTA), Freund's complete adjuvant, Freund's incomplete adjuvant, Trypsin, Gel filtration molecular weight markers, SDS-PAGE molecular weight Markers (M.SDS-200), Sodium dodecyl sulfate, TEMED, p-dimethyl amino benzaldehyde (PDMAB) (Sigma, U.S.A.).

Ammonium persulfate, Ethylenediaminetetraacetic acid (EDTA), Glycine, Magnesium chloride, Potassium chloride, Potassium dihydrogen phosphate, Sodium azide, Sodium phosphate (monobasic), Sodium phosphate (dibasic), Sodium hydroxide, Sodium chloride (Merck, India).

Bromophenol blue, Barbituric acid, Barbitone sodium, Boric acid, Gelatin, Inulin, Polyethylene glycol-6000, Potassium metabisulphite (BDH, U.K.).

Acrylamide, Agarose-M, orcinol (SRL-INDIA). Acetic acid (glacial), Hydrochloric acid, Methanol, Iso-prophyl alcohol 70% (Polypharm). Agar (Difco). DEAE-Sephadex-A-50, CM-Sephadex-C-50, Sephadex-G-200 (Pharmactia, Sweden).

METHODS

Note : For details of reagents, solutions and buffers used in the present study, see Appendix.

C.3 PRODUCTION OF ANTISERA

C.3.1 Production of Anti-Sheep Whole Sera

Anti sheep whole sera (ASWS) was raised in adult male New-zealand white rabbits according to the following schedule :

Day	Inoculum	Route
Zero	1 ml fresh sheep serum + 1 ml FCA	Multiple 1/m
26	1.5 ml fresh sheep serum + 1.5 ml IFA	Multiple 1/m
40	-do-	-do-
48	Test bleeding	Intra cardiac

Rabbits were test bled and sera obtained was tested against sheep whole serum by Immunoelectrophoresis (IEP). Those rabbits showing high antibody response, were subsequently bled and serum obtained was decomplemented (56°C for 30 min) and stored at -20°C in sterile vials (1.5ml) till used.

C.3.1.1 Production of Anti-C_3/inulin (Anti C_3/I) Sera

C_{3b} adsorbed inulin suspension was prepared following the method of (Gorman et al., 1981) with modifications.The inulin suspension (100mg/ml) in Veronol buffer saline (VBS) was sonicated for one minute. Freshly drawn sheep serum was fortified with 10mM EGTA and 10mM Mg^{++} ion. Equal volumes of inulin suspension and sheep serum (EGTA/Mg^{++}) were mixed and incubated for 90 minutes at 37°C with intermittent stirring. The whole suspension was centrifuged in cold (4°C) at 4000 rpm and the pellet was washed thrice in PBS, twice in 2M NaCl and once again in PBS. The final pellet was suspended in 1 ml of PBS.

Four male Newzealand rabbits were used for immunization as per the protocol below.

Day	Inoculum	Route
Zero	1ml C_3b/I suspension in PBS + 1 ml FCA	Multiple i/m(hind leg)
24	1ml C_3b/I suspension in PBS + 1 ml IFA	Multiple i/m(hind leg)
32	Test bleeding	Intra cardiac

Serum obtained from the inoculated rabbits was subjected to titer testing by Ouchterlony method. Rabbits showing high titer were bled intracardiac and serum after collection was decomplemented (56°C for 30 min) and stored in aliquotes of 1.5 ml in disposable vials at -20°C till used.

.4 IMMUNOPRECIPITATION TECHNIQUES

All immunoprecipitation procedures were performed according to ne methods described by **Hudson** and **Hay** (1986) and **Talwar** (1983). gar/agarose solutions were prepared in Tris-borate buffer (0.05M pH 6.8) ontaining 5mM EDTA and the same was also used as electrode buffer in IEP.

;.4.1 Ouchterlony Method, Doubleimmunodiffusion (DID)

DID was performed in 1% agar coated glass slides (2.5 x 7 cm) using 20/ul of antigen and 20/ul of antisera in each wells. The development of Immunodiffusograms was carried out in humid chamber at 37°C and observed after 12 hrs.

C.4.2 Immunoelectrophoresis (IEP)

The antigen (20/ul) was subjected to electrophoresis at a constant current of 7mA/150V for 70 min. in 1% agarose gel on glass slide (2.5 x 7.5 cm). Development was carried out in a humid chamber at 37°C using 250/ul of previously tested antisera placed in the cut our trough. Results were interpreted after 12 hrs.

C.4.2.1 Comparative Immunoelectrophoresis (COIEP)

This technique was utilised for checking the stepwise purification procedure. It was performed using more than one antigen against a monospecific, high titer antisera on the same agarose coated plate (7.5 x 8cm). The antigens (pooled elutes from different purification steps), 20/ul each, were subjected to electrophoresis in 1% agarose gel at a constant current of 10mA for 80 minutes. Developing was done with 250/ul of antisera placed in each cut out trough in a humid chamber at 37°C for 12 hrs.

C.5 PURIFICATION OF C_3

C.5.1 Collection of Sera

Adult sheep clinically in a disease free state and maintained on ad-lib ration, were used for collection of blood. Sheep blood collected by jugular venepuncture was allowed to clot in sterilised glass tubes for 40 min. at room temp (25-30°C) and subsequently transferred to 4°C for 90 minutes. Sera was latter harvested at 4°C using refrigerated centrifuge (Hermile, BGH) and stored at -20°C till used with 0.01% sod. azide.

C.5.2 *Polyethylene Glycol Precipitation*

Pooled sheep serum (150 ml) was adjusted to pH 7.4 with 0.5N HCl, 2mM EDTA and 0.3mM PMSF. Polyethylene glycol-6000 (PEG) was added to a final concentration of 7% (w/v) resultant mixture was stirred gently by a sterilized glass rod at 4°C for 40 minutes. The precipitate was collected by centrifugation at 4°C (5000 RPM x 20 min.) and the precipitate was washed thrice with 0.1M phosphate buffer, pH 7.5 containing 7% PEG-6000 (w/v) and 0.15 mM PMSF. Finally the washed precipitate was dissolved in about 140 ml of 25mM phosphate buffer, pH 7.0 having 25 mM EDTA, 20 mM EACA. The solution was assayed for C_3 presence by Ouchterlony method. Protein was assayed by the method of Lowry et al. (1951).

C.5.3 Anion Exchange Chromatography

A (2.5 cm x 46 cm) column was prepared with DEAE-Sephadex-A50 after swelling the powder in the equilibrating buffer (P.B., 25 mM, pH 7.0, 5 mM EDTA) for 72 hrs; the bed was thoroughly washed with the starting buffer.

The post PEG precipitate (140 ml) was applied to the column and washed with the equilibrating buffer. It was developed with a linear gradient of NaCl upto 0.45 M at a flow rate of 50ml/hr. Fractions of 8 ml each were collected and monitored in a LKB chromatography unit (2201

COMBICOLDRAC II. LKB with fraction collector : 2070 Ultro RACII.LKB, peristaltic pump : 2120 Varioprpex.LKB, a uv monitor unit : 2138 UVICORD.LKB, with a recorder : 2201-1-channel.LKB).

The fractions of various peaks were tested for C_3 by Ouchterlony method and the fractions reacting with anti C_3/I serum were pooled, concentrated in a Vac-concentrator (SAVANT) and stored at -20°C till use.

C.5.4 Cation Exchange Chromatography

A (2.5 x 60 cm) CM Sephadex-C 50 column was prepared with CM Sephadex-C50 powder in the equilibrating buffer (Tris acetate 20 mM, pH 6.6). The post DEAE -pool was dialysed against the starting buffer for 24 hrs at 4°C and applied to the column. The column was initially washed with the equilibrating buffer at the flow rate of 40 ml/hr. Fractions of 6 ml were collected. A linear gradient of NaCl to 0.35M NaCl was used for elution of the bound protein. The fractions of different peaks were tested for C_3 by Ouchterlony method using C_3/I antisera. Reacting fractions were pooled and concentrated in Vac-concentrator (SAVANT) and stored at -20°C till use.

C.5.5 Gel Filtration

A (90 x 2.5 cm) Sephadex-G-200 column was made as per the instruction of (Pharmacia fine chemicals). The column was throughly washed by the equilibrating buffer (PB.25 mM pH 7.0, 25 mM EACA, 5mM EDTA, 150mM NaCl), Post CM-Sephadex pool was applied in aliquotes of 5ml. The column was eluted at a flow rate of 10 ml/hr. and fractions of 2.65 ml were collected. Initially V_o was calculated by using Blue-Dextran 5ml (20mg). The elution pattern was monitored at 280 nm in the LKB chromatography unit as mentioned earlier. The C_3 containing fractions obtained from gel filtration column were taken, and concentrated after assaying each fractions for the presence of C_3 by Ouchterlony method using anti C_3/I serum. Fractions reacting with anti C_3/I serum were subjected to IEP.

All the chromatogrphic procedures were performed at 4°C and all buffers contained 0.03% sodium azide and 5mM EDTA.

C.6 CHARACTERIZATION OF PURIFIED C_3

C.6.1 Simple PAGE

Polyacrylamide gel electrophoresis (PAGE) in tubes under non *denaturing and non reducing conditions* was performed according to **Davis** (1964) to check the charge homogeneity of the purified protein. Post-PEG precipitate, aliquots of post DEAE-Sephadex pool, post CM-Sephadex pool and Sephadex G-200 pool fractions were loaded separately in gel tubes (8.0cm x 0.5). The sample (100 ug) corresponding to each purification step was mixed with sucrose solution (400g/l) containing bromophenol blue (10mg/l) and applied. The electrode buffer was Tris glycine (pH 8.6). The current was set at 2.5 mA/tube for first 30 min, later raised to 5 mA/tube. After the run monitered by tracking dye, the gels were stained and destained by procedure explained elsewere.

C.6.1.1. Staining and Destaining of Gels

The gels were fixed in a fixative solution at 37°C and then stained by 0.1% Coomassie brilliant blue staining solution for 4 hrs at 37°C. Destaining was done at room temperature in a destaining solution with quick changes initially and leaving overnight in destainer later.

C.6.1.2 Staining Purified C_3 for Glycoprotein - (PAS-Staining)

The polyacrylamide (tube) gel after electrophoresis of purified C_3 (simple PAGE) were stained for glycoprotein adopting the procedure of Fairbanks et al. (1977).

Gels were removed from the tubes and kept in 7.5% v/v acetic acid for 1 hr at room temp, then transferred successively in to 0.2% (v/v)

periodic acid and Schiffs reagent, subsequently kept in the latter for 45 minutes at 4°C, the gels were destained at room temperature making several changes with 10% (v/v) acetic acid.

C.6.2 SDS-PAGE

Polyacrylamide gel (slab) electrophoresis under denaturing conditions, with reducing and non reducing environment was done. SDS-PAGE was carried out in 7% slab gel (0.15 x 14 x 18 cm) adapted from the procedure described by **Laemmli** (1970) in a pharmacia gel electrophoresis apparatus (GE-2/4-LS). A 7% separating gel with a 4 cm overlay of 4.0% stacking gel were prepared as per the procedure.

Purified-C_3 (2-3 mg) was equilibrated with **Laemmli** sample buffer and denatured at 100°C for 2 min.; same treatment was adopted for standard protein markers (2 mg each) viz. B Galactosidase 116 KD, Phosphorylase-B 97.4 kD, Bovine albumin 66 kD and Carbonic anhydrase 29 kD.

A modification effected, by mixing purified C_3 (2 mg) in **Laemmli** sample buffer devoid of mercapto-ethanol was incubated at 37°C for 30 min before heating at 100° for 2 min, for observing C_3 character under SDS-PAGE in non-reducing condition.

Samples (40-50 µl) were loaded in separate wells and electrophoresis was conducted initially at 25mA for 30-40 min. later increased to 45 mA. Temperature during the run was maintained at 20°C by using the electrophoresis chamber connected to cooling unit (JULABO). After the run the slab was stained and destained as described earlier.

C.6.2.1 Molecular Weight Determination by SDS-PAGE

For determining the M.W. of purified C_3 by SDS-PAGE, the standard proteins were run along with the test sample. The relative mobility (Rm) of all proteins were recorded. The distance of each band was taken

from top of separting gel to the centre of protein band. Relative mobility was calculated as :

$$Rm = \frac{\text{Distance of protein migrating after destaining}}{\text{Distance of tracking dye migration}}$$

A standard curve was plotted against Rm and Log M.W. of Marker proteins.

C.6.3 Carbohydrate Estimation of Purified C_3

C.6.3.1 Aminosugars (Hexosamine)

Hexosamine content of purified C_3 was determined according to the procedure of Davidson (1966).

Purified C_3 (in 0.02% SDS) was hydrolysed in sealed tubes with concentrated (12N) HCl at 85°C for 8 hr in a hot air oven. After hydrolysis, the contents were transferred to small beakers and HCl removed by drying the beakers in a dessicator in presence of flakes of sodium hydroxide; aliquotes of hydrolyzed samples were dissolved in distilled water and transferred into capped tubes and volume adjusted to 1.0 ml. One ml of acetyl acetone reagent (2 ml Acetylacetone dissolved in 98ml of 1 N Sodium carbonate) was added to the tubes and heated in a water bath at 90°C for 45 min. and then cooled in running tap water; to this 4 ml of 95% Ethanol and the contents mixed. One ml of p-dimethyl amino benzaldehyde (PDMAB) reagent was added and tubes vortexed. Incubation for 1 hr. was done at room temperature (30-32°C) and the colour developed was read at 540 nm. N-acetyl-glucosamine was used as the standard.

C.6.3.2 Neutral Sugars (Total Hexoses)

The neutral sugar content of the purified C_3 was estimated by the method of Johansen et al. (1960). A mixture of glucose and galactose was used as standard. To one ml of sample (500 µg), 8.5 ml of orcinol sulfuric

acid reagent was added; the mixture was heated for 45 min at 80°C in a water bath, cooled to room temp. and absorbance read at 505nm.

C.6.4 Effect of Trypsin

To elucidate the action of trypsin on purified C_3, IEP of trypisinised C_3 protein was performed. IEP of C_3 solution and control was done according to procedure described earlier. 100/ul of C_3 solution (1 mg/ml) was incubated at 37°C for 2 min, with a solution of 1% trypsin at a substrate enzyme ration of 1:1 v/v. The reaction was stopped by adding 0.5 ml of 1N HCl The same material without trypsin treatment served as control.

C.6.5 Cross Reactivity of C_3 to Other Species

C.6.5.1 Raising Anti C_3 Serum

The purified ovine C_3 was tested for antigenic cross-reactivity with other species. Anti-C_3 serum was raised in Newzealand rabbits according to the following protocol described for bovine C_3 by Menger and Auston (1984).

Date	Inoculum	Route
Zero	500/ug of C_3 in saline + FCA	1.5 ml emulsion S/c
11	200/ug of C_3 in IFA	-do-
22	-do-	-do-

After the final booster the animal was rested for 10 days and test bled for titer checking. IEP was performed to test the monospecificity of anti serum.

