Influence of serum factors on lymphocyte adhesion to material and biological surfaces

A thesis submitted to the University of St. Andrews (Department of Biochemistry) for the degree of Master of Science

by

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CONTENTS

	PAGE
Declaration	(i)
Certificate	(ii)
University Record	(iii)
Acknowledgements	(iv)
Summary	(v)
Introduction	1
Aims	29
Materials and Methods	30
Results	55
Discussion	132
References	152

DECLARATION

I hereby state that this thesis and the research work reported in this thesis is my own composition as a result of research conducted in the Department of Biochemistry, United College of St. Salvator and St. Leonard, University of St. Andrews under the direction of Dr G.D. Kemp. No part of this work has previously been presented for a higher degree.

C E R T I F I C A T E

I hereby certify that MOHAMMAD AKHTAR ANWAR has spent seven terms engaged in research work under my guidance, and that he has fulfilled the conditions of General Ordinance No. 51 (Resolution of the University Court), and that he is qualified to submit the accompanying thesis in application for the degree of Master of Science.

Supervisor

UNIVERSITY RECORD

I graduated with the BSc pass degree from the University of Essex in June 1981. I matriculated as a research student in the Department of Biochemistry, United College of St. Salvator and St. Leonard, University of St. Andrews in October 1981.

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Summary

1) The effects of calf blood serum on the lymphocyte adhesion to glass and to two-dimensional collagen films have been examined, Serum potently inhibits lymphocyte adhesion to glass. It has also been observed that serum enhances lymphocyte adhesion to collagen but, however, a slight inhibitory effect was observed at high serum concentrations.

2) An attempt was made to purify serum factors which are the cause of inhibition. Gel filtration and electrophoresis studies suggest that four factors of molecular weights 400000, 230000, 100000 and 45000 daltons may be involved, and the 400000 band is the α_2 macroglobulin (α_2M) dimer. Hence, α_2M or an α_2M-associated substance has been implicated as an adhesion-inhibitor. Further purification studies utilising ammonium sulphate precipitation, ion-exchange and gel filtration indicate one factor of molecular weight 100000. Polyethylene glycol studies confirm the presence of the inhibitory factor in blood serum. It should be emphasized that the adhesive inhibition is relatively specific, that is the mere presence of protein alone is not sufficient.

3) Lymphocyte adhesion follows Michaelis-Menten type kinetics, and the serum acting as an inhibitor gives a straight line form of the Dixon plot, showing non-competitive form of inhibition.

Introduction

		Page
1.	Lymphocytes	2
1.1	Lymphocyte Plasma membrane	2
1.2	Types of Lymphocytes	3
2.	Adhesion	7
2.1	Types of Interactions	7
2.2	Lymphocyte properties associated with adhesion	8
2.3	Extracellular proteins involved with adhesion	13
3.	Serum Inhibitors	22
2.1	Alpha-2-macroglobulin	23
2.2	Serum Albumin	25
2.3	Assay and mechanism of adhesion	26

1. LYMPHOCYTES

The importance of lymphocytes is well recognised, especially by haematologists, immunologists, and pathologists amongst others. A lymphocyte, is 5-8µm in size (depending on the animal), and has a very high nucleus/cytoplasmic ratio. It is capable of RNA and protein synthesis, and contains enzymes for glycolysis and oxidative respiration, with significant amounts of hydrolytic, proteolytic and lipolytic enzymes.

1.1 Lymphocyte Plasma Membrane

The integrity of the plasma membrane is reflected by the part it plays in controlling the cell's behaviour. Such a role is implicated in several aspects of cell biology, as a consequence of structural glycoproteins, embodying such characteristics as recognition during differentiation, cell adhesion, contact inhibition, intercellular communication and the migration peculiarities of lymphocytes (Gesner and Ginsberg, 1964; Woodruff and Gesner, 1968; Cook and Stoddart, 1973), in recognising and regulating the response to hormones and diverse secreted factors and in conducting the transport of ions and other small molecules. Moreover, the lymphocyte cell surface has the function of recognising antigen, which normally results in lymphocyte growth, division and differentiation to immunocompetent memory and effector cells (see Johnstone and Crumpton, 1982 for review).

According to Warren et al, (1975), these biological functions are attributable to glycoproteins and lipids are requisite for their support and activity. Plasma membrane structure of lymphocytes is similar to the model proposed by Singer and Nicolson (1972). It has been suggested that the physiological state of the membrane lipids is one of the factors controlling cell adhesion (Ueda et al., 1976). Recently, Sage et al. (1982) have elucidated on porcine lymphocytes the existence of three different species of plasma membrane glycolipids that bind soybean agglutinin (lectin): trihexosyl ceramide, globoside and ganglioside G_{M2} - in order of decreasing affinity.

The structure and composition of the myriad of proteins located on the surface of lymphocytes is slowly being established.

1.2 Types of Lymphocytes

Two functional types of lymphocytes exist, depending on the place of production, bone marrow-influenced lymphocytes (B lymphocytes) and thymus-influenced lymphocytes (T lymphocytes) - (Goldstein and White, 1973; Greaves et al., 1973; Katz, 1977).

The B lymphocyte population in the bloodstream consists of 20-30% of the total pool of lymphocytes. Located on the surface of each B lymphocyte are a varying number of antigenic receptors from 50000 to a 100000 strong. These antigenic receptors consist of light and heavy chains of

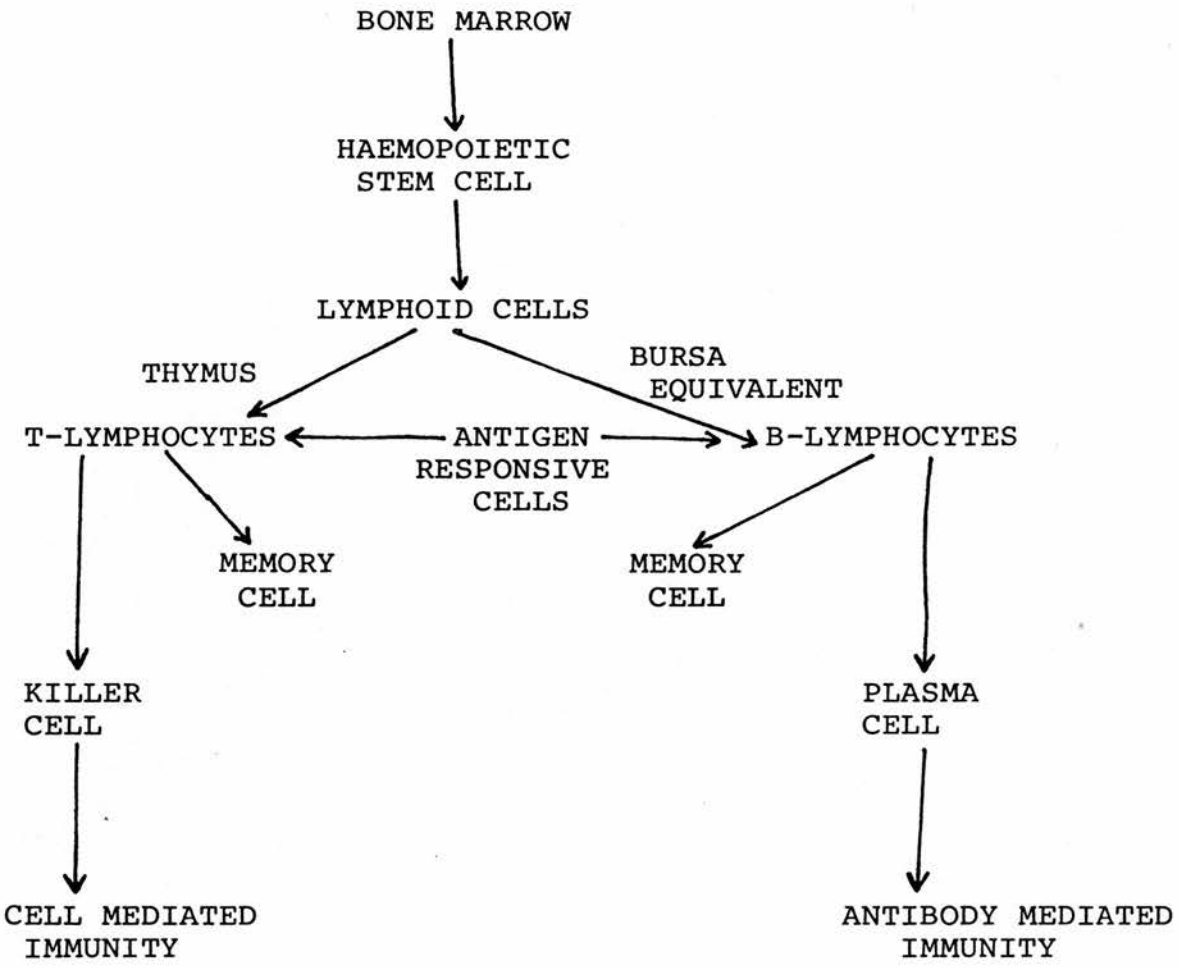


Figure 1

DEVELOPMENT OF LYMPHOID SYSTEM BY DIFFERENTIATION OF STEM CELLS

immunoglobulin, peculiar to the B lymphocyte, and identical to the immunoglobulin synthesised by the cell. Thus it can be seen that the B lymphocytes are characterised by such surface immunoglobulin receptors. Also, the B lymphocytes possess a receptor for the complement factor C3 (the third component of the complement cascade), and a receptor for Fc region of the antibody molecule.

During the establishment of the state of immunity, B lymphocytes develop into plasma cells which actively synthesise and secrete antibody. This is reflected by the increasing presence (in these cells) of the well-developed rough endoplasmic reticulum. The induction of an antibody response to many antigens, however, requires a collaborative interaction between B and T lymphocytes (Miller and Mitchell, 1968; Claman and Chaperon, 1969; Feldmann, 1972).

Approximately four-fifths of the lymphocyte population is attributed to T lymphocytes in the circulation. The T lymphocyte population is responsible for cell-mediated immune reactions. These reactions comprise protection against foreign bodies, cell-mediated delayed hypersensitivity, and a hand in graft rejection. A subset of T lymphocytes, the helper cells, augment the manufacture of antibody by B lymphocytes (Cantor and Boyse, 1975). In humans, T cells can be identified by their property of spontaneously binding sheep erythrocytes to produce so-called, E rosettes. Most of them also stain specifically with the enzyme α-naphthyl acetate esterase (ANAE). Furthermore, T lymphocytes have

a comparatively long life (5-10 years). This may be responsible for immunological memory (Figure 1).

2. ADHESION

Cell adhesion is an indispensable property of multicellular organisms. Cell recognition and formation of specific adhesions of cells from conception right up to adult life is essential for ontogenesis. During this course intercellular and cell-substrate contacts are involved in the control of processes such as cellular motility and growth. Hence, adhesion underlies several lymphocyte properties, including lymphocyte-antigen interaction and lymphocyte recirculation.

2.1. Types of interactions

Two types of adhesive interactions are possible; either one or both may be involved. The mechanisms for both types of interactions are slowly being defined.

2.1.A. Cell-substrate interaction

This type of adhesive interaction is displayed by many *in vitro* experiments, for example, cells adhering to glass or plastic surfaces as employed in tissue culture and other such similar techniques. *In vivo*, blood cells (e.g. lymphocytes) have the capacity to adhere to collagen of blood vessel walls (type III collagen being predominant, type IV collagen of basement membranes may also be of significance in cell attachment). The interaction of lymphocytes with foreign materials within the body in the production of antibodies is also of significance.

2.1.B. Cell-cell interaction

Intercellular interaction may lead to aggregation. Lymphocytes can adhere to cells that have surface antigens, and cooperation between specific antigen-recognising and antigen-binding cells is often necessary for the commencing of the immune responses (Feldmann, 1972; Rosenstreich and Mizel, 1978; Persson et al., 1978). Aberrant cellular behaviour observed in this type of adhesion is of significance in certain pathological conditions, especially tumour growth.

Tissue specificity of cell adhesion is at the most limited to some combinations (Pessac et al., 1979), and it has been suggested that it may be species specific.

A mechanism for this type of interaction has been postulated by Roseman (1970), who suggested that intercellular adhesion is mediated by enzyme-substrate recognition, in that a glycosyl transferase on the surface of one cell binds specifically to its substrate oligosaccharide on a neighbouring cell. However, there is no convincing experimental backing.

2.2 Lymphocyte properties associated with adhesion

Lymphocytes can be considered to be adherent cells under the appropriate in vitro conditions. Lymphocyte aggregation and adhesion to material surfaces such as glass and tissue culture plastic was observed by Kellie (1980) when serum was excluded. Moreover, Barker et al. (1981)

demonstrated that T lymphocytes adhere firmly to solid substrates in the absence of serum. Nonetheless, other authors have tended to disagree (Pulvertaft and Weiss, 1957; McGregor et al., 1978; Grinnell, 1981).

Shortman et al. (1972) investigating the adhesion of lymphocyte subpopulations to glass bead columns, found that subpopulations of cells which were rich in dividing and immature cells were more adhesive to this substrate than were non-dividing cell populations. These adherent populations were of low density and contained both dividing T cells and dividing B cells.

Recently, Kellie (1980) has shown that sensitised lymphocyte cells (with Oxazolone) are more adhesive to each other and to substrates than unsensitised cells. Kellie and Evans (1981) also found that the adhesiveness of these stimulated cells to a glass substrate in the presence of serum was also significantly higher than that of unstimulated lymph node cells, due possibly to an increase in the number of lymphoblasts in addition to an increase in the adhesiveness of small lymphocytes. Earlier, Curtis and de Sousa (1973) had found that oxazolone-stimulated lymphocytes were less adhesive than unstimulated lymphocytes.

Cell-substrate and intercellular adhesion of lymphocytes may be the central event, and other activities may be dependent on this process, including antibody production (e.g. lymphocyte-macrophage recognition and cluster formation), lymphoid cell mediated cytotoxicity (Martz, 1975), migration and recirculation.