C.6.5.2 Cross Reaction

Simple immunodiffusion in 1% agar was done using 20/ul serum of goat, dog, ox, rabbit and human in the center well, and anti C_3 serum (20/ul serially diluted from 1:0 to 1:8 titer) in the surrounding wells. Veronal-buffer-saline (VBS) pH 7.4 containing 5mM EDTA was used for agar preparation and dilution of serum. Immunodiffusion was carried out as per the procedure described earlier.

C.6.6 Absorption Spectrum Scan

C_3 purified protein, was scanned in uv range for its peak maxima in (DU-7 Beckmann, USA) spectrophotometer. For this sample (500/ug) was appropriatly mixed in VBS buffer and the same buffer served as background.

C.6.7 Determination of Protein Concentration

Protein concentration was determined by the method of **Lowry** et al. (1951) or by spectrophotometric method of **Warburg** and **Christian** (1941) using; $mg/ml = 1.55 \times A_{280} - 0.76 \times A_{260}$.

C.6.8 Hydrodynamic Parameters

C.6.8.1 Caliberation of Sephadex G-200 Column

The sephadex G-200 column (90x2.5) was caliberated by determining the elution volume of proteins of known M.W. and stokes radii. The standard protein markers used were - cytochrome-C, Ovalbumin, Bovine serum albumin, and Alcohol dehydrogenase in the concentration of 4 mg/ml and a total of 5 ml of each was loaded. Fraction volume and flow rate were kept identical to that used in purification of post CM-Sephadex pool.

C.6.8.2 Stokes Radius

For purified C_3 this was calculated from their gel filteration data obtained after calibrating Sephadex G-200 column. Treatment of gel filtration

data according to Laurent and Killander (1964) was adopted to calculate stokes radius of purified C_3.

C.6.8.3 Intrinsic Viscosity

Intrinsic viscosity (n) for purified C_3 was calculated from the following equation (Tanford 1961) :

$$a = \left[\frac{3\ Mn}{10\ \pi\ N} \right]^{1/3}$$

Where -

N = Avogadro number = 6.023×10^{23}

a = Stokes radius

M = Molecular weight of C_3

C.6.8.4 Diffusion Coefficient

The following equation was used for calculating diffusion coefficient :

$$D = \frac{KT}{6\ \pi\ n\ a}$$

Where -

K = Boltzmann constant (1.386×10^{-16} erg/degree)

T = Absolute temperature (298°K)

n = Viscosity in poise (0.01 P)

a = Stokes radius

RESULTS

D. RESULTS

D.1 ANTI SERA CHARACTERIZATION

D.1.1 Anti C_3/I

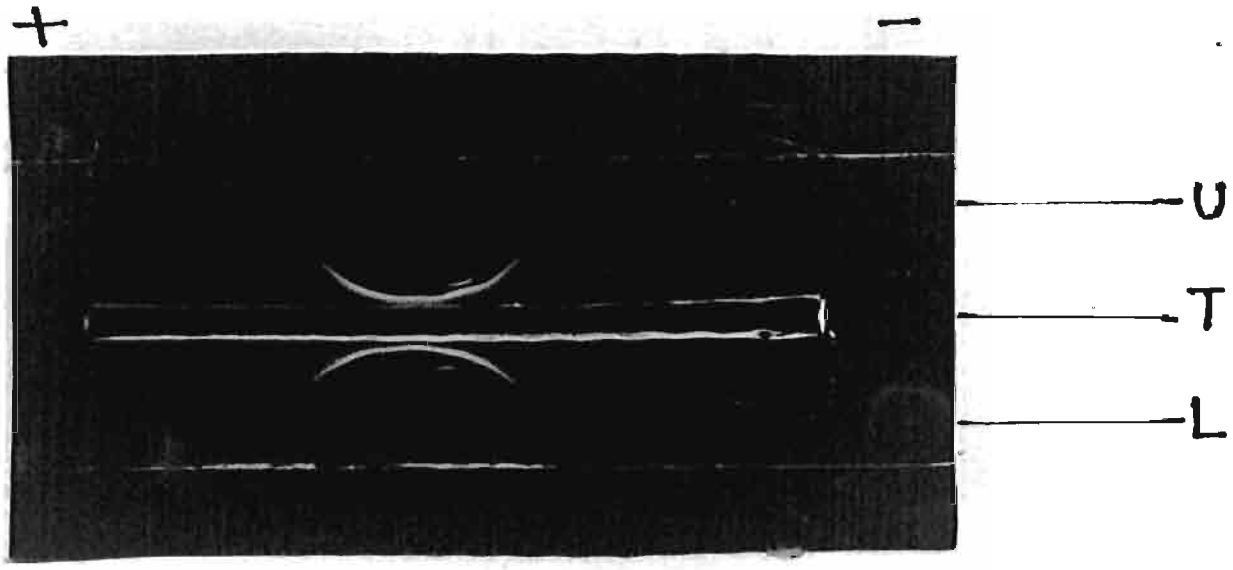
Anti C_3/I sera raised by Inulin method in a group of four New-zealand white rabbits was titer tested by Ouchterlony (DID) method. Two rabbits showed an antibody titer of (1:32); sera from these was harvested and after decomplementing stored for subsequent use. C_3/I was characterized by Immunoelectrophoresis (IEP) against sheep whole serum (PT-1A), a single precipitation arc was observed confirming its monospecificity.

D.1.2 Anti sheep whole sera (ASWS)

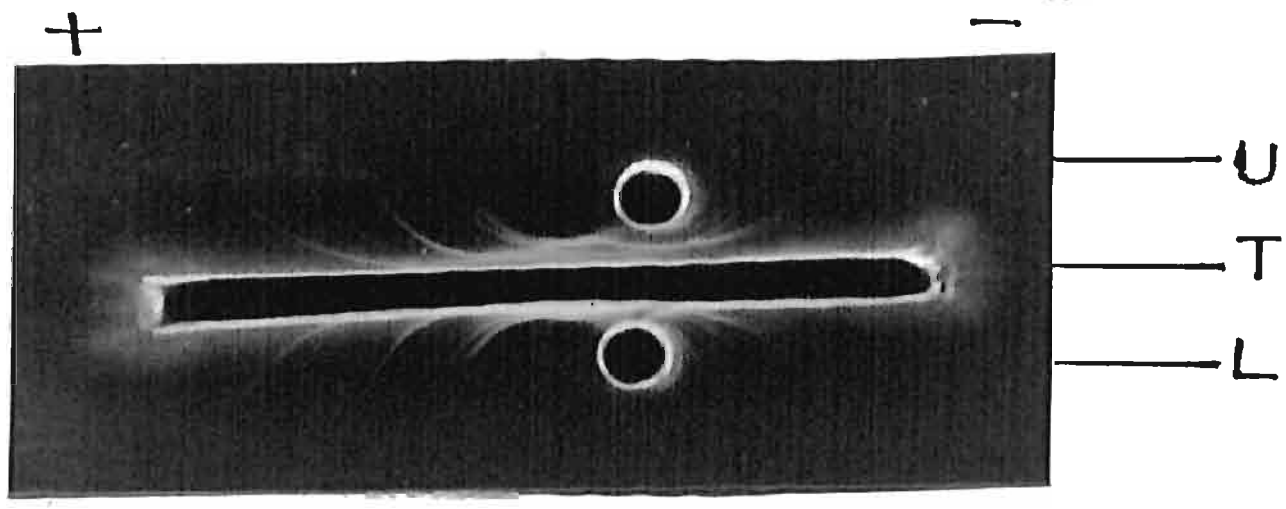
ASWS raised in rabbits was tested in similar manner by DID and characterized by IEP. This (PT-1B) revealed numerous precipitiating arcs as expected; ASWS with high antibody titer (1:24) was stored after decomplementing for subsequent use.

D.1.3 Anti purified C_3

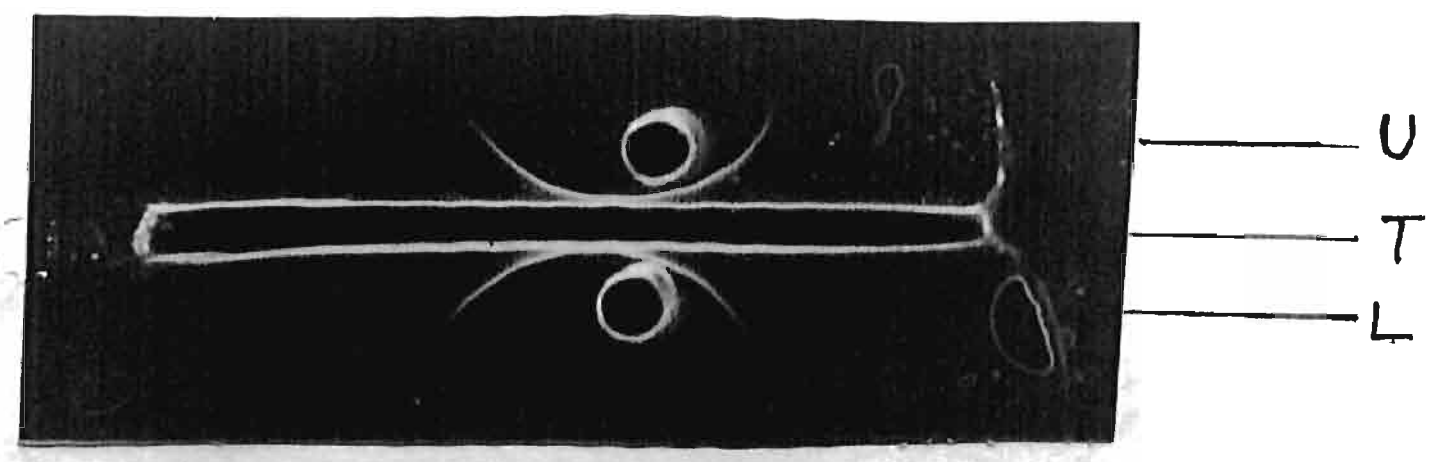
Purified C_3 obtained after final purification step was used for raising antisera in one rabbit. Titer testing by DID showed a titer of 1:20, IEP against sheep whole sera showed a single sharp precipitating arc, (PT-1C) revealing the recognition of a single protein from sheep whole sera.



PT-1A



PT-1B



PT-1C

D.2. PURIFICATION OF SHEEP C_3

D.2.1 Precipitation by PEG-6000

Fresh sheep whole serum (150 ml) used for purification of C_3 had a protein concentration of 65.4 mg/ml. On addition of PEG-6000 (7% w/v) the precipitate which was obtained was dissolved in 140 ml of 25 mM phosphate buffer, pH 7.0 containing 20 mM EACA and 2.5 mM EDTA. The post-PEG-precipitate thus obtained was assayed for C_3 presence by DID using anti C_3/I sera. A total protein of 2.4 gm was recorded as C_3 containing post PEG-precipitate.

D.2.2 DEAE-Sephadex (A-50) anion exchange chromatography

The post PEG-6000 precipitated material (140 ml) was loaded in a DEAE-SEphadex-A-50 column (2.5 x 46). A linear gradient of NaCl upto 0.45M was used for elution (820 ml total) after the initial wash. The elution profile (Fig.1) showed four peaks, the last peak fraction number 156 to 166 (88ml) reacted positively to anti C_3/I sera in DID, these fractions were eluted out by the salt gradient range of 0.24M NaCl to 0.29M NaCl. These fractions were pooled and concentrated to 53 ml; protein content of post-DEAE-Sephadex pool was recorded as 136 mg.

D.2.3 CM-Sephadex (C-50) cation exchange chromatography

The pooled and concentrated post DEAE-material was loaded in a well equiliberated CM-Sephadex C-50 column (2.5 x 60) after exhaustive dialysis of the Post-DEAE-Pool against the eluting buffer (Tris-acetate 20mM, pH 6.6, 5mM EDTA). The elution profile (Fig.2) revealed two peaks joined together. The anti-C_3/I reacting fraction number 22 to 30 (54 ml) were found in the unbound portion of elution profile. No C_3 presence could be confirmed in the fractions obtained after using linear gradient of NaCl to 0.3M (940ml total).

The C_3 positive fractions (54ml) were subsequently pooled and concentrated to 30 ml; a protein concentration of 1.51 mg/ml and total of 45.3 mg was recorded.

TABLE 1 : PROTEIN CONCENTRATION DURING - PURIFICATION OF OVINE C_3

Purification step	Protein concentration mg/ml	Volume ml	Total Protein mg
PEG-6000 treatment	18.723	140	2641
DEAE-Sephadex anion exchange chromatography	2.575	54	136.475
CM-Sephadex cation exchange chromatography	1.510	30	45.3
Sephadex G-200 gel filtration	0.735	20	14.715

FIG.-1 Anion exchange chromatography of PEG-6000 precipitate

140 ml of PEG precipitate (2641 mg protein) was applied to DEAE-Sephadex (2.5 cm x 46 cm) column equilibrated with 25mM phosphate buffer pH 7.0 the column was eluted at a flow rate of 50ml/hr. 8 ml fraction were collected and monitored at 280 nm O——O. Fractions were assayed for C_3 presence by Ouchterlony method |——|.