Lymphocytes are actively mobile (33μm min^{-1}), however, according to Lewis (1933), the average rate of locomotion is approximately 16μm min^{-1}. Hence, both adhesive and adhesive-inhibitory processes may presumably be involved in lymphocyte traffic. The two processes of adhesion and adhesion-inhibition in vivo are part of the homeostatic mechanism, and the interplay of both is of significance for normal function.

Two types of lymphocyte traffic have been observed (Gowans, 1959; Ford and Gowans, 1969; Weir, 1973; De Sousa, 1981);

a) The movement of lymphocytes from the bone marrow to lymph nodes, with or without passage through the thymus. This migration, during which the cells are maturing and proliferating, probably takes a period of weeks.

b) Recirculation of small lymphocytes (non-dividing population with potential life span of many months) between the peripheral blood and various tissue compartments takes place in hours.

Both B and T lymphocytes possess this ability to recirculate from the blood to lymph and it is considered that they take the identical path although T lymphocytes recirculate more rapidly than do B lymphocytes which traverse the lymph nodes and spleen at distinctly slower

speeds (Ford, 1975). After utilising a common path of entry into lymphoid tissue, T and B cells sort out into defined regions of tissue; this is possibly due to different adhesive properties.

The mechanism of transfer in the lymph nodes has been studied using the electron microscope. Early studies suggested that small lymphocytes actually penetrate and traverse the cytoplasm of the cuboidal endothelial cells of the post-capillary venules (Marchesi and Gowans, 1964). However, Schoefl (1972) demonstrated that lymphocytes adhere to endothelial cells in specific regions of the microvasculature and that extravasation of adherent lymphocytes into the perivascular tissue space takes place between the endothelial cells.

The biological value of recirculation has become established in recent years as insight into the cellular events of immune responses grew.

Advantages of the recirculation process include (Weir, 1973):

a) During the course of an infection, the perpetual migration of lymphocytes would capacitate numerously different lymphocytes to have access to the antigen with the consequence that a lymphocyte with the reciprocating antibody receptor for the particular antigen would come across the antigen and initiate an immune reaction.

b) Lymphoid tissues can replenish lymphocytes from the recirculating pool, for example, the spleen which may have been depleted as a result of infection, trauma or X-rays.

c) The passage of lymphocytes through an area where antigen had been localised and concentrated on the dendritic processes of macrophages might facilitate the induction of immunity.

The recirculation of lymphocytes between the blood and other tissues, and the migration of antigenically activated lymphocytes into certain tissues has been reviewed comprehensively by Ford (1975).

Differences in adhesion may affect migratory patterns, since Evans and Davies (1977) have proposed that sub-populations of thymocytes with different intercellular adhesiveness possess different migratory behaviour in syngeneic recipients. This indicates that transitory changes in adhesiveness are perhaps responsible for migratory patterns. Lymphocyte movement may be the outcome of adhesion-inhibition of adhesive cell-cell and cell-substrate interactions. Immunological reactions as a consequence of migration comprise auto-allergies, chronic inflammation, delayed hypersensitivity, homograft rejection and tumour regression. Other factors, which are perhaps relevant during recirculation and migration apart from adhesion, include the cell surface characteristics of

lymphocytes, chemotaxis, release of enzymes by macrophages and release of substances by other lymphocytes (Alnasiry and Greally, 1982; Dunn and Halliday, 1981) and are all likely to influence lymphocyte recirculation.

2.3 Extracellular Proteins Involved In Adhesion

Both collagen and fibronectin have been implicated in cellular adhesion, as both are part of the tissue stroma. The nature of the substratum plays an important role in controlling the migratory behaviour of a number of cell types (Hay, 1981). Collagen is a major component of the extracellular tissue matrix through which lymphocytes migrate *in vivo*. Therefore, it is appropriate to give background information on the two proteins.

2.3.A. Collagen

Collagen is the main component of the extracellular matrix (Miller, 1977; Bornstein and Ash, 1977) which also contains different glycoproteins (Bornstein and Ash, 1977) and a variety of proteoglycans (Hascall, 1981). It has become apparent that the extracellular matrix does not function merely as an inert structural support, but plays an important role in the control and integration of cell behaviour in multicellular organisms.

Briefly, the collagen monomer (previously called Tropocollagen) has a molecular weight of about 300000, and has a rod-like shape with dimensions of 300nm in length and

1.5nm in width. It consists of three individual polypeptide chains. Each of the three polypeptide chains is in a helical conformation. Furthermore, the three helical chains are coiled around each other to form a triple helix. Comprehensive literature exists on the biochemistry of collagen (Ramachandran and Reddi, 1976; Bornstein and Traub, 1979).

Collagen is unique in that every third amino acid residue is glycine in each of the three polypeptide chains (located inside of the helix, the small size of glycine is appropriate). Hence, the primary sequence of $(Gly\text{-}X\text{-}Y)_n$ is very common. The high content of imino acids (proline and hydroxyproline) is a characteristic feature, which together constitute about 15-30% of all residues. Hydroxyproline occurs mainly as 4-Hydroxyproline in collagens, and is placed exclusively in the third position (i.e. Y) in vertebrate collagen. Recently, it has also been recognised that it confers greater stability on the triple-helix conformation than does proline in this position. Hydroxyproline has been used as a criterion for identifying members of this class of proteins. Hydroxylysine is another unusual amino acid which occurs in collagens, though in smaller amounts than hydroxyproline. It is important as an attachment site for carbohydrates and also because of its involvement in covalent cross-linking.

The amount of attached carbohydrate varies greatly in mammalian collagen, the contents ranging from 0.4% in skin to about 4% in cartilage, to some 12% in basement membrane.

Type of Collagen	Tissue Distribution	Monomer Constituents	Glycoprotein	Characteristics
TYPE I	Skin, bone, tendon, dentin, ligament, blood vessels	$[\alpha 1\ (I)]_2 \alpha 2$	Fibronectin, instrumental in cell adhesion	Hybrid molecule consisting of $2\alpha 1(I)$ subunit chains plus a more basic and hydrophobic $\alpha 2$ subunit chain. Low hydroxylation of lysine (15%) Low carbohydrate content.
TYPE II	Cartilage	$[\alpha 1\ (II)]_3$	Chondronectin, aids cell adhesion	Intermediate (50%) hydroxylation of lysine, all hydroxylysines are glycosylated.
TYPE III	Skin, blood vessels, fetal skin, gastro-intestinal tract, and including a number of other soft connective tissue	$[\alpha 1\ (III)]_3$	Fibronectin, mediates cell adhesion	Relatively high levels of 4-hydroxyproline and glycine as well as cysteinyl residues/subunit chain (thus disulphide bonds present). Low (15%) hydroxylation of lysine.

(continued overleaf)

Table 1. Collagens

Type of Collagen	Tissue Distribution	Monomer Constituents	Glycoprotein	Characteristics
TYPE IV	Basement Membrane	$[\alpha 1\ (IV)]_3$ - with hesitancy, since the basement membrane may contain more than one type and that the compositions of basement membrane may vary in different tissues	Laminin, Entactin. Assists in cell adhesion.	High 3 - Hydroxyproline (1%); contains cysteine; most lysines hydroxylated; all hydroxylysines glycosylated; low alanine. Carbohydrate content not limited to glucose and galactose.
TYPE V	First located in pepsin - solubilised extracts of tissues that were rich in basement membranes. Since then, it has been located in placenta, lung, muscle.	Consists of 3 different chains $\alpha 1(V)$, $\alpha 2(V)$, $\alpha 3(V)$. However, conflicting evidence supports the existence of $[\alpha 1\ (V)]_2 \alpha 2(V)$, $[\alpha 1(V)]_3$ and $\alpha 1(V)[\alpha 2(V)]_2$	?	?