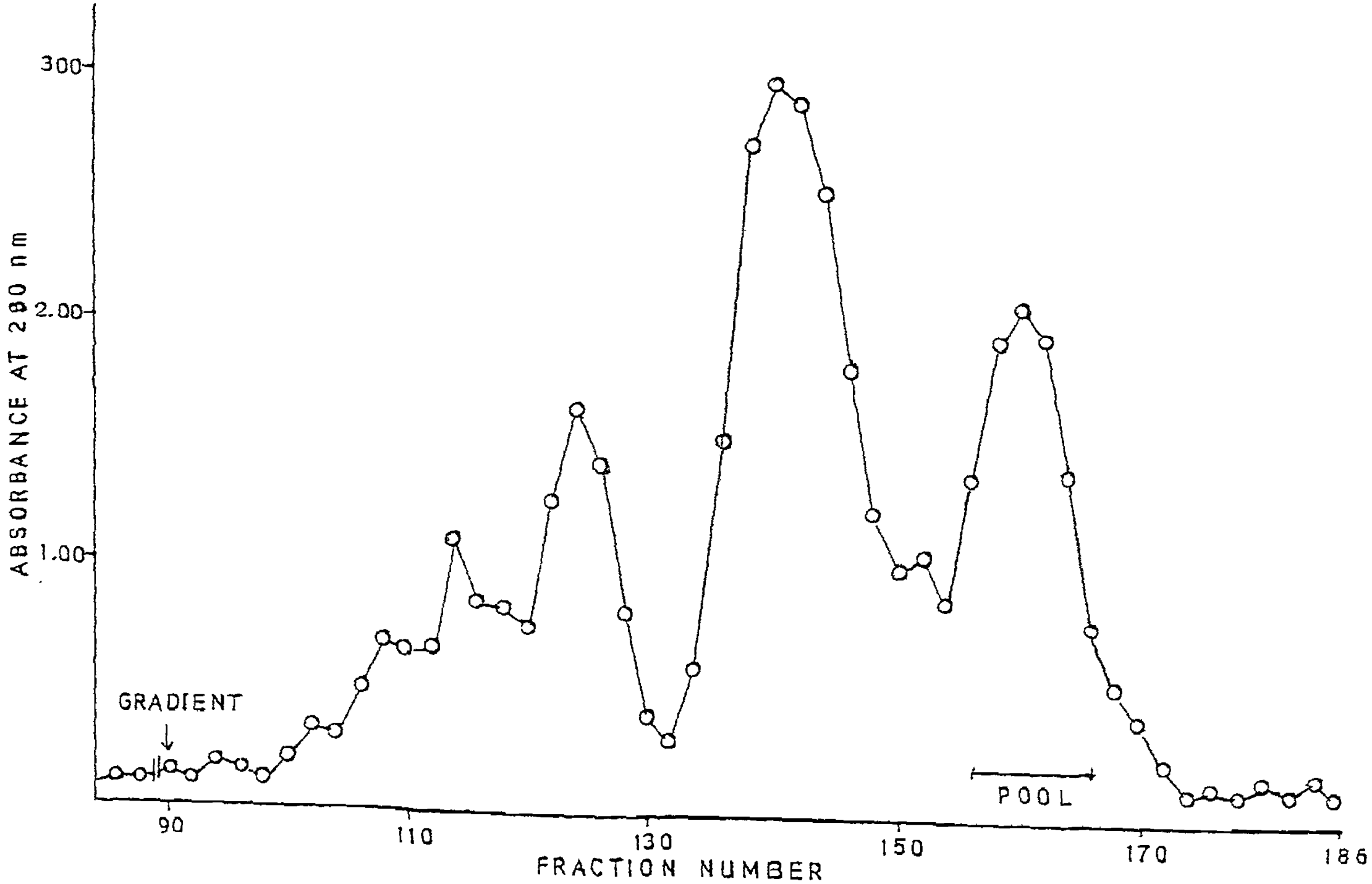

ABSORBANCE AT 280 nm
3.00
2.00
1.00
GRADIENT
POOL
90
110
130
150
170
186
FRACTION NUMBER

Fig _ 1

FIG.-2 Cation exchange chromatography of post DEAE-pool.

53 ml of post DEAE pool (136.47 mg protein) was applied to CM-Sephadex column (2.5 cm x 60 cm) equilibrated with 20mM Tris acetate pH 6.6 buffer. The column was eluted at a flow rate of 40 ml/hr. 6 ml fractions were collected and monitored at 280 nm O——O. Fractions were assayed for C_3 presence by Ouchterlony method |——|.

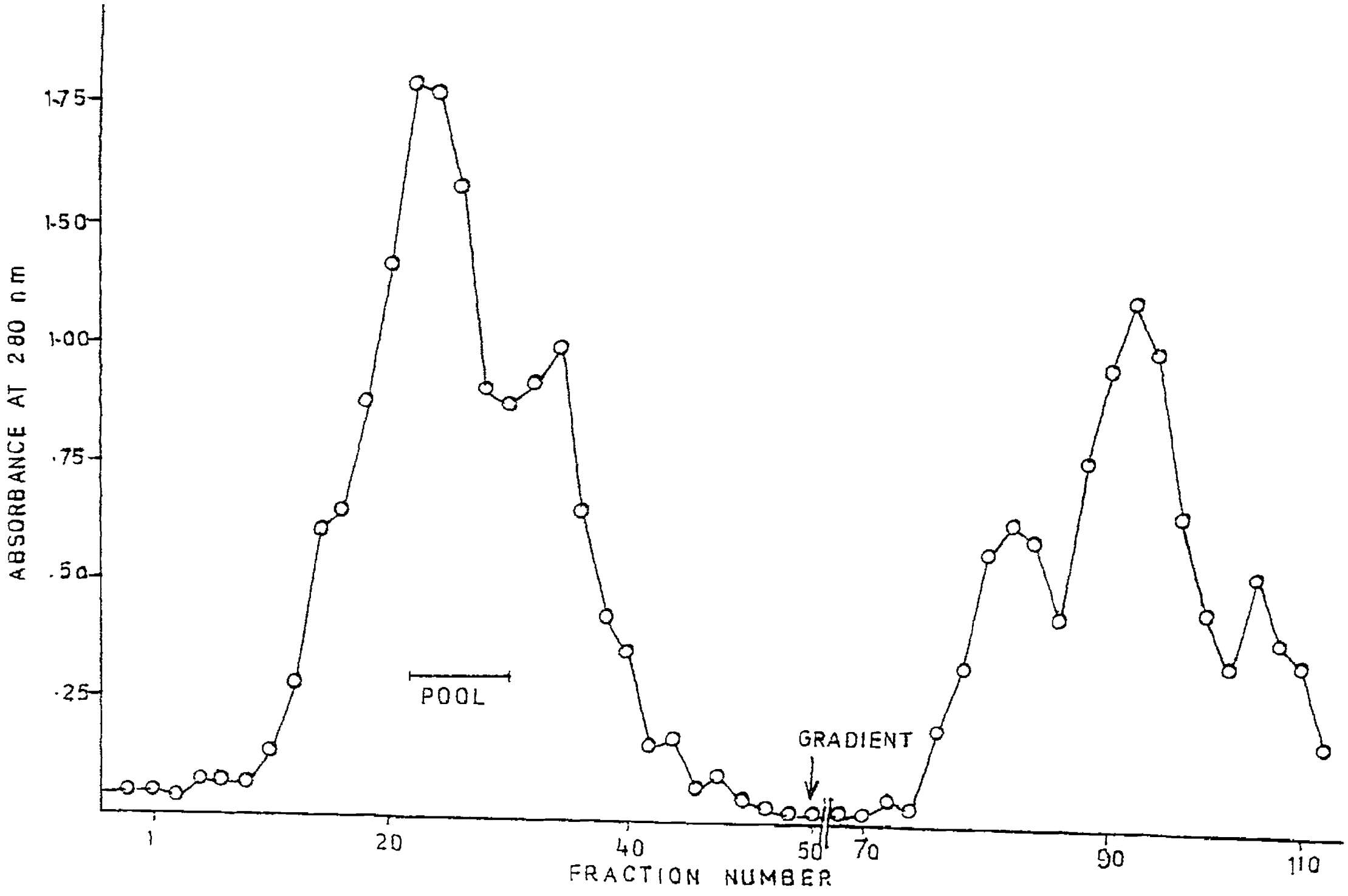

ABSORBANCE AT 280 nm
1·75
1·50
1·00
·75
·50
·25
POOL
GRADIENT
1
20
40
50
70
90
110
FRACTION NUMBER

Fig - 2

D.2.4 Sephadex G-200 gel filtration

The Post-CM-Sephadex pool of 30ml was further fractionated in a column (90 x 2.5) of Sephadex-G-200. The elution profile (Fig.3) indicated two sharp peaks I and II. Fractions 72-80 (21ml) from Peak I and fractions 86-90 (13 ml) from Peak II both gave precipitating line in DID against anti C_3/I. These fractions of peaks I and II were pooled separately and concentrated to 5 ml and 4 ml respectively. In contrast to DID, IEP of Peak II Pool revealed a cleaved arc and a separate shifted arc (PT-3B) while the peak I pool gave a single sharp arc against anti sheep whole sera, in IEP. The protein concentration of peak I pool (henceforth called purified C_3) was 0.735 mg/ml and a total of 14.2 mg was recorded (the protein yield of peak I pool recorded, corresponded to six runs of 5ml each aliquote from CM-Sephadex pool).

D.3 CHARACTERIZATION OF PURIFIED C_3

D.3.1 Immunoelectrophoresis (IEP)

The purified C_3 when subjected to IEP using anti-purified C_3 serum (ASWS), recognised a single protein from ASWS as evident from a single sharp precipitation arc (PT-1C). Purified ovine C_3 was also run in IEP against anti-C_3/I serum were it developed a sharp arc (PT-3A) that was positionally comparable with the arc developed by sheep whole serum against anti-C_3/I.

D.3.2 *Comparative immunoelectrophoresis*

This was performed to monitor the stepwise purification of C_3, which is quite evident by the reduction in precipitating arcs as the purification proceeded and finally a single arc was obtained with the post-G-200 (Peak I) pool. ASWS (250ml) was used for development in each trough (PT-2B).

FIG.-3 Gel filtration elution profile of post CM-Sephadex pool.

30 ml of post CM-Sephadex pool (45.3 mg protein) was applied to Sephaxed-G-200 column (90 x 2.5 cm) equlibrated with phosphate buffer. 25 mM pH 7.0 with 25mM EACA 5mM EDTA 150mM NaCl. The column was eluted at a flow rate of 10 ml/hr fraction of 2.65ml collected and monitored at 280 nm O——O; C_3 presence was assayed by Ouchterlony method |——|.