Glucose and galactose are the principal carbohydrate components of collagen, but there is also evidence for the binding of other sugars. Carbohydrates are apparently attached preferentially to hydroxylysines situated between arginine and the hydrophobic residues.

At present, there are at least five genetically and structurally distinct collagen types known (Fietzek and Kühn, 1976; Miller, 1976; Bornstein and Sage, 1980; Miller and Gay, 1982). Several glycoproteins are now identified that mediate cell adhesion to collagen (Aplin and Hughes, 1982). The following description of collagen types has been adapted from Bornstein and Traub (1979) and is given in Table 1.

The main function of collagen, which is universally recognised, is that of support. However, the idea that collagen is an inert structural protein no longer applies, since enough evidence exists to support the view that collagen may play an active part in:

1) Developmental processes (Gey et al., 1974; Bunge and Bunge, 1978).
2) Cell attachment (Meier and Hay, 1975).
3) Migration (Bard and Hay, 1975) and chemotaxis.
4) Binding of antigen-antibody complex.

The mechanisms mediating the collagen effects have still to be ascertained, but Balleisen et al. (1975) studying collagen type I mediated adhesion and spreading of platelets

discovered that the following structural parameters were of significance:

1) triple-helical conformation
2) non-triple-helical regions
3) charged amino acid chains
4) some activity was also detected for cyanogen bromide peptides of collagen α1 chain.

Some interest attends the possibility that adhesive specificity exists between cell types and their corresponding collagens which may be lost in oncogenic transformation (Aplin and Hughes, 1982). Hence, it was thought appropriate to look at the effect of serum on lymphocyte adhesion to collagen. Substrates of collagen prepared in a variety of ways have been generally regarded as the closest convenient in vitro approximation to the in vivo growth surface (Kleinman et al., 1981).

2.3.B. Fibronectin

Fibronectins are a group of glycoproteins present in connective tissues, on cell surfaces (secreted by cells such as fibroblasts, endothelial cells and hepatocytes), and in the plasma (Yamada and Olden, 1978; Mosher, 1980; Ruoslahti et al., 1981; Grinnell, 1981).

Two forms of fibronectin exist; the cellular form of fibronectin differs from the plasma form of fibronectin in molecular weight as well as in several biochemical and

biological properties (Yamada and Kennedy, 1979). Both forms of fibronectins possess a molecular weight in the range 420000-500000. Plasma fibronectin exists mainly as dimers, whilst cell surface fibronectin exists both as disulphide-bonded dimers and as multimers. Vuento et al. (1980) have proposed that fibronectins have a tendency for self-association (see Figure 2).

Evidence accumulated during the last decade suggests that they may play an important role in mediating cellular interactions of different cellular types with inert material surfaces (Grinnell, 1978) and with many other macromolecules, such as collagen (Klebe, 1974; Pearlstein, 1976) and fibrin (Stemberger and Hörmann, 1976; Grinnell et al., 1980). It has also been suggested that fibronectins have the ability to selectively recognise and bind denatured proteins and hence mediate their uptake by the reticuloendothelial system (Saba and Jaffe, 1980; Vuento et al., 1982).

The most prominent change occurring upon transformation of fibroblasts or myoblasts by biological or chemical modification is the loss of or reduction in the amount of fibronectin.

Fibronectin added to transformed cells binds to them in a fibrillar array very similar to that seen on normal cells. It causes detached cells to attach to the substratum and causes attached cells to spread out, elongate, and align with each other (Ali et al., 1977).

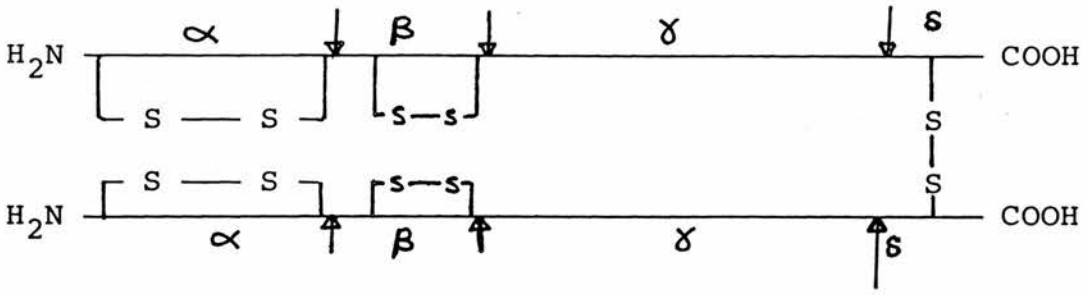


Figure 2.

Model of Fibronectin with functional domains*

↑ - proteolytic cleavage sites

Domains:

α - Binds and cross-links with fibrin, also self-associating area (M.Wt.: 30000).

β - Collagen binding domain (M. Wt.: 35000)

γ - cell-binding and heparin-binding area (M.Wt: 150000)

δ - Area where two chains of fibronectin are joined (M.Wt.: 10000)

* - adapted from Engel et al. (1981).

All five known mammalian collagen isotypes bind fibronectin with varying affinity, with type III collagen being the most effective native collagen in binding plasma fibronectin. Denatured collagen types I, II and III are substantially more effective in binding plasma fibronectin than native collagens. The interaction of denatured collagen with fibronectin promotes adhesion (Engvall and Ruoslahti, 1977; Engvall et al., 1978; Jilek and Hormann, 1978).

Fibronectin binds strongly between the amino acid residues 568-835 of the α1 subunit chain of type I collagen (Kleinman et al., 1976), plus several other weaker sites. According to Hughes and co-authors (1979), collagen-fibronectin binding is relatively weak and is stabilised by a large number of interactions between each cell and the substratum, for example baby hamster kidney (BHK) cells make about 50000 such contacts.

Schor et al. (1981) report that a fibronectin-collagen gel complex inhibits the migration of human skin fibroblasts and stimulates the migration of syrian hamster melanoma cells into the gel matrix. Kleinman et al. (1979) communicated that gangliosides inhibited fibronectin-mediated cell adhesion to collagen by binding to fibronectin, with the oligosaccharide of the ganglioside being the most potent binding region.

3. SERUM INHIBITORS

The role of serum proteins has been controversial, since several authors have demonstrated that serum promotes the substrate adhesion of various cell types (Weiss, 1961; Klebe, 1975; Grinnell, 1978), whereas results from other laboratories reveal that serum inhibits the adhesion of some cell types (Taylor, 1961; George et al., 1970; Witkowski and Brighton, 1972; Harris, 1973).

Recently, Kellie (1980) has affirmed that serum inhibits both lymphocyte-lymphocyte and lymphocyte-substrate adhesion *in vitro*. The inhibition by serum of glass-lymphocyte adhesion has been verified by Wayman (1981). These findings are perhaps of significance *in vivo*, when correlated with the observations of Evans and Proctor (1978). These authors relate that maximum aggregation of lymphocytes was obtained in serum-free conditions, at a shear rate of about $100S^{-1}$ in Couette viscometers. Note that shear rates of between $100\text{-}1000S^{-1}$ usually occur in the capillaries, but aggregation of lymphocytes does not generally occur *in vivo*. Hence, the serum may be inhibiting the intercellular interaction.

Musson and Henson (1979) have reported that autologous serum or heat-inactivated plasma inhibited the adherence of both human monocytes and lymphocytes to material surfaces. Fractionation by gel permeation on a column of Sephacryl S-200 revealed three peaks of inhibitory activity, coinciding with molecular weights of 230000, 90000 and 14000 daltons.

An attempt was made in this thesis to purify adhesion-inhibitors of lymphocytes, utilising the conventional techniques of isolation and purification. The possible serum proteins that are implicated as inhibitors are described, with substantiation of this perception in the discussion.

3.1 Alpha-2-Macroglobulin (α_2M)

Evidence is presented later by gel filtration studies, to indicate that α_2M may possibly be a lymphocyte adhesion inhibitor, therefore it is important to give details on α_2M.

α_2-globulins constitute approximately 8-9% of the human serum, and α_2-macroglobulin is part of this fraction (2-5mg/ml). α_2M is a glycoprotein (about 9% by weight) with a molecular weight of 725000 daltons and an isoelectric point of 5.4 (Laurell and Jeppsson, 1975; Harpel, 1976). The pH stability range of α_2M is limited to pH5-8.4, and the protein is unusually sensitive to ammonium ion.

The molecule of α_2M consists of four identical subunit chains, and each protomer has a molecular weight of 185000 daltons (termed band I). Two subunits of each of the dimers are held together by disulphide bonds, and both of the dimers are united by non-covalent interactions (Harpel, 1973; Harpel et al., 1979; Hall and Roberts, 1978).

Furthermore, the α_2M protomers can produce two additional polypeptide chains, at high temperature and under alkaline conditions in SDS of apparent molecular weights 139000 (termed band II) and 68000 daltons (band III) (Barrett et al., 1979; Harpel et al., 1979). The point of cleavage is approximately one-third of the way along the chain. As β-mercaptoethanol is requisite for this peptide cleavage, it can be assumed that the alkali-labile bond is bridged by an intra-chain disulphide bond. The generation of these fragments is enhanced by increasing the time of incubation. Alternatively, cleavage of an α_2M-subunit at the proteinase-susceptible site (the "bait domain") produces fragments of molecular weight 111000 and 98000 daltons (Barrett et al., 1979).

Barrett and Starkey (1973) have pointed out that this serum constituent is a potent endopeptidase inhibitor (thrombin, plasmin and kallikrein), and proposed the "entrapment" hypothesis to interpret the formation of the α_2M-protease complex. The fate of the α_2M complexes formed was traced to the reticuloendothelial cells in the liver, spleen, and bone marrow. The half-life of the complexes (trypsin and chymotrypsin) was about 8 min. (Ohlsson, 1971), and the half-life for the free molecule is about 10 days. The short half-life is reasonably related to the conformational change caused by the endopeptidases. The uptake of complexes of proteinase with serum α_2M via receptors by macrophages has been demonstrated <u>in vitro</u> (Debanne et al., 1975; Kaplan and Nielson, 1979).

α_2M may also have a transport function for various hormones and for metals. About a third of the zinc in plasma is bound to α_2M, and nickel is attached to it at least in rabbits.

The immunosuppressive activity both in vitro and in vivo of α_2-macroglobulin has been demonstrated (inhibiting antibody production), and transplantation centres have disclosed that blood transfusion accorded to kidney recipients are favourable to subsequent graft survival. A large proportion of this suppressive activity was lost from the plasma with the removal of α_2M (Mowbray, 1963; Barta, 1983). Recently, experiments by Koo (1983) have implicated α_2M or an α_2M-associated substance as a natural inhibitor of both human and murine tumours, and its effect is irreversible.

3.2 Serum Albumin

Albumin is the most abundant serum protein (4g/100ml.) and is one of the few plasma proteins that is not a glycoprotein. Bovine serum albumin has a molecular weight of 68000 daltons, and consists of a single polypeptide chain of 580 amino acid residues with 17 intrachain disulphide bonds, and one free sulphydryl group. The conformation of the molecule is such that 3 structural domains and 9 subdomains exist.

Apart from maintaining osmotic pressure, albumin acts as a carrier for the transport of such diverse substances as

calcium, fatty acids, amino acids and steroid hormones. Moreover, recently denatured human serum albumin has been identified as an adhesion-inhibitor of lymphocytes (Haston et al., 1982), and as a complex with 1gG in the inhibition of platelet aggregation (Sharma et al., 1981).

3.3 Assay and mechanism of adhesion

Adhesion is not a simple contact process but a physiological event, and it could be examined by one of the two methods:

a) measure the strength of cell adhesion

b) measure the rate of adhesion

The method employed in the current study to assay lymphocyte adhesion can be utilised to encompass both of the above two options. However, it was constantly used to measure the rate of adhesion, yet, it could have easily been utilised to evaluate the strength of adhesion, as was done by Kellie (1980), and he calculated the force of detachment (F_d) on a single lymphocyte adhering to glass to be 1.015×10^{-13}N. Briefly, a diffusion chamber ring, sealed on both sides with glass coverslips, was used to measure the number of adherent lymphocytes to a glass coverslip. It can be stated that the present method of assay is both convenient and reproducible for monitoring inhibitory activity. The assay system is a detachment method (involving the force of gravity, which acts in an opposite

direction to the adhesive force) and entails the inversion of the substrate to which the cells are adhering, and the subsequent counting of the adherent cells.

Since serum contains inhibitors of lymphocyte adhesion, it seems logical to purify these factors. However, the inhibitory activity to date has been measured in terms of percentage adherent cells; this is an arbitrary unit (Kellie, 1980; Hassan, 1981; Wayman, 1981). Hence an attempt was made to quantify the inhibitory activity in terms of a defined activity unit (see discussion).

The mechanism of the adhesion process is still largely unknown, but is slowly being elucidated. However, the results (see later) illustrate that lymphocyte-glass adhesion follows Michaelis-Menten type kinetics, and adhesion could be interpreted by a two step process. The effect of serum as an inhibitor is also being analysed.

Cellular interactions with artificial or biological substrates, and with other cells involve the interplay of the following factors amongst others:

a) Electrostatic bonds

b) Hydrogen bond

c) Long-range forces

d) Bridging mechanisms

For an extensive survey on the various factors which may contribute to attachment see Curtis (1973). Presumably, the same forces may have to be broken for inhibition to take

place. However, it is noteworthy to point out that Bell (1978) has related that "the force required to separate two cells is greater than the expected electrical forces between cells, and of the same order of magnitude as the forces required to pull gangliosides and perhaps some integral membrane proteins out of the cell membrane".