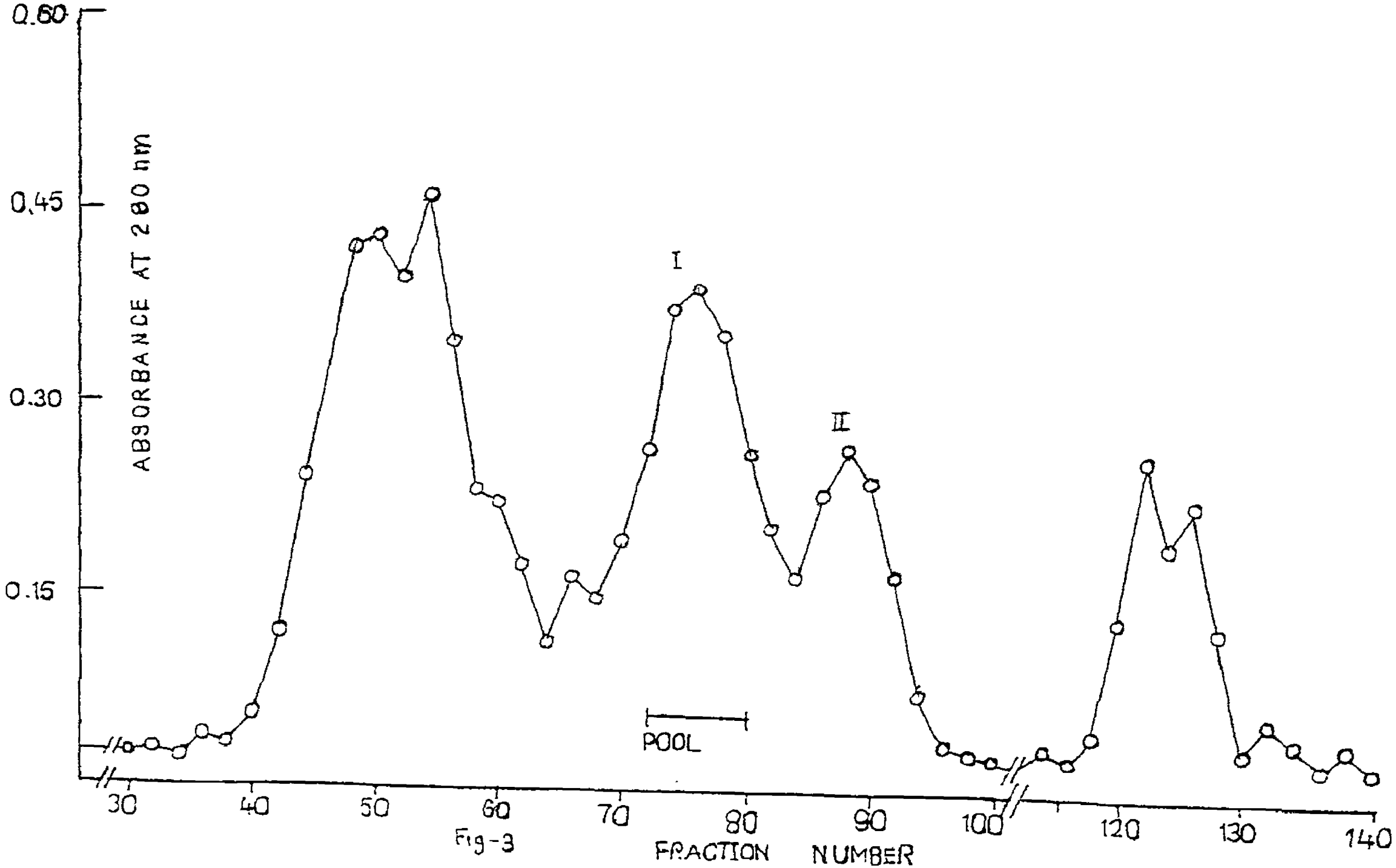

0.60
0.45
0.30
0.15
ABSORBANCE AT 280 nm
I
II
POOL
30
40
50
60
70
80
90
100
120
130
140
FRACTION NUMBER

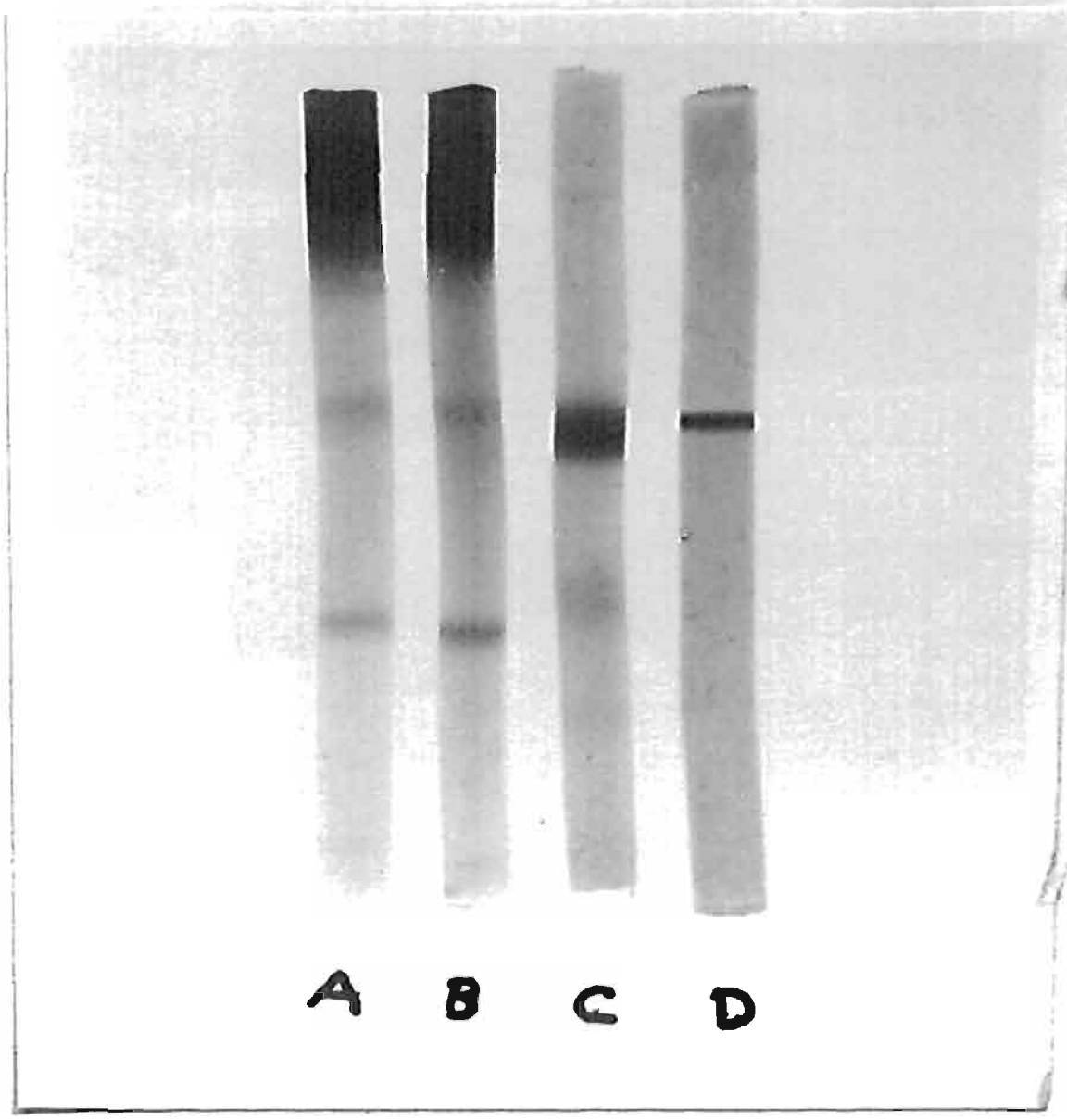
Fig-3

MONITORING OF PURIFICATION PROCESS BY PAGE AND IEP

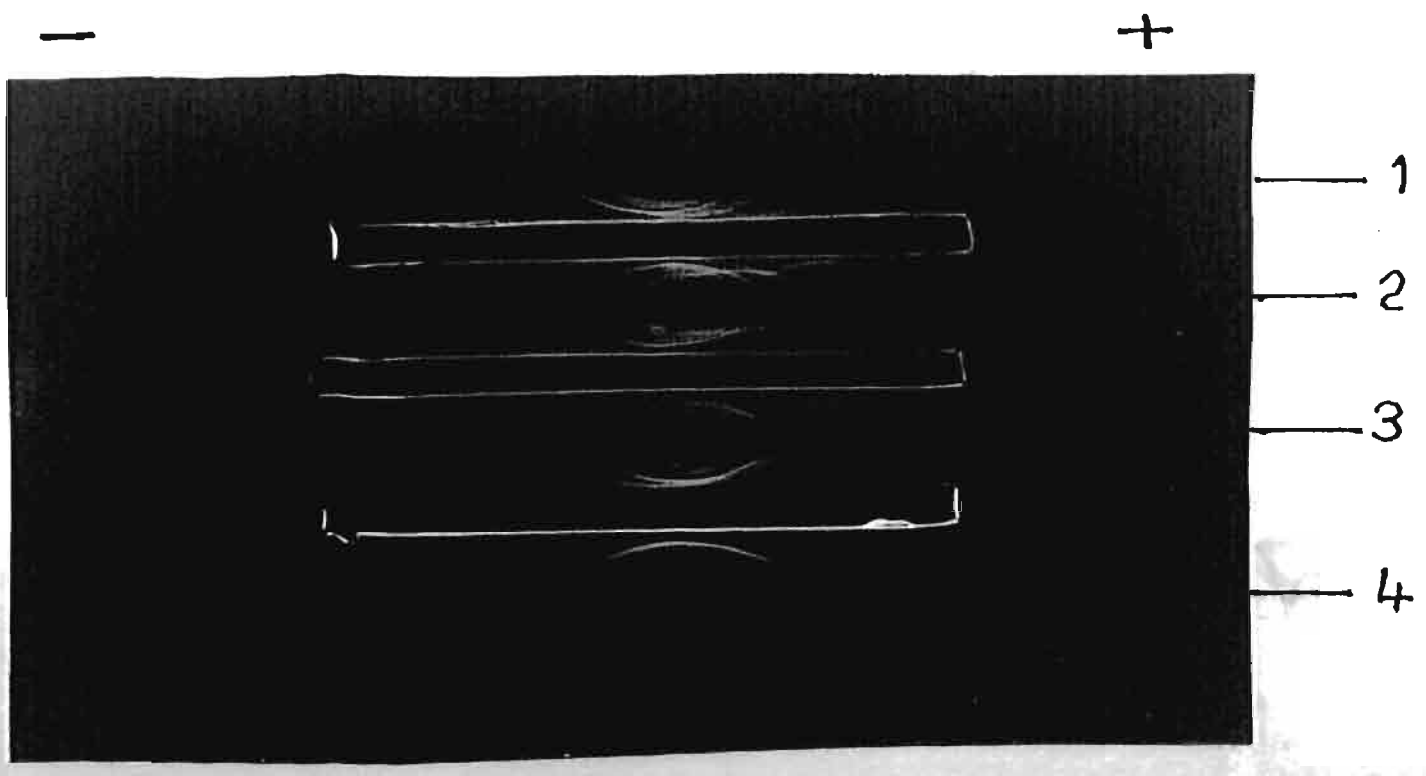
PT-2A Simple PAGE of (A) PEG precipitate (B) Post DEAE Sephadex Pool (C) Post CM-Sephadex Pool (D) Post G-200 (Peak I) Pool.

PT-2B Comparative IEP

1 - PEG precipitate
2 - Post DEAE-Sephadex pool
3 - Post CM-Sephadex pool
4 - Post G-200 Peak I pool.



PT – 2A



PT – 2B

PT-3A IEP-Pattern of purified C_3 and sheep serum against Anti-sheep C_3 serum.

U - Purified C_3 (Peak I)
T - Anti-purified C_3
L - Sheep whole serum

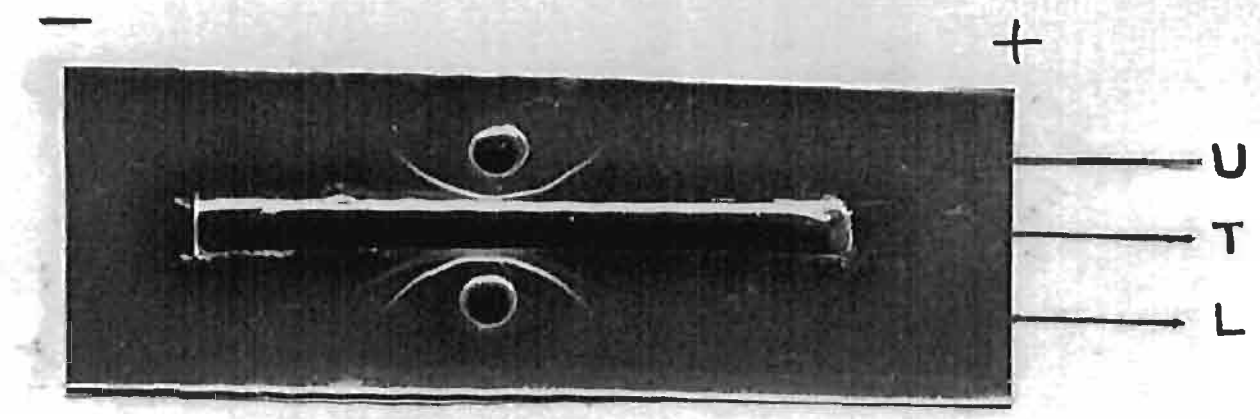
PT-3B IEP-Pattern of G-200-peak II anti-C_3/I reacting G-200 pool

U and L - Peak II sample

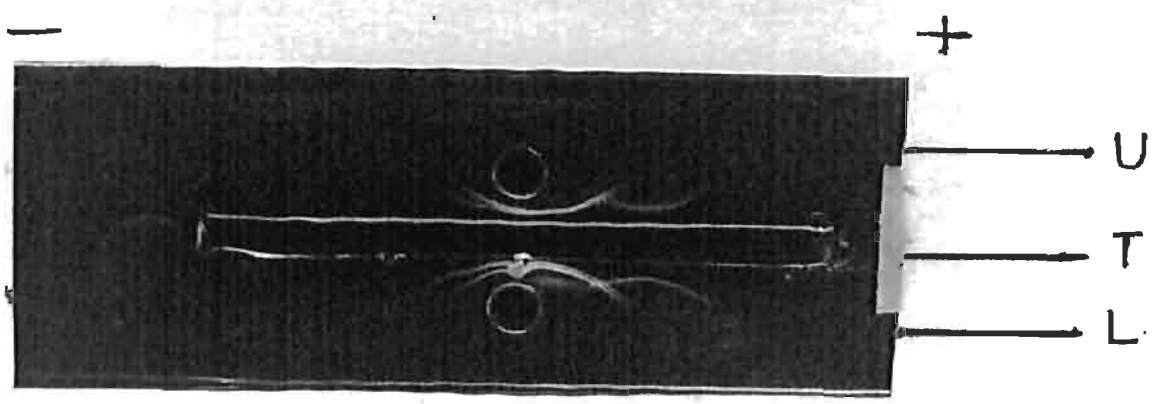
T - ASWS

PT-3C SDS-PAGE (R) - (Peak II) post G-200 fraction,

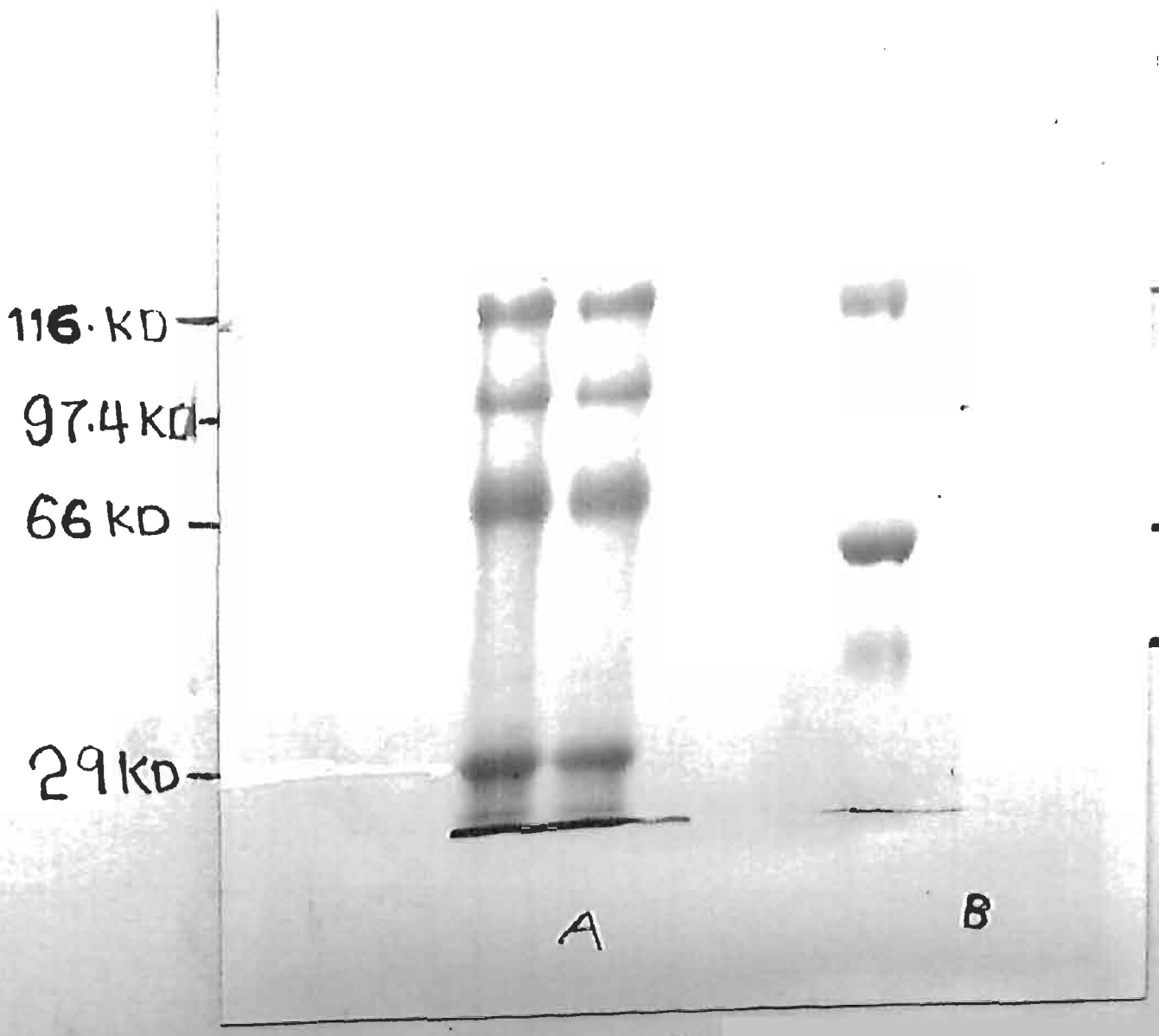
A - SDS-PAGE Markers
B - Peak II - Bands



PT - 3A



PT - 3B



PT - 3C

D.3.3 Polyacrylamide gel electrophoresis (PAGE)

D.3.3.1 Simple PAGE

Disc electrophoresis using 7.5% polyacrylamide gel, was conducted for pools obtained after each purification step. There was progressive reduction of the number of bands, indicating removal of undesired proteins and a sharp band observed for post G-200 (Peak I) pool, confirmed the charge homogeneity of the purified protein (PT-2A).

D.3.3.2 Sodium dodecyl-sulfate (SDS) PAGE

To probe the existence of disulphide linkage and number of polypeptide chains in the post-G-200 (Peak I) C_3/I reacting pool, SDS-PAGE was run using 7% Lamelli slab gel. A similar run was conducted for peak-II pool also.

A single band (PT-4A) was observed in the non reducing-condition for the (Peak I) Post G-200 pool. Under reducing conditions peak I gave two bands (PT-4B) and peak II pool showed three bands (PT-3C).

D.3.4 Molecular weight determination of purified C_3

D.3.4.1 SDS-PAGE

Protein markers of known molecular weight (SDS-PAGE grade) were run in the slab (7%) gel, samples of purified C_3 (Peak I and Peak II) were also run in the same slab in separate well spaced wells. The relative mobility of each marker protein band and bands obtained from sample tracks was calculated (Table-2).

A graph was plotted with log MW of marker proteins against their R_m value. A linear cuve (Fig.4) was obtained by least square method which followed the equation :

$$\text{Log M.W.} = 5.542 - 1.23 \times R_m \text{----------(1)}$$

FIG.-4 **Determination of molecular weight of purified C_3 by SDS-PAGE.**

The relative mobility of SDS-protein markers in SDS-PAGE were plotted against logarithm M.W. of protein marker (1) Carbonic Anhydrase 29 kD (2) Bovine Serum Albumin 66 kD (3) Phosphorylase-B 97.4 kD (4) B Galactosidase 116 kD.