Kellie (1980) has reported that the adhesion of thymocytes to glass in the absence of serum is metabolism and divalent cation independent and trypsin resistant. Adhesion is dependant, however, on ionic environment and is likely to be mediated to a large extent by hydrogen bonding.

Aims

Workers in different laboratories have observed that serum acts as an inhibitor of intercellular and cell-substrate adhesion. The objectives of the present study were to:

1) Follow the effects of serum on lyphocyte adhesion to glass, and to both native and denatured collagen two-dimensional films.

2) purify and isolate the factors responsible for inhibition of cell adhesion to glass,
   a) initially by gel filtration
   b) develop conditions for other methods of isolation and purification - techniques of differential solubility by using ammonium sulphate and polyethylene glycol, and ion-exchange for separation
   c) using SDS-polyacrylamide gel electrophoresis and analytical ultracentrifuge to determine homogeneity

3) examine the kinetics of cell adhesion by following:
   a) the initial rate of adhesion on cell concentration
   b) at a fixed cell concentration, the dependence of the initial rate of cell adhesion on the percentage serum concentration.

Materials and Methods

MATERIALS AND METHODS

	page
1. Chemicals	31
2.1 Animals	32
2.2 Serum	32
3. Preparative Procedures	32
3.1 Gel filtration	32
3.2 Ammonium sulphate precipitation	35
3.3 Ion-Exchange	36
3.4 Polyethylene glycol fractionation	39
3.5 Collagen preparation	39
4. Analytical Procedures	42
4.1 Assay	42
4.2 Cell viability	45
4.3 S.D.S. polyacrylamide gel electrophoresis	45
4.4 Analytical Ultracentrifugation	53
4.5 Protein determination	53
4.6 Amino acid analysis	54
4.7 Iso-electrofocusing	54

1. Chemicals

Sepharose 4B was purchased from Pharmacia (Great Britain) Ltd., Middlesex, England. CM-cellulose was supplied by Whatman Chemical Separation Ltd., Maidstone (Kent), England, and so were the columns for Gel-filtration and Ion-exchange. The following were acquired from B.D.H. Chemicals Ltd., Poole, Dorset, U.K.: Acrylamide; Ammonium sulphate; Chloramine T; Cupric sulphate, 4-Dimethylaminobenzaldehyde; Disodium hydrogen phosphate; 2-mercaptoethanol; N,N′-methylene bisacrylamide; N,N,N′,N′-tetramethylethylene diamine (TEMED); Sodium hydroxide; and Sodium dodecyl sulphate (sodium lauryl sulphate). Sodium chloride and potassium dihydrogen phosphate were purchased from Fisons Scientific Apparatus Ltd., Loughborough (Leicestershire), England. Protein standards (Phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, myoglobulin) and Hydroxy-L-Proline (4-Hydroxy-L-proline) were obtained from Sigma Chemicals Co. Ltd., London-England. All other chemicals were of Analar grade from B.D.H. Chemicals Ltd., U.K.

2.1. Animals

An inbred strain of mice (C57BL6 Ola or C57BL10 S_CS_N) were used, which were acquired from the Bute Medical Building Animal House, University of St. Andrews. The mice were fed and watered ad libitum.

2.2. Serum

Calf serum was purchased from Flow Laboratories Ltd., Scotland.

3. Preparative Procedures

3.1 Gel Filtration

This is generally considered to be an extremely useful purification technique, and can be applied to separate molecules of varying molecular size by the use of columns packed with gels. The matrix used in this study is

Sepharose 4B with molecular weight separation range from 6×10^4 to 20×10^6 daltons. (Pharmacia - Gel filtration; Ackers, 1975).

3.1.A Buffer

Dulbecco's phosphate buffered saline (Dulbecco's PBS) was used for dialysis of samples, equilibration of the Sepharose 4B column, and elution of the samples. The constituents of the saline are listed below for a litre of water (final pH 7.3):

$CaCl_2$ - 100mg
KCl - 200mg
KH_2PO_4 - 200mg
$MgCl_2.6H_20$ - 100mg
NaCl - 8g
Na_2HPO_4 - 1.15g

3.1.B Fractionation of Serum by Gel Filtration

3.1.B.I. First Run

5-6 mls of serum were applied to a Sepharose 4B gel filtration column (50 x 2.2cm.) at room temperature, which had previously been equilibrated with Dulbecco's PBS. Once the serum had adsorbed onto the gel surface, the sample was eluted with Dulbecco's PBS and fractions collected. The absorbance of the eluant fractions was measured at 280 nm in a Cecil Spectrophotometer (CE272 Linear Readout U.V.) using silica cuvettes. See Elution profile in results (Fig. 10).

3.1.B.II Second Run

Fractions 14 to 21 on the first run (see Fig. 10) on the gel filtration column were pooled (1 ml. of each fraction was saved for molecular weight and assay). The pooled sample was concentrated either by freeze-drying (a) or by ultrafiltration (b).

a) Freeze-Drying Method

The concentrated pooled sample was dialysed with water for 24 hr. at 4°C., and further dialysed for 6 hr. at 4°C against fresh distilled water. The dialysed pooled sample was freeze dried, reconstituted in 6ml. of distilled water and dialysed against Dulbecco's PBS overnight at 4°C. The dialysis was repeated for a further 6 hr. at 4°C with fresh Dulbecco's PBS. The concentrated sample was applied to the Sepharose 4B column and fractions collected. Subsequently, the absorbance of the fractions was measured.

b) Ultrafiltration Method

The pooled fractions were concentrated by ultra-filtration using an Amicon XM50 membrane with a cut-off molecular weight value of 50000 daltons. The concentrate (5ml.) was dialysed overnight with Dulbecco's PBS at 4°C, and then fractionated by gel filtration at room temperature, and the optical density of the collected fractions was read on a Cecil spectrophotometer.

Note that alternate fractions from the two runs were assayed for inhibitory activity, and used to determine the molecular weights by .S.D.S. Polyacrylamide gel electrophoresis.

Furthermore, the above two runs were repeated with nine pure serum samples (5-6 mls.) as this was requisite for the eventual determination of molecular weights in the ultracentrifuge.

3.2 Ammonium Sulphate Precipitation

Neutral salt precipitation is a simple and useful method for protein separation.

A 100% saturated ammonium sulphate solution of molarity 4.10 was prepared at room temperature and adjusted to pH 7.2 with ammonium hydroxide solution (Segel, 1976).

18ml. of the saturated ammonium sulphate solution was added to 22ml. of pure calf serum so as to give a final concentration of 45% (V/V), and stirred at room temperature for 30 min. The precipitate was centrifuged at 10,000 r.p.m. for half an hour. The pellet acquired after centrifugation was dissolved in water and dialysed for 24 hr. twice against Dulbecco's PBS at 4°C. Ammonium sulphate concentration of the supernatant was raised to 68%, and the procedure above repeated for both the precipitate and the supernatant. Hence, three serum fractions were obtained:

α) the initial precipitate with 45% saturation

β) precipitate with 68% saturation

γ) supernatant from 68% precipitate

An adhesion inhibition activity assay was performed on these three fractions. Previously, the optical density was measured on a U.V. spectrophotometer.