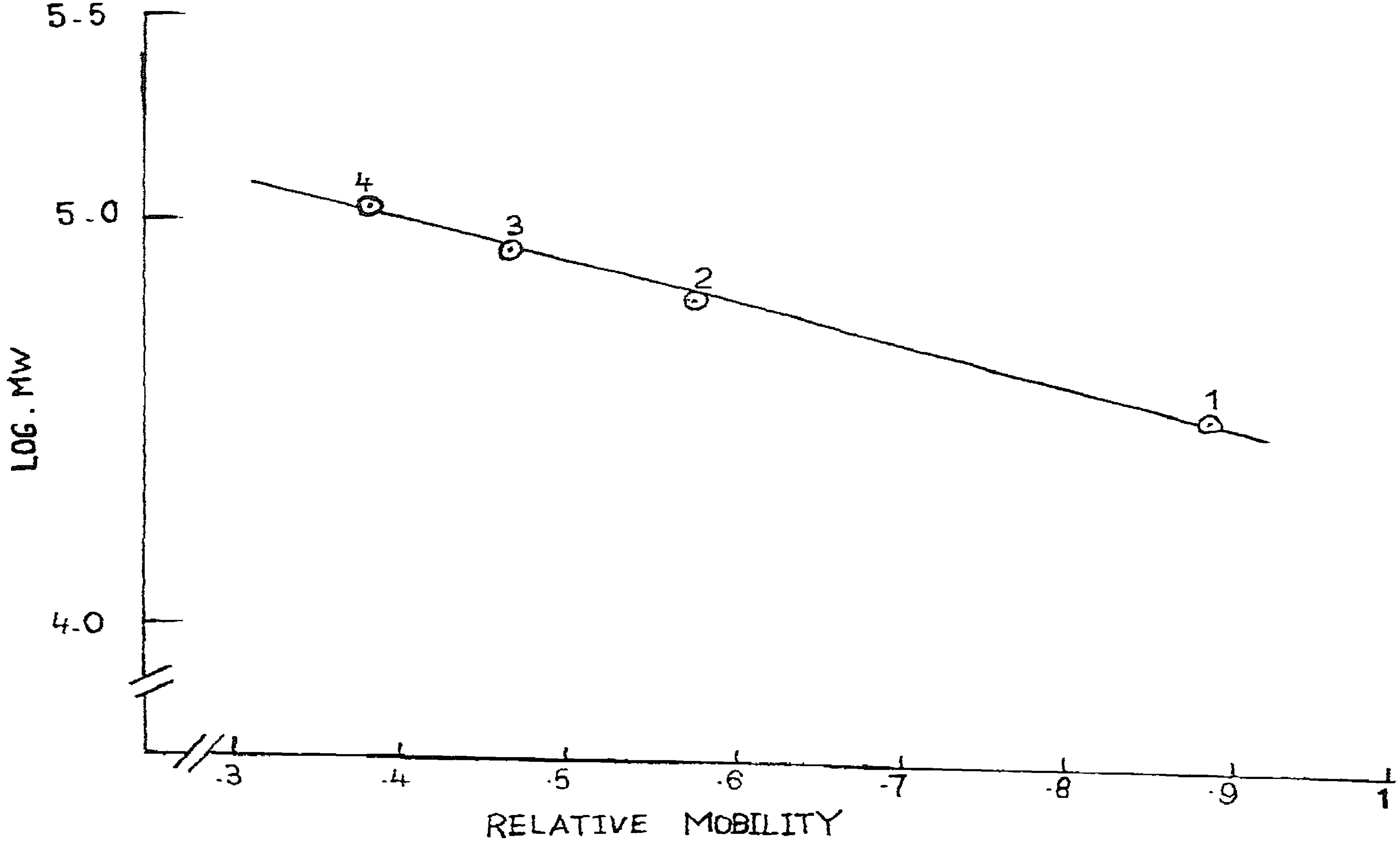


Fig-4

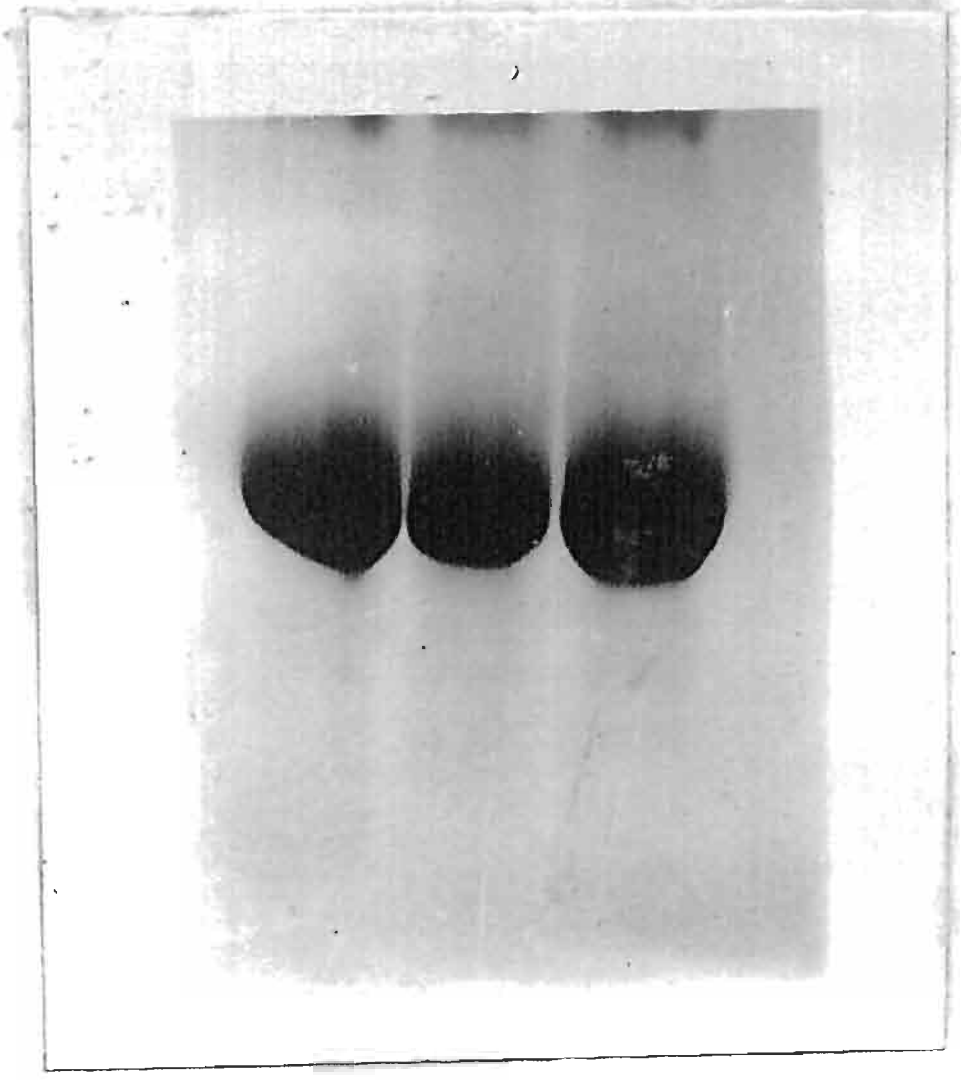
SDS-PAGE OF PURIFIED C_3

PT-4A SDS-PAGE under non-reducing conditions (NR) - All three Lanes had purified-C_3

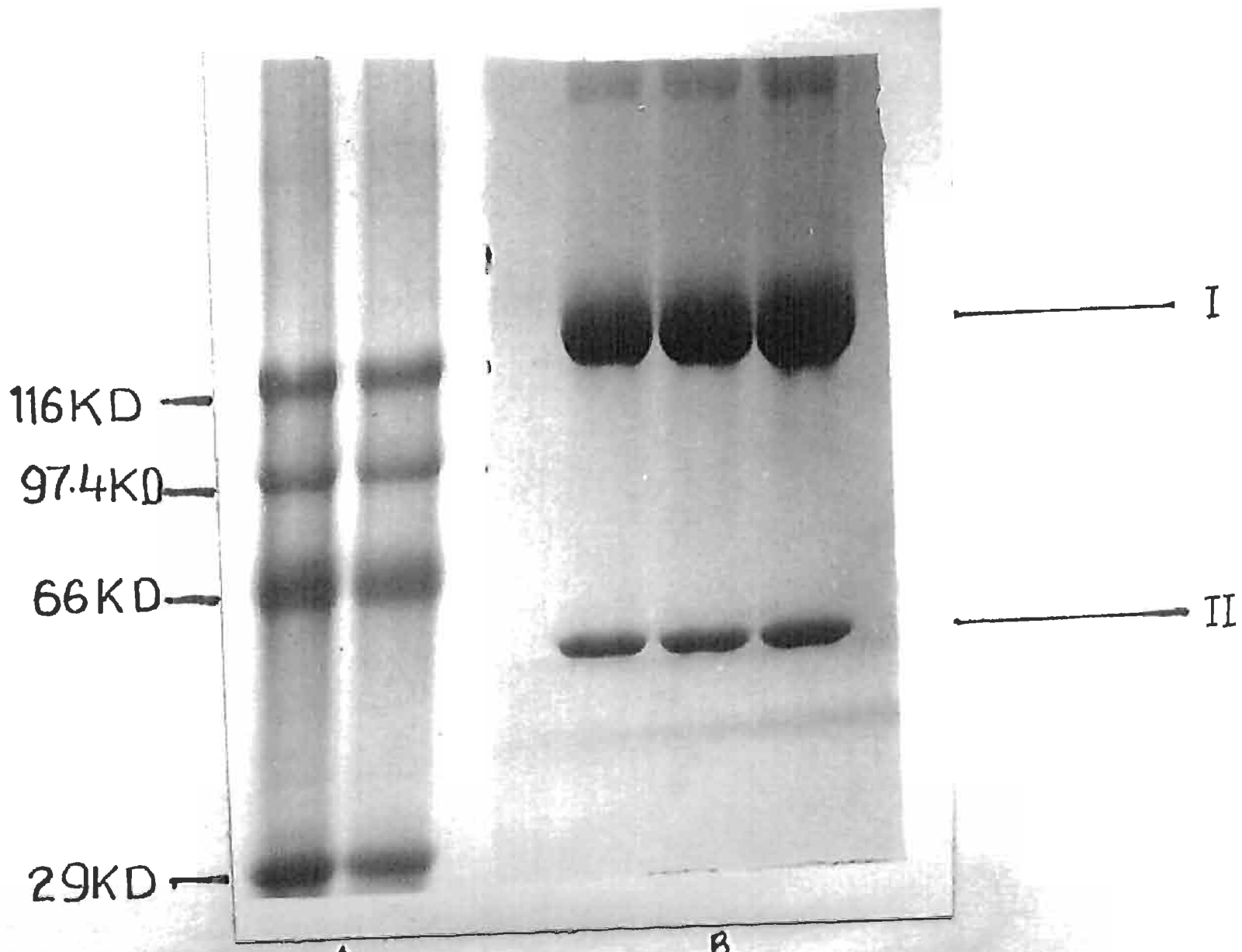
PT-4B Purified C_3 pattern on SDS-PAGE under reducing condition

A - M.W. markers

B - All 3 Lanes - Purified C_3.



PT-4A



PT-4B

TABLE 2 : RELATIVE MOBILITIES AND MOLECULAR WEIGHTS OF MARKER PROTEINS/PURIFIED OVINE C_3

Protein	M.W. dalton	Log M.W.	Distance covered (cm)	R_m
B. Galactosidase	116000	5.064	5	0.384
Phosphorylase-B	97400	4.986	6	0.461
Bovine serum albumin	66000	4.819	7.5	0.576
Carbonic Anhydrase	29000	4.462	11.5	0.884
Sample (C_3)				
Peak I				
I Band (Upper)	128176*		4.60	0.353
II Band (Lower)	54803*		8.50	0.653
Peak II	98453*		5.90	0.438
	43022*		9.60	0.738
	35431*		10.5	0.8076

*Calculated from equation - (1)

From equation (1) using the R_m calculated for the two bands for (peak I) purified C_3, MWs of 128176 dalton for the upper band (I) and MW of 54803 dalton for the lower band (II) were calculated giving a aggregate MW of 182979 dalton to the purified C_3.

Similarly for the three bands (I,II,III) of peak II pool, MWs of 96562, 43022 and 35431 dalton, respectively were calculated, giving an aggregate of 175015 dalton.

D.3.4.2 Mol. wt. determination by gel filtration

D.3.4.2.1 Caliberation of Sephadex G-200 column

The total volume of (90 x 2.5 cm) Sephadex G-200 column was calculated as 441 ml ($V_t = \prod r^2 h$). Void volume V_o was found to be 138 ml, as determined by gel filteration behaviour of Blue dextron-2000.

The column was caliberated by passing four protein markers (gel filteration grade) of known molecular weight and Stokes radii. The elution volume V_e of each marker was obtained (Table 3). A constant column loading volume of 5 ml, flow rate of 10 m/hr and fraction volume of 2.65 ml were faithfully followed for all the samples.

D.3.4.2.2 Molecular weight calculation

A graph was plotted for Log MW of marker proteins against their V_e/V_o ratio. A linear curve (Fig.5) was obtained by least square method which obeyed the equation.

$$\text{Log M.W.} = 6.548 - 0.860 \times V_e/V_o \text{--------(2)}$$

From the V_e/V_o ratio of purified C_3 (V_e for C_3 taken as 202 ml, corresponding to fraction number 76, having the maximum absorbance at 280 nm) a MW of 186595 dalton was calculated for purified C_3 (Peak I) from equation (2).

TABLE 3 : GEL FILTRATION CALIBRATION DATA FOR MARKER PROTEIN/PURIFIED OVINE C_3 IN SEPHADEX G-200 COLUMN.

Protein	M.W. dalton's	10g M.W.	V_e ml.	V_e/V_o
Cytochrome-C	12,400	4.093	394	2.855
Ova albumin	45,000	4.653	304	2.210
Bovine serum albumin	66,000	4.819	277	2.007
Alcohol dehydrogenase	150,000	5.176	220	1.594
Purified C_3	186,595*		205	1.485

*Calculated from equation (2).

FIG.-5 **Determination of M.W. by gel filtration.**

20 mg of each protein marker in 5 ml elution buffer was applied to Sephadex G-200 column (90 x 2.5 cm), Equilibrating buffer (Legend FIG.-3) flow rate of 10 ml/hr and fractions of 2.6ml was throughout maintained. From V_e of markers the ratio V_e/V_o was plotted against log M.W. of respective protein marker (1) cytochrome C 12.4 kD (2) Ovalbumin 45 kD (3) Bovine serum albumin 66 kD (4) Alcohol dehydrogenase 150 kD.

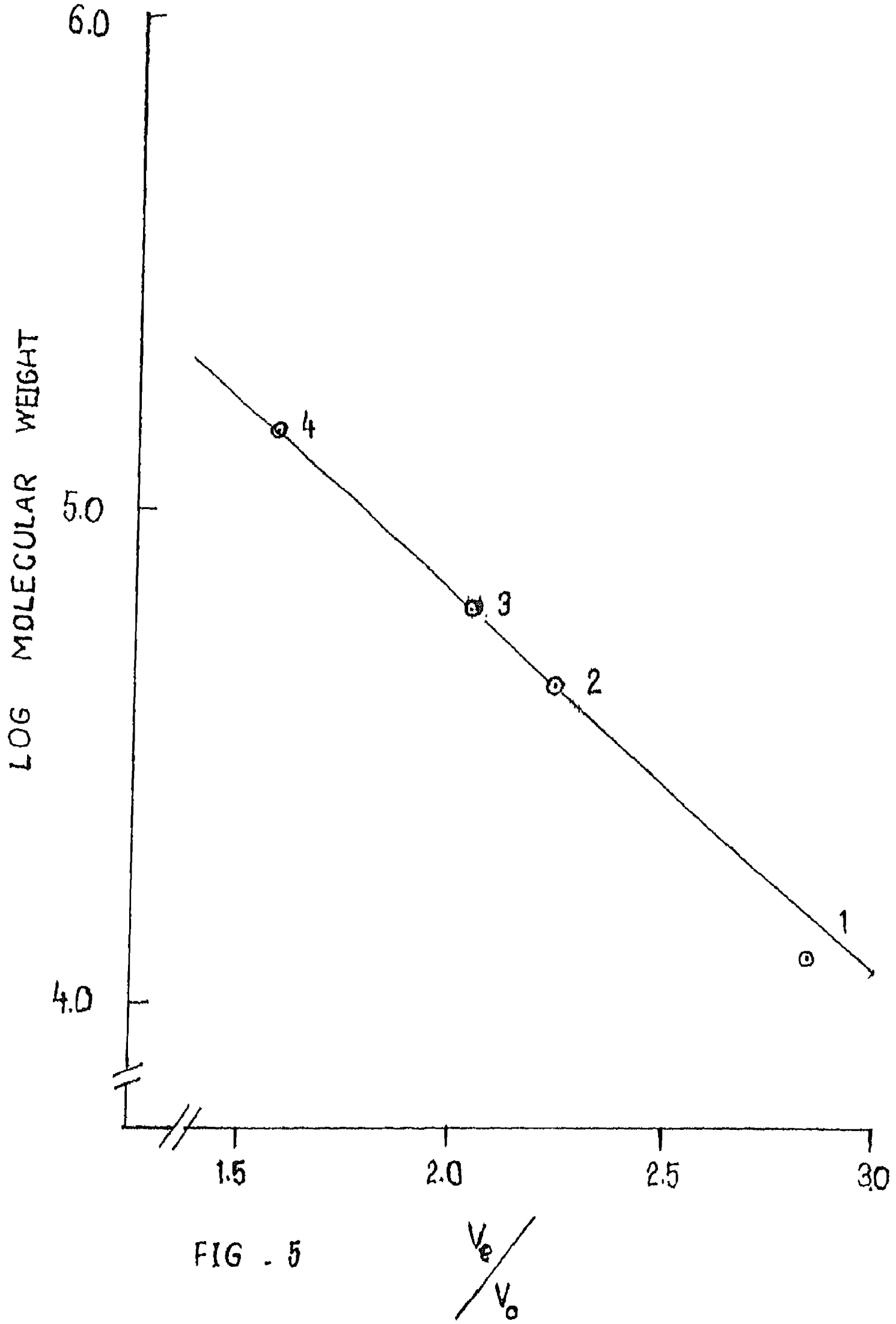


FIG . 5

D.3.5 Carbhohydrate estimation

D.3.5.1 Amino sugars (Hexosamine)

The purified C_3 protein did not show a positive test for the presence of amino sugars when tested by Davidson (1966) method.

D.3.5.2 Neutral sugar's (Hexoses)

The presence of neutral sugars in purified C_3 was confirmed, when tested for the same by Orcinol-sulphuric acid method. Glucose and Galatose 1:1 v/v mixture was used as standards, neutral sugar content of purified C_3 was 2.2%.

D.3.5.3 PAS staining

That the purified ovine C_3 is a glycoprotein was demonstrated by PAS staining after polyacrylamide gel electrophresis of native protein. A faint pink coloured band against pinkish background was observed.

D.3.6 Action with trypsin

IEP of the trypsinised sample of purified C_3 showed characteristic drift of the precipitated arc towards anode when compared with the control (PT-5A

D.3.7 UV absorption spetra

The absorption spectrum of the purified ovine C_3 between 250-320 was scaned. The absorption maxima was found to be at 281 nm in the form of a sharp peak.

D.3.8 Cross reactivity of purified C_3

Cross reaction by sera of five different species viz. goat, ox, dog, rabbit and man to the purified C_3 was ascertained by DID. Positive cross

TABLE 4 : CROSS REACTIVE OF DIFFERENT SPECIES TO OVINE C_3.

Species	Anti-purified C_3 serum dilution	Result
Goat	1:0	+
	1:1	+
	1:4	+
	1:8	+
Ox	1:0	+
	1:1	+
	1:4	+
	1:8	-
Human	1:0	+
	1:1	-
	1:4	-
	1:8	-
Dog	1:0	-
	1:1	-
	1:4	-
	1:8	-
Rabbit	1:0	-
	1:1	-
	1:4	-
	1:8	-

PT-5A IEP-Pattern of trypsinized C_3/control against anti-C_3/I serum

U - Trypsinized C_3

T - Anti-C_3/I serum

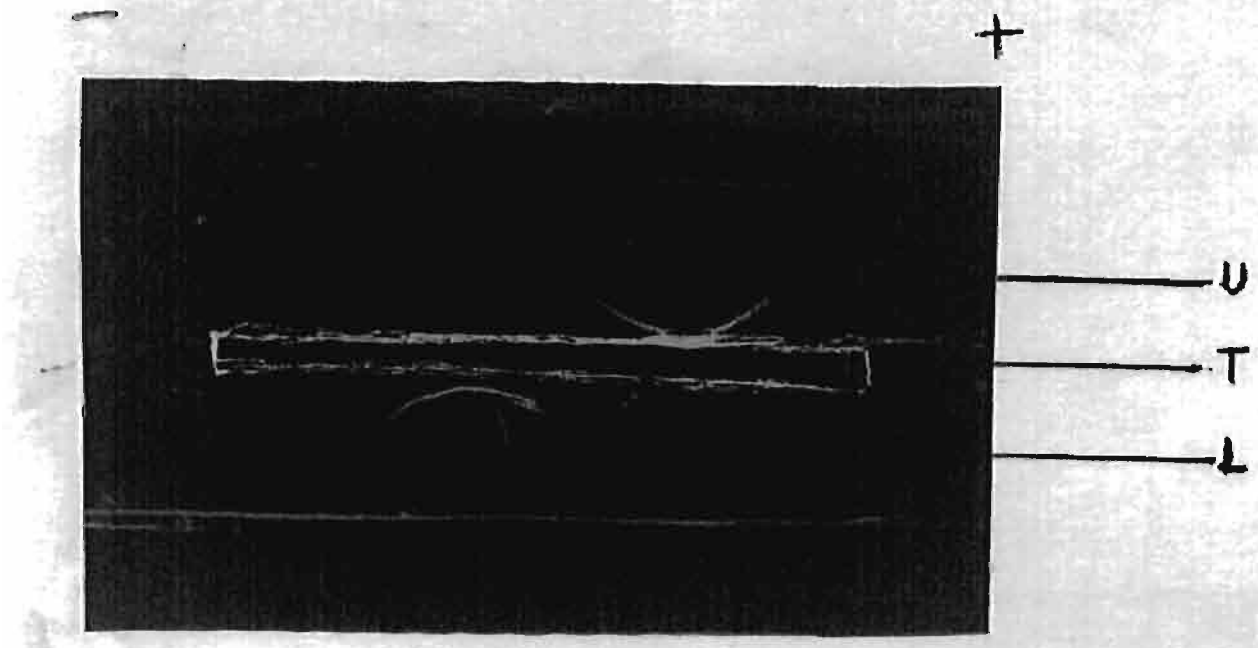
L - Un Trypsinized control

PT-5B Cross reactivity of anti-purified C_3

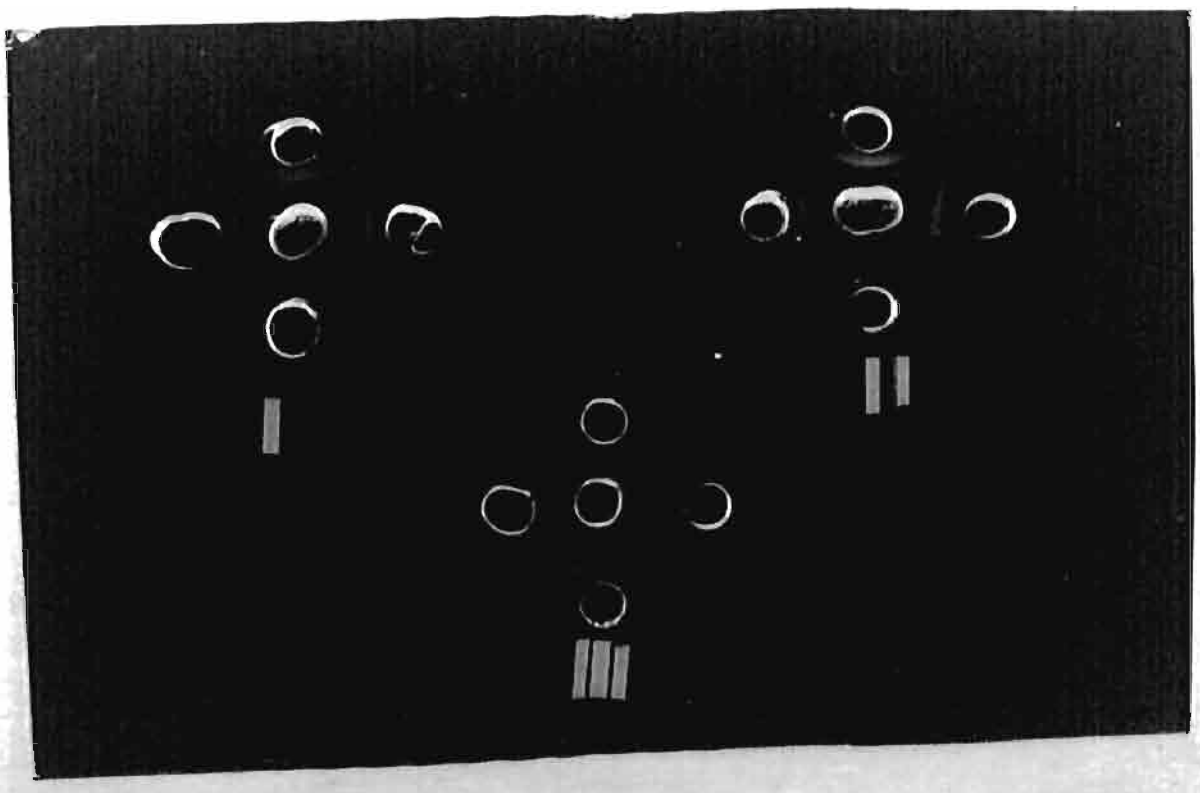
I - Against goat serum (Centre well)
Anti-purified C_3 (Surrounding wells)

II - Against cattle serum (Centre well)
Anti-purified C_3 (Surrounding wells)

III - Against human serum (Centre well)
Anti-purified C_3 (Surrounding wells)



PT-5A



PT-5B

reaction was seen (PT-5B) in case of goat and ox, while human sera gave positive reaction with undiluted antipurified C_3 only (1:0) and no reaction was observed against the sera of dog and rabbit (Table 4).

D.3.9 STOKES RADIUS

Stokes radius of purified C_3 was determined from their gel filtration behaviour on caliberated Sephadex G-200 column.. Values of (-Log K_{av}), stokes radius are given in Table 4.

Treatment of gel filteration data according to Laurent and Killander (1964) yeilded a curve (Fig.6) when the values of $(-10g\ K_{av})^{\frac{1}{2}}$ were plotted against stokes radii of marker protein. By least square analysis equation that obey the curve was

$$(-10g\ K_{av})^{\frac{1}{2}} = 0.016 \times (\text{Stokes Radius} + 0.023 \quad \text{-------(3)}$$

The Stokes radius of 49.19 A° was calculated for purified C_3 from equation (3).

D.10 Intrinsic viscosity

Values of intrinsic viscosity for purified C_3 was calculated from the following equation (Tandford, 1961).

$$a = \left[\frac{3\ M}{10\ \pi\ N}\right]^{1/3} \quad \text{--------(4)}$$

Where N is Avagadro number (6.02×10^{23}) "a" Stokes radius, and M is molecular weight of C_3 purified. The n (intrinsic viscosity) for purified C_3 was obtained as 4.01 gm/ml.

FIG.-6 Determination of Stokes radii of C_3 by Laurent and Killander (1964).

The protein markers used were (1) Cyctochrome C (2) Ovalbumin (3) Bovine serum albumin (4) Alcohol dehydrogenase.