3.3 Ion-Exchange

3.3.A Initial experiment to determine starting pH and ionic strength

Preliminary experiments were performed with the Whatman cation-exchanger CM-cellulose 52 in order to select the pH at which the active components are adsorbed.

The starting buffer chosen was sodium acetate titrated with acetic acid to the required pH (Pharmacia - Ion-exchange chromatography), and the pH range was selected from pH 3.5 to 6 with pH intervals of 0.5pH units. A series of six test-tubes (15 ml.) were placed in a test-tube rack, and each tube was marked with the specific pH in the pH range. One gram of CM-cellulose was added to each tube, and each was equilibrated with 0.5M sodium acetate buffer with its specified pH by washing several times with 100ml. of the buffer. Subsequently, the exchanger in each tube was equilibrated by washing with 50ml. of 0.05M buffer of the same pH as labelled on the tube. 2ml. of serum was added to each tube, and the exchanger in each tube was shaken for 6 min. The gel was allowed to settle,

and the supernatant was assayed for adhesive inhibitory activity.

Once the starting pH of the buffer had been established, the next step involved the selection of the starting ionic strengths. Four different buffer solutions were prepared, with the following constituents:

a) 0.05M NaOAc, 0.5M NaCl at pH5 (and at pH3.5)

b) 0.05M NaOAc, 1M NaCl at pH5 (and at pH3.5)

and the procedure was as follows:

1) A test-tube with a gram of cation-exchanger was equilibrated with the sodium acetate buffer (0.05M) at pH5 save sodium chloride

2) 2ml. of serum was added to the tube, shaken gently for 10 min., allowed to settle and the supernatant discarded

3) The gel was washed with 5x10ml. of sodium acetate buffer (0.05M NaOAc, 0.5M NaCl) at pH5 and the supernatants were pooled and assayed for adhesive inhibitory activity.

4) The gel was again washed with sodium acetate buffer (0.05M NaOAc, 1M NaCl) at pH5 and the process in (3) was repeated.

5) Steps (1) to (4) were repeated with resin equilibrated at pH 3.5.

3.3.B. Actual Experiment

The ammonium sulphate precipitation experiment gave adhesive inhibitory activity with the 45% precipitate, and it was decided to combine this procedure with the Ion-exchange purification step. The precipitate from the ammonium sulphate salt precipitation was dissolved in water and dialysed with 0.05M sodium acetate buffer (pH 5) overnight at 4°C, and further dialysed with fresh buffer for 24 hr. at 4°C.

Initially, the ion exchanger was prepared as outlined in the Whatman Booklet on Ion-exchange (that is, precycled, degassed and equilibrated).

The dialysed sample was applied to a column (1.6 x 40cm.) containing CM-cellulose 52 ion-exchanger, which had previously been equilibrated with 0.05M sodium acetate buffer (pH5). Once the sample had been adsorbed by the gel, the column was eluted with the equilibration buffer, and the flow rate was maintained by a peristaltic pump and the absorbance of the eluate was monitored at 280nm. Penultimately, the column was eluted with the equilibration buffer containing 0.1M NaCl, at the same flow rate and again the absorbance was monitored at 280nm. Finally, a linear concentration gradient (0.1M-0.6M NaCl) in 0.05M sodium acetate buffer was applied to the column, maintained at the same flow rate and absorbance of the fractions monitored at 280nm. Aliquots removed from the eluted fractions were examined for adhesive inhibitory activity

and the molecular weights determined by SDS-polyacrylamide gel electrophoresis.

3.4 Polyethylene glycol (PEG) Fractionation

PEG separation is an extremely useful technique for the precipitation of proteins since all the labile proteins remain protected under these conditions.

This partial purification is a two step process (Barrett et al., 1979; Hao et al., 1980):

a) 5.6ml. of 25% ($^{W}/V$) PEG 6000 in 50mM trizma adjusted to pH 7.2 with HCl was slowly added to 20ml. of serum and stirred for 30 min. at room temperature, so as to bring the final concentration of PEG to 5.468%. The mixture was centrifuged at 15000 r.p.m. for half-an-hour at 4°C and the pellet discarded.

b) 14.4ml. of 25% P.E.G. was mixed with the supernatant and the procedure in (a) was repeated, except for the fact that the supernatant was discarded and the precipitate formed at a final concentration of 12.5% P.E.G. was dissolved in 2ml. of 0.05M trizma buffer at pH 7.2. This was dialysed for 24 hr. with two changes of Dulbecco's PBS, and assayed for inhibitory activity.

3.5 Collagen Preparation

Rat tail tendons were used for collagen extraction using the method of Steven and Tristram (1962). The long