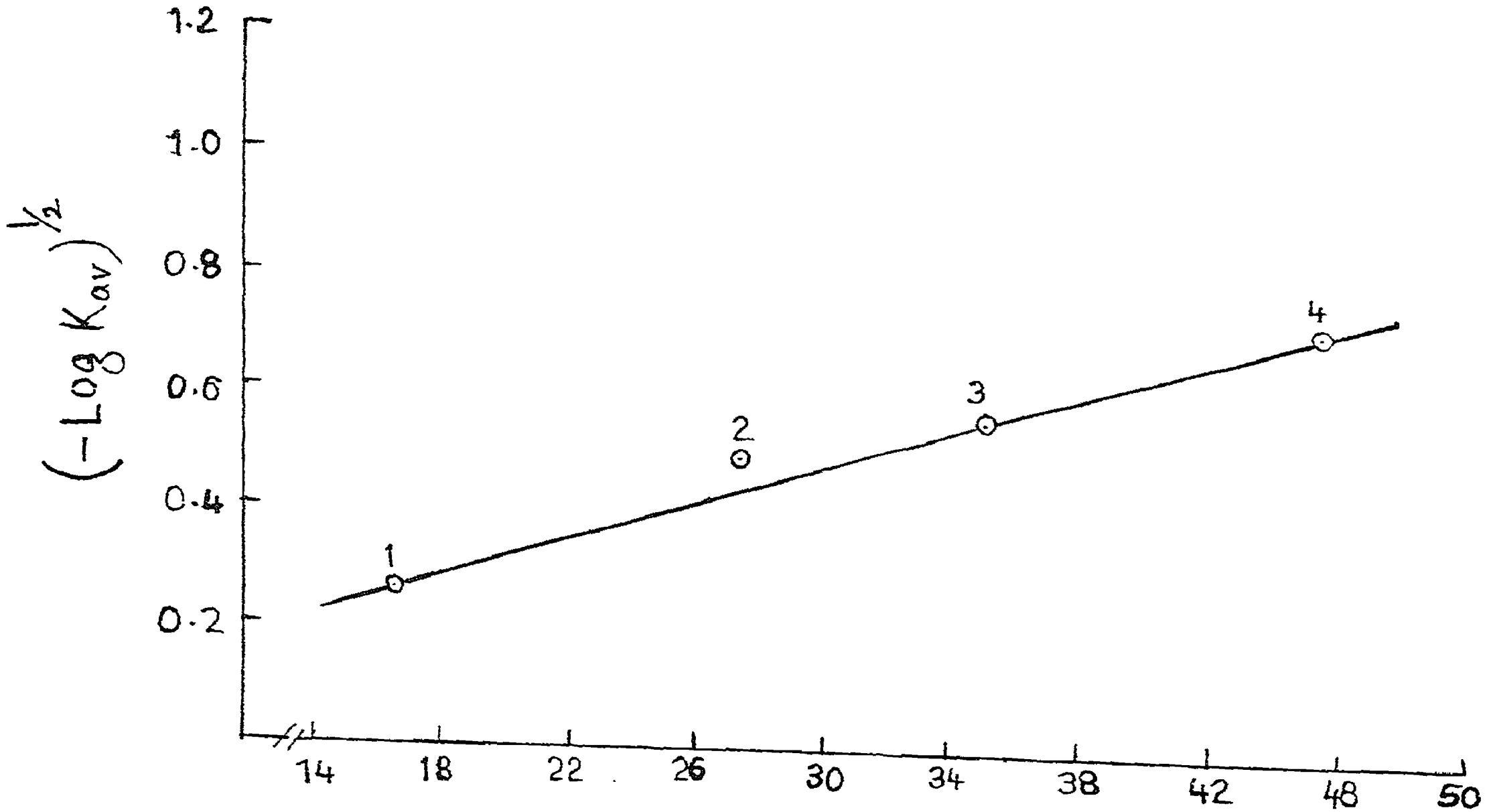

$(-\text{Log } K_{av})^{1/2}$
1.2
1.0
0.8
0.6
0.4
0.2
1
2
3
4
14
18
22
26
30
34
38
42
48
50
STOKES RADII

Fig - 6

D.3.11 Diffusion coefficient

Value for diffusion co-efficient were calculated from the following equation

$$D = \frac{KT}{6 \prod n a}$$

Where K is Boltzmann constant (1.38×10^{-16} erg/deg)

T is absolute temperature (298°K)

n is the viscosity in poise (0.01 p)

"a" is the Stokes radius of purified C_3

The value of diffusion coefficient for purified C_3 was calculated as

4.460×10^{-16} m^2/sec

TABLE 5 : VALUES OF STOKES RADIUS AND CORRESPONDING PARAMETERS FOR VARIOUS MARKER PROTEINS AND PURIFIED OVINE C_3.

Protein	Stokes radii A^0	K_{av}	$(-\log K_{av})^{\frac{1}{2}}$
Cytochrome-C	16.4	0.844	0.271
Ovalbumin	27.3	0.547	0.511
Bovine serum albumin	35.3	0.458	0.582
Alcohol dehydrogenase	45.0	0.270	0.754
Purified C_3	49.12*	0.221	0.809

*Calculated from equation (3)

DISCUSSION

E. DISCUSSION

The review of literature has already shown that information on the structure and biochemistry of third component of complement in human and some other species is available, but scanty reports are available for C_3 in common domestic species in general and sheep in particular; thus prime objective of the investigations carried out and presented in this study was to provide some initial information on ovine C_3.

The procedures followed for purification of ovine C_3 did not differ much from the procedures used by other workers in other species; however enough care was taken to secure the isolation of C_3 in its native form, as such all the procedures regarding purification were carried out at 4°C and use of protease inhibitors was rigorously followed. The relative ease with which this component of complement can be purified is attributable, in part, to its high concentration in serum.

The assay for C_3 during purification process has traditionally been followed by hemolytic assays (**Barta** and **Hubbert** 1978) based on the classical pathway of complement activation. These assays are performed by using various cellular intermediates like EAC_1 guinea pig, C_4 human, C_2 guinea pig and functionally purified C_5 to C_9 (**Daha** et al., 1979; **Tack** and **Prahl** 1976; **Shin** and **Mayer** 1968). These assays are tedious due to their inherent complexity, time consuming and require functionally pure intermediates of complement components. On the other hand they have the advantage of monitoring the purification process quantitatively.

An innovative procedure, basically a hemolytic assay for C_3, wherein the alternate pathway of complement activation is utilized was developed by

Jessen et al. (1982), here methylamine treated plasma serves as the source of complement components depleted of C_3 activity and rabbit RBC as complement activator. This procedure sounds a definite improvement over the traditional hemolytic assay.

Also available now are specific and rapid immunochemical techniques that can be easily adopted for C_3 identification and thus monitor the purification process. Immunochemical techniques primarily led to the perfection in raising of monospecific anti-C_3 sera (Henson 1977; Kai et al., 1983; Gorman et al., 1981). The identification of C_3 by use of anti-C_3 sera raised in a distant heterologous species, even though being a highly specific and simple technique is only qualitative in nature.

Notwithstanding this, the C_3 identification in the present study was carried out by the use of a monospecific anti-ovine C_3 serum. Monospecific anti-C_3 serum was raised by the use of Inulin method (Harrison and Lachmann 1986). Inulin, biochemically a polysaccharide without any sialic acid, has the ability to serve as an activator surface by triggering the positive feed back loop of ACP through favouring the binding of factor B in preference to factor H, to the solid phase C_{3b} (Muller- Eberhard 1988) resulting in rapid and irreversible covalent 'sticking' of sufficient number of C_{3b} molecules on to the Inulin particle. For this process, only ACP activation was ensured by the use of EGTA, which blocked any complement activation via classical route by preferential chelation of Ca^{++} ions rather than Mg^{++} ions (Bryant and Jenkis 1968; Fine et al., 1972; Ferrone et al., 1973; Platts-Mills and Ishizaka 1974) as Ca^{++} ions are essential for complement activation via classical pathway. Blocking of classical pathway was necessary so as to prevent its activation and subsequent covalent sticking of C_4 component on inulin particles, since this complement component (C_4) also possess a metastable thioester bond (Tack 1983). While raising the anti-C_3/I serum in rabbits the inulin suspension was throughly washed with PBS so as to ensure removal of non-C_3 proteins adhering to inulin particles by non covalent attractions. The characterization of anti-C_3 serum raised by either Inulin or zymosan method is usually done by adopting Ouchterlony method and/or IEP (Henson 1977; Gorman et al., 1981). However Kai et al. (1983)

characterized anti C_3/zymosan serum by crossed-IEP also. In the present study anti C_3/I serum was characterized by Ouchterlony method and IEP. By Ouchterlony method, using fresh ovine serum and anti-C_3/I serum revealed a single precipitation line as well as a line, of identity. In IEP against sheep whole serum anti-C_3/I serum was able to recognize a single protein, as reflected by the formation of a single sharp precipitation arc in the beta-region of Immunodiffusogram (PT-3A), out of a host of proteins present in the ovine serum. Similar findings were reported in cannine and feline systems by Gorman et al. (1981). These observations indicated that anti-C_3/I serum was monospecific and could identify the C_3 in both native and cleaved form.

The present purification scheme for C_3 differ very little from the procedures adopted for earlier purification protocols of C_3 in other species. This is a slightly modified version of purification proceudre used for bovine C_3 (Menger and Aston 1985). The purification procedure adopted utilized initial precipitation of serum by PEG 6000, 7% (w/v) followed by ion exchange chromatography on anion exchanger and cation exchanger, finally gel filtration on Sephadex G-200 was done.

One of the major practical problem faced during the purification of ovine C_3 is activation/cleavage of native C_3 molecules . This was limited and to some extent rectified by the use of protease inhibitors, ion chelators and carrying the purification work at 4°C. Use of EDTA, PMSF and EACA in buffers for column chromatography and initial precipitation had found approval with other workers also (Kai et al., 1983; Menger and Aston, 1985; Shin and Mayer 1968; Tack and Prahl 1976). In the present study also, EDTA, PMSF and EACA were used in comparable molarities; complement activation pathways were blocked by the use of divalent cation chelator EDTA, while the use of PMSF and EACA ensured the inhibition of serine class of serum proteases and plasmin (Bokisch et al., 1969) respectively.

As described earlier the serum after initial precipitation by PEG-6000 was fractionated on DEAE-Sephadex anion exchanger. The elution profile

obtained by the salt gradient upto 0.45M revealed three major peaks. C_3 was found only in the third peak by Ouchterlony method. The salt molarity responsible for eluting the bound C_3 roughly ranged from 0.24M to 0.29M NaCl. Similar observation for Bovine C_3 (**Menger** and **Aston** 1985), Guinea pig C_3 (**Shin** and **Mayer** 1968), Human C_3 (**Tack** and **Prahl** 1976) and Canine C_3 (Johnson et al., 1985) are reported. In all these cases C_3 was found to bind to anion exchanger and eluted out only after use of salt gradient. On the other hand in case of quail (**Kai et al.**, 1983) C_3 could be eluted out from DE-52 cellulose column as a first peak with a buffer having molarity ranging approximately from 0.1M to 0.15M NaCl. Mouse C_3 was also eluted after the use of salt gradient from a stronger anion exchanger viz. QAE-Sephadex (**Gyongyossy et al.**, 1977). Thus the binding of ovine C_3 to DEAE-Sephadex column under the buffer conditions employed in the present study draws a clear homology with the C_3 behaviour in anion exchanger column from other species.

The post DEAE-Sephadex pool was next fractionated on a CM-Sephadex cation exchanger, the elution profile revealed a forked peak in the unbound region and three small peaks after using the salt gradient. Under the buffer conditions employed ovine C_3 did not bind to the negatively charged resin, but eluted out in the fork region of the exclusion peak. None of the peaks eluting after application of the linear gradient of 0.3M NaCl reacted with anti C_3/I serum. This behaviour of ovine C_3 is in contrast to C_3 behaviour of cattle, which was eluted out after the use of linear gradient under similar conditions of pH and ionic strength (**Menger** and **Aston** 1985). Rat C_3 was found to bind to CM-Cellulose column by **Daha et al.** (1979) as also guinea pig and mouse C_3 to respective cation exchangers viz. CM-Cellulose and SP-Sephadex (**Shin** and **Mayer**, 1968, **Gyongyossy et al.**, 1977). The behaviour of ovine C_3 in CM-Sephadex column bears similarity to the behaviour of hemolytically active canine C_3 in CM-Sepharose CL-6B column (**Johnson et al.**, 1985). Which was reported to elute out in the unbound region of elution profile.

The post CM-Sephadex pool was finally purified to homogeneity by gel filtration on Sephadex G-200. The elution profile revealed four major peaks out of which the second and portion of the third peak reacted

positively with anti C_3/I serum in DID. Only one of the peaks (Peak I, Fig.3) was subsequently shown to contain intact C_3 while the (Peak II, Fig.3) had either a fragmented C_3 or a contamination that reacted positively with anti C_3/I serum

The purified C_3 (Peak I) was subjected to IEP where it developed a single sharp precipitating arc against ASWS, thus the ability of ASWS to specifically recognise a single protein, confirmed the antigenic homogeneity of the purified ovine C_3. Similar observation have been reported in rats (Daha et al., 1979), cannine (Johnson et al., 1985) and guinea pig (Shin and Mayer, 1968).

Bovine C_3 (Menger and Aston, 1985) also showed a similar two peak profile and both reacting with bovine anti-C_3/I serum in CM-Sepahdex elution.

But IEP of fractions reacting with Anti C_3/I serum (Peak II) pool showed a clear contrast to peak II pattern in IEP when run aginst ASWS; a cleaved arc and a separate arc on anodal side were observed, a similar behaviour exhibited by inactive forms of human C_3 was reported by Henson (1977). This led to the conclusion that the peak II anti-C_3/I reacting fractions could be a form of ovine C_3 that is fragmented. This was further substantiated by SDS-PAGE character of peak II pool.

Purified ovine C_3 when run in IEP against anti-C_3/I serum gave a sharp arc (PT-3A) which was positionally comparable with the arc developed by sheep whole serum against anti C_3/I serum. Thus it could be safely assumed that the purified C_3 was the same protein which anti C_3/I recognised from sheep serum. As, a slight positional shift towards anode by purified C_3 could be interpreted as fragmented C_3 (Henson 1977; Gorman et al., 1981; Pepys et al., 1977).

Comparative immunoelectrophoresis was performed in order to show the step wise purification of C_3 and to know the changes in its electropho-

retic mobility during purification steps. The aliquotes from different purification steps were run against ASWS. It was evident by successive reduction of precipitating arcs that the contaminating proteins were reduced as the purification process procceded till a single precipitating arc corresponding to Sephadex G-200 Peak I was reached. The electrophoretic mobility of the ovine C_3 was same throughout the whole procedure, thus indicating that the native configuration of the C_3 protein was maintained.

Henson (1977) reported a change of electrophoretic mobility of native human C_3 towards anode when inactivated/cleaved. The anodal drift of C_{3b}/C_{3c} relative to the native C_3 has been well recorded in human, mice, feline and cannine species (McConnel et al., 1978; Henson 1977; Pepys et al., 1977; Gorman et al., 1981).

To follow the progress of purification, polyacrylamide gel electrophoresis of different aliquotes from various purification steps was conducted. The gels showed a progressive reduction in the number of bands as the purification progressed. Finally a single discrete band is clearly observed for the purified C_3 (Post-G-200 Peak I aliquote). Thus confirming charge homogeneity of the purified C_3, a similar observation was reported for guinea pig C_3 (Shin and Mayer 1968).

As already mentioned, the purified C_3 was also analysed by SDS-PAGE under both reducing and non-reducing conditions. This provided information about the sub unit structure and existence of disulfide linkage, if any. Both the fraction viz. Peak I and Peak II obtained after final gel filtration step were analysed.

Under the non reducing conditions a single band was observed, though this was not sharp but a diffused one; nevertheless the homogeneity of purified C_3 under denaturing conditions can safely be assumed. Similar, single band SDS-PAGE pattern for purified C_3 under non reducing condition is a well established (Daha et al., 1979; Aston and Menger 1985; Gynogossy 1977).

SDS-PAGE of purified C_3 (Peak I) under reducing condition revealed two bands, corresponding to two polypeptide chains linked by disulphide linkage. The M.W. of chains i.e. chain I and chain II was determined as 128.17 kD and 54.80 kD respectively, giving an aggregate of 182.9 kD for the purified C_3.

The two chain structure of the purified ovine C_3 bears homology with the C_3 molecules from the other species which show a similar two chain structure; but on the basis of molecular weight of purified C_3 and its subunits the closest parallel can be drawn with bovine C_3 having aggregate M.W. of 185 kD (Mayer and Aston 1985). For sub units, the MW for chain I of ovine C_3 is closest to cannine C_3 α chain which has a MW of 126 kD (Gorman et al., 1981) but chain II of ovine C_3 has a lower MW compared to the β chains of other species, which range from 65 kD for Dog β chain (Johnson et al., 1985) to 76 kD for Rat β-chain (Daha et al., 1979).

The SDS-PAGE (reducing) pattern of the peak II anti-C_3/I reacting material gave three bands, corresponding to the MW of 98.45 kD, 43.02 kD and 35.43 kD with an aggregate of 176.90 kD. Similar finding was reported by Molenaar et al. (1975) for human C_3 where C_{3b} on reduction gave four chains and C_{3c} gave two chains. Also for bovine C_3, Menger and Aston (1985) reported some anti-C_3/I reacting fractions in CM-Sephadex elution profile that on reduction gave three bands of 69 kD, 40 kD and 29 kD. It was reported to be C_{3c}, on the basis that the largest chain had identical MW to beta chain of the purified native C_3 and MW of other two chains were similar to those of alpha chain fragments.

However in the present study, establishing similar analogy can prove faulty and further investigations are needed to reveal the actual nature of the peak II protein. It can be, of course assumed that the peak II protein is a fragment of native C_3 with or without contaminating proteins.

Mol. wt. of purified ovine C_3 was also established by gel filtration method and a MW of 186.59 kD was calculated. This is very much comparable with the MW obtained by SDS-PAGE.

The glycoprotein nature of the ovine C_3 was established by PAS-staining following gel electrophoresis. Analysis of the carbohydrate moiety was also carried out. The presence of amino sugar could not be detected by the procedure of **Davidson** (1966). However, the neutral sugar content of the purified C_3 was found to be 2.2% (w/w).

The glycoprotein nature of human C_3 was reported by *Hirani* et al. (1986) and a 2% of total mass of human C_3 was attributed to carbohydrate content. C_3 was found to contain N-acetylglucosamine and mannose in the ratio of 1:4; a comparable report for human C_3 carbohydrate content was also reported by **Tack** and **Prahl** (1976), a total 1.5% (w/w) carbohydrate content (0.3% amino sugar and 1.2% neutral sugar) was reported. No report about the presence of aminosugar in purified C_3 from other domestic species is available.

Purified ovine C_3 showed the expected anodal drift on trypsinization relative to the untrypsinized control. This behaviour of ovine C_3 was similar to that of human C_3 (**Tack** and **Prahl** 1976). The change in electrophoretic mobility could be due to cleavage of α chain or β chain of the native C_3 (*Gorman* et al., 1981; **Minta** et al., 1977).

In view of a relatively high degree of homology of C_3 over species, simple DID was conducted to assess the ability of ovine anti-purified C_3 serum to react with whole serum from other species. The results showed that anti-purified C_3 reacted with the serum of ruminants (goat and cattle) in almost similar dilution of anti-purified C_3 serum. However, human sera elicited positive reaction only with undiluted antisera. Dog and rabbit serum failed totally to react with the anti-purified C_3 serum.

Tosic et al. (1989) reported that anti-human C_{3b} monoclonal antibodies cross reacted with primate derived C_{3b}, but failed to give a positive reaction with non primate derived C_{3b}. **Kai** et al. (1983) showed the cross reactivity of anti quail C_3 serum with only quail and chicken sera but not with mammalian sera. It is quite indicative from these studies that cross

reactivity of C_3 over the species is phylogeny related. The results of the present study in this regard seem to substantiate the above fact, though the method adapted for cross reactivity test is not as sensitive as a technique like indirect ELISA etc.

The values of hydrodynamic parameter viz. stokes radius, intrinsic viscosity and diffusion coefficient suggest that the purified ovine C_3 has a compact, globular configuration (**Gosting** 1956; **Yang**, 1961).

The results, pertaining to the hydrodynamic features of the purified ovine C_3, however, cannot be compared with a similar data from other species as no published report is available pertaining to these data.

In conclusion the present study has established the ovine C_3 to be a disulphide linked two chain glycoprotein with a MW of 182±5 kD and bears homology with other species in some aspects while stand unique in others. This study will pave way for characterization of other complement component of ovine species which would facilitate further investigation into the molecular organisation and function of ovine complement system.

SUMMARY

F. SUMMARY

Complement is a complex series of some twenty proteins which form one of the classical example to triggered enzyme complex, when activated. Complement system comprises of more than twenty chemically and immunologically distinct plasma proteins which have the capacity of interacting among themselves as well as with antibody and cell membranes. Once activated, this system gives rise to a wide spectrum of biochemical activities, that terminate with the lysis of different cells, bacteria or viruses.

C_3 is one of the thirty complement proteins recognised to date and has been the focus of intensive studies due to its important role in complement activation pathways. In human, this 185 kD protein is the most abundant among the complement proteins in serum (1-2 $mg.ml^{-1}$). It is comprised of two polypeptide chains, a 110 kD α chain and a 75 kD β chain linked together by one disulfide bond. Molecular weight of guinea pig C_3 is reported as 180 kD, while as rat C_3 M.W. is 187 kD. Dog, cat have MW of 179 kD and 197 kD respectively, in each case the C_3 molecule comprises of two chains.

The literature available regarding C_3 from human and some other species is enormous, while as scanty reports are available for C_3 in species of domestic animals.

The third component of complement has been purified from fresh sheep serum employing an initial fractionation with polyethylene glycol followed by sequential depletion of contaminating serum proteins by ion exchange chromatography on DEAE-Sephadex and CM-Sephadex followed by gel filtration on Sephadex G-200.

Final concentration of purified protein was 0.735 mg/ml approximately. During purification C_3 was monitored by immunochemical techniques using anti-C_3/I serum raised in rabbits.

Purified C_3 when subjected to SDS-PAGE revealed two bands corresponding to two chains of MW 128.17 kD and 54.80 kD with an aggregate of 182 kD. MW of purified C_3 as deduced by gel filtration was calculated to be 186 kD.

The purified C_3 was also confirmed to have neutral sugar moeities to 2.2% w/w, while no amino sugar could be confirmed. Trypsinization of purified C_3 elicited the expected anodal drift and in this regard was very much homologous with C_3 from other species.

Cross reactivity of anti-purified C_3 gave positive results with ruminant serum only, substantiating the hypothesis that C_3 cross reactivity across the species is phylogeny related.

Biophysical parameters like Stokes radius (49.12 A°) diffusion coefficient (4.460×10^{-16} m^2 sec) and intrinsic viscosity (4.04 gm/ml) was calculated from the caliberation data of purified C_3 on G-200 column which indicated C_3 to be a compact globular protein molecule.

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APPENDIX

H. APPENDIX

II.1 Reagents used for Production of Anti C_3/I Serum

a) Veronal buffered saline (VBS) (5x) pH 7.4 according to Mayer, (1961).

Solution A :	Barbituric acid	:	5.75	g
	Hot D.W.	:	500	ml
Solution B :	NaCl	:	85	g
	Sodium barbitone	:	3.75	g
	D.W.	:	1500	ml

Solutions A and B were mixed and pH adjusted to 7.4.

b) Phosphate buffered saline (PBS) pH 7.2

NaCl	:	8.0	g
KCl	:	0.2	g
Na_2HPO_4	:	1.15	g
KH_2PO_4	:	0.2	g
D.W.	:	1000	ml

H.2 Buffer for Immunoprecipitation Techniques Tris-borate Buffer (pH 8.6, 0.05M)

0.05M Tris was made, of the required volume, subsequently titrated with boric acid dissolved in warm D.W. pH 8.6 was adjusted at 25°C.

H.3 Buffer's used during Purification Procedure

H.3.1.1 Phosphate Buffer (PB) Stock Solution 0.2M, pH 7.0

Solution A : 0.2M Monobasic Sodium Phosphate
Solution B : 0.2M Dibasic Sodium Phosphate

Solution A and B were mixed in the ratio 39:61 to get 100 ml of PB 0.2M, pH 7.0.

H.3.1.2 Phosphate Buffer (PB) 0.1M pH 7.5, 7% PEG 6000 and 0.15mM PMSF

PB stock was diluted 1:1 with distilled water and made 7% with PEG 6000 and 0.15mM PMSF. The pH was adjusted to 7.5 at 25°C.

H.3.1.3 PB, 25mM, pH 7.0, Containing 20mM EACA, 25mM EDTA

PB stock was diluted 1:7 with D.W.; this buffer was made 5mM EDTA, 20mM EACA, by adding required amounts of respective chemicals and final pH was adjusted to 7.0 at 25°C.

H.4 Buffer used in Chromatographic procedures

H.4.1 DEAE- anion exchange chromatography (PB 25mM, 5mM EDTA pH 7.0)

Starting buffer : PB stock was diluted 1:7 with D.W. and then brought to 5mM EDTA and 0.03% sodium azide concentration, pH adjusted to 7.0 at 4°C.

Final buffer : Same as the starting, but was brought to 0.45M NaCl.

H.4.2 Buffer used in CM Sephadex Cation exchange Chromatography

Stock buffer : Tris acetate 0.1M pH 6.6 0.1M.Tris solution was prepared in D.W., and titrated against acetic acid at 4°C until pH reached 6.8.

Starting buffer : Tris acetate 20mM pH 6.6 5mM EDTA 0.03% Sodium azide. Stock buffer was diluted 1:4 by D.W. and resulting buffer brought to 5mM EDTA, 0.03% sodium azide. pH 6.8 was adjusted at 4°C.

Final buffer : This was same as the starting buffer but contained 0.30M NaCl.

H.4.3 Buffer used in, Sephadex G-200 exclusion chromatography

PB 25mM pH 7.0 containing 25mM EACA, 5mM EDTA, 150mM NaCl and 0.03% Sodium azide.

PB stock was diluted 1:7 with D.W., resulting buffer brought to 5mM EDTA, 25mM EACA, 150mM NaCl and 0.03% sodium azide, by adding required amount of respective chemical; pH was adjusted to 7.0 at 4°C.

H.5 Reagents used in Polyacrylamide Gel Electrophoresis

H.5.1 Simple PAGE (Davis, B.J., 1964)

Solution A (Separating Gel Buffer)

1NHCl	:	48	ml
Tris	:	36.6	gm
Temed	:	0.23	ml

D. water added to make up 100 ml.

Solution B :	N, N-methylene bisacrylamide	:	0.735	g
	Acrylamide	:	28.0	g

D.W. added to make up 100 ml. and stored in brown bottle at 4°C, the solution was filtered before use.

Solution C : Ammonium per sulfate 0.2 gm dissolved in 100 ml. D.W. (prepared immediately before use).

Electrode buffer :

Tris	:	0.60	g
Glycine	:	2.88	g
D.W.	:	1000	ml

Staining solution :

Comassie brilliant blue	:	0.1	g
Glacial acetic acid	:	10.0	ml
Methanol	:	45.0	ml
D.W.	:	45.0	ml

Fixative solution :

Glacial acetic acid	:	10.0	ml
Methanol	:	45.0	ml
D.W.	:	45.0	ml

Destainer solution :

Glacial acetic	:	10.0	ml
Methanol	:	10.0	ml
D.W.	:	80	ml

H.5.2 **Reagents for SDS-PAGE according to (Laemmli 1970).**

Solution A : Separating Gel Buffer (5x)

5 [0.0375 M Tris HCl buffer pH 8.8 with 0.1% SDS]

OR

Tris	:	22.71	g
SDS	:	0.1	g

D.W. added to make final volume of 100 ml after pH adjusted to 8.8, with HCL, at 20°C.

Solution B : Stacking Gel Buffer (5x)

5 [0.125 M Tris.HCl, pH 6.8 with 0.1% SDS]

OR

Tris	:	7.57	g

SDS : 0.1 g

D.W. added to make final volume of 100 ml after pH adjusted to 6.8 with HCl at 20°C.

Solution C : Stock acrylamide/Bisacrylamide solution

Acrylamide : 29.2 gm
BIS : 0.8 gm
D.W. added to 100ml (stored in brown bottle at 4°C).

Solution D : Sample buffer

Tris : 0.75 g
SDS : 1 gm
Sucrose : 5 gm
Beta Mercaptoethanol : 2.0 ml
Bromophenol blue : .001 gm

D.W. added to make final volume of 50 ml pH 6.8 adjusted with HCl at 20°C. Stored in aliquotes, at 4°C till used.

Solution E : Electrolyte buffer (0.025M Tris, pH 8.3, Glycine 0.192 M, SDS- 0.1%)

Tris : 3.028 g
Glycine : 14.4 g
SDS : 1.0 g
D.W. added to make up final volume of 1000 ml.

Solution F : TEMED : 0.025% solution (v/v)

Solution G : Ammonium per sulfate : 0.25% (w/v).

Solution H : Fixative solution

Glacial acetic acid : 10.0 ml
Methanol ; 50,0 ml
D.W. : 50.0 ml

Solution I : Stainer solution

Coomassie brilliant blue	:	0.1 gm
Glacial acetic acid	:	10.0 ml
Isopropyl alcohol	:	50.0 ml
D.W.	:	50.0 ml

Solution J : Destainer solution

Glacial acetic acid	:	10.0 ml
Isoprophyl alcohol	:	10.0 ml
D.W.	:	80.0 ml

H.5.2.1 Composition of separating gel

10% acrylamide 2.5% BIS for every 10.5 ml gel

Solution C	=	2.5 ml
Solution A	=	2.0 ml
Solution G	=	1.0 ml
Solution F	=	0.25 ml
D.W.	=	4.25 ml

H.5.2.2 Composition for stacking gel

4% acrylamide; 2.7% BIS for every 8.5 ml of stacking gel.

Solution C	:	1.0 ml
Solution B	:	2.0 ml
Solution G	:	2.0 ml
Solution F	:	0.25 ml
D.W.	:	3.25 ml

H.6 Reagents used in Carbohydrate Estimation and Identification

H.6.1 Hexozamine estimation

PDMAB reagent :

P-dimethyl amino benzaldehyde	:	677.5 mg
Ethanol : Concentrated HCL solution:		25 ml

H.6.2 Neutral sugar estimation

Orcinol-sulfuric acid reagent

H_2SO_4; D.W. mixture (3:2 v/v)	:	7.5 ml
Orcinol solution 1.6% (w/v)	:	1.5 ml

H.6.3 PAS staining reagents

Schiff's reagent

1 g of basic fuchsin dissolved in 200 ml of boiling distilled water; solution was stirred and cooled to 50°C and filtered. To the filtrate, 20 ml of 1 N HCL was added. The solution was cooled to 25°C and 1 g of potassium metabisuphite was added. This solution was kept in dark for 24 hours. The solution was shaken for one min after mixing with 2 g of activated charcoal. This mixture was filtered and the filterate was stored at room temperature.

H.7 Reagents for Trypsin action on purified C_3

Tris Borate buffer pH 7.4 (0.05M) as explained elsewere (H.2) used to make 1% trypsin solution.

H.8 Reagents used in protein estimation

Lowry's Method

Stock solutions

Solution A :

(1) 2% (w/v) sodium potassium tartarate
(2) 1% (w/v) copper sulphate solution
(3) 2% (w/v) sodium carbonate in 0.1 N sodium hydroxide.

(1), (2) and (3) were mixed in the ratio of 1:1:100 (v/v/v).

Solution B (Folin-phenol reagent)
Commercially avaliable.

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