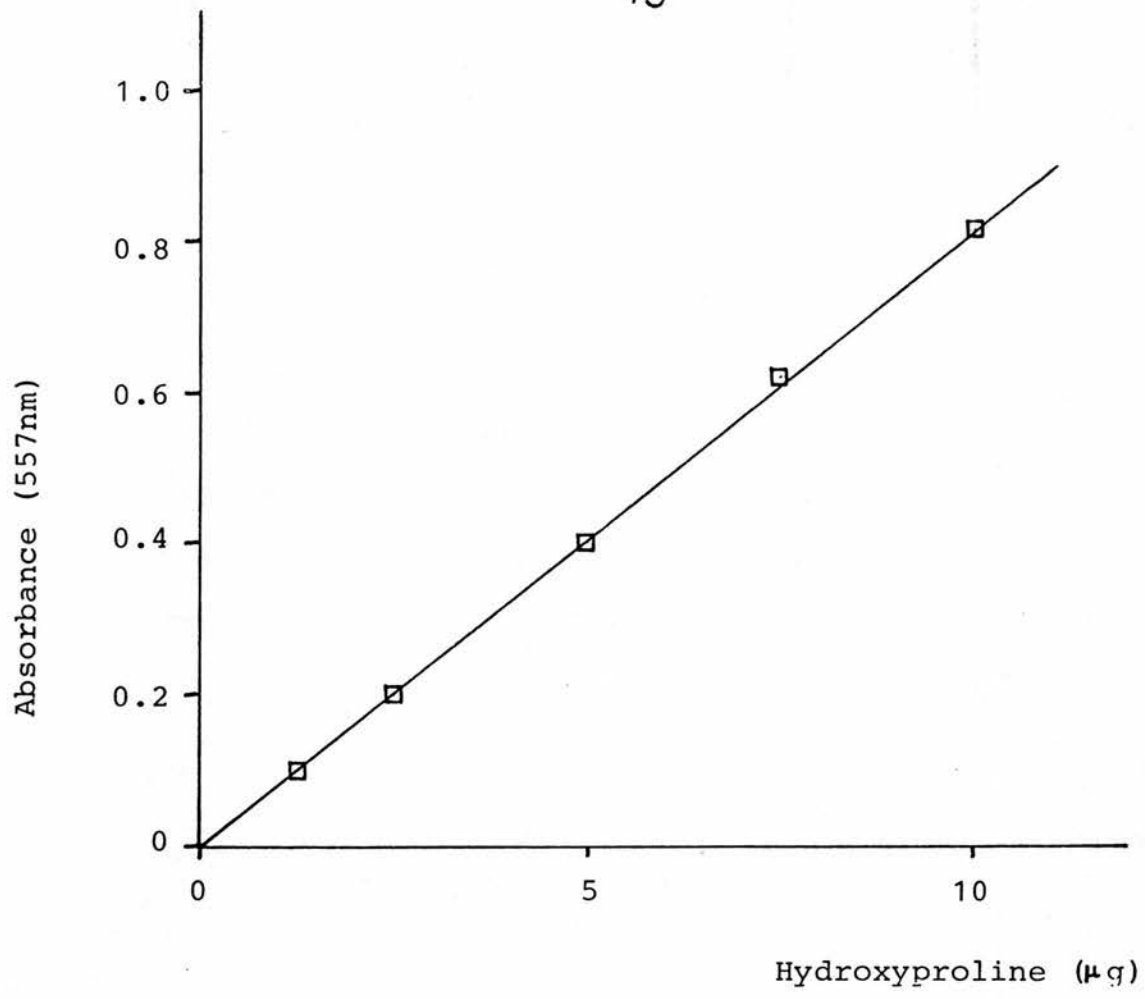


Figure 3

Hydroxyproline standard curve

silvery tendons were freed from the tails, and cut into small pieces and suspended in 0.2M disodium hydrogen phosphate at room temperature for 18 hr.; a couple of drops of toluene were added to prevent bacterial contamination. The insoluble residues were separated by centrifugation at 6000 r.p.m., and further reduced in size by disintegration in a Waring Blender. The insoluble material was suspended in 0.1M acetic acid for 24 hr. at room temperature, and centrifuged at 6000 r.p.m. for 30 min. to separate the acid soluble collagen from the remaining solid residue. The insoluble residue was resuspended in acetic acid and incubated for a further period of 24 hr. at room temperature, and centrifuged again for 30 min. at 6000 r.p.m. The two supernatants were pooled, and collagen was precipitated by the dropwise addition of 30% ($^{W}/V$) NaCl solution with continuous stirring, precipitation of protein being complete at a final concentration of 7% sodium chloride. After centrifugation, the precipitate was redissolved in 0.1M acetic acid, and the protein was again salted out by the addition of sodium chloride. This procedure was repeated thrice over to attenuate the non-collagenous contaminants.

The hydroxyproline content was determined by the method of Stegeman (1958), see Figure 3. The conversion factor, 7.46, was used as determined by Neuman and Logan (1950). Concentration of collagen in the extract was 1.3 mg/ml.

The collagen was subsequently dialysed against double distilled water for 24 hr. in the cold room, and was then stored in the refrigerator. This is referred to as "native collagen". A portion (20ml.) of the collagen was heated in a water bath kept at 60°C for 30 min., and subsequently stored in the refrigerator. This is referred to as "denatured collagen".

Glass coverslips (13mm. diameter) were coated with 60µl of native collagen and dried for 2hr. in an incubator at 37°C, and the assay described in the next section was enacted. The process was repeated with denatured collagen.

4. Analytical Procedures

4.1 Assay

4.1.A. Cell Preparation

Mice were killed by prolonged ether inhalation.

Mesenteric lymph nodes were removed from these mice and placed in RPMI 1640 (Gibco-Biocult Ltd.) supplemented

with 2mM glutamine. The mixture of RPMI 1640 and glutamine is referred to as a Medium. A cell suspension was prepared by teasing apart the lymph nodes with a scalpel. The cell suspension was then filtered through a 20µm. nylon mesh (Begg Cousland Ltd.) to remove the solid residue, centrifuged at 1500 r.p.m. for 3 min., and decanted. The cells were then resuspended in 1ml. medium, counted, and subsequently diluted to a concentration of approximately 6×10^6 cells/ml. with the ice cold medium.

The number of cells in the suspension were counted by diluting a 40µl. aliquot of the suspension in 20ml. Isoton (Coulter Electronics Ltd.) and counting in a Coulter electronic particle counter (Model ZB) linked to a channelyzer (Model C1000). The settings written below were used on the counter:

Aperture Current (I) = 0.117mA

Amplitude (A) = 1

Threshold Limit (Tl) = 10

4.1.B. Determination of Cell-Substrate Adhesion

Two 1.3cm. diameter glass coverslips (Chance Propper Ltd.) were cleaned by washing in 50% HCl for 1 hr. thoroughly rinsed in double distilled water, subsequently rinsed in acetone and air dried in a warm cabinet. The coverslips were fixed onto both sides of a 1cm. diameter diffusion Chamber ring (Millipore Ltd., Cat. No. PR0001401) with a mixture of paraffin wax and vaseline.

Employing a 1ml. syringe with a small bore needle, approximately 0.17ml. of cell suspension was introduced into the chamber through an aperture in the side, until the chamber was completely filled. The apertures were sealed with the wax/vaseline mixture. Chamber rings thus prepared were then incubated at 37°C for 1 hr., inverted for 5min. (30 min. for collagen as substrate), and placed onto a glass slide on a microscope stage. Cells adherent to the upper coverslip were counted using phase contrast optics, with a x40 objective lens and a gridded eye-piece (0.023mm^2). Under these conditions, it was difficult to differentiate between monocytes and lymphocytes, however, polymorphonuclear leukocytes could be identified by their lobed nuclei.

Usually, triplicate chambers were produced per sample and three fields of view in each chamber were counted. Initially, serial dilutions were performed on pure serum, which were assayed for inhibitory activity, and the buffer was used as a control. However, assays carried out on fractions collected from gel filtration and Ion-Exchange columns were performed at 50% dilution with the medium.

The percentage number of adherent cells was calculated as follows:

$$\frac{\text{No. of cells adhering to glass with serum fraction}}{\text{No. of cells adhering to glass with buffer}} \times 100$$

4.2 Cell Viability

Cell viability was tested by using one part of cell suspension mixed with one part of 0.2% trypan blue (BDH) solution in Dulbecco's phosphate-buffered saline. The mixture was placed in a haemocytometer and over 100 cells counted. Living cells were able to exclude the dye whereas the dead cells permitted the dye to enter easily. The viability was consistently approaching 85%, and was calculated as shown:

$$\frac{\text{Total cells - dead cells}}{\text{Total cells}} \times 100\%$$

4.3 Sodium dodecyl sulphate (S.D.S) - Polyacrylamide gel electrophoresis (P.A.G.E.): SDS-PAGE

This method used to determine molecular weights is extremely useful, since it is easy to perform, and is both reliable and reproducible, using minute quantities of protein. Also, the method was used to assess the homegeneity of the fractions collected from the preparation. S.D.S. is an amphiphilic substance, and forms a water-soluble complex with proteins. Hence, therefore, the complexes are differentiated according to their molecular sizes during electrophoresis (Shapiro et al., 1967; Weber and Osborn, 1969), Reynolds and Tanford (1970) experimented with the interaction of SDS with proteins, and concluded:

1) The association of SDS and protein is non-specific for virtually all water-soluble proteins, and 1.4g

of SDS is bound per gram of protein. Note that, however, there are exceptions to this figure (Takagi et al., 1975).

2) Association occurs in two steps.

3) Only the monomeric form of SDS interacts with protein, not the micellar form.

4) The association between the complex is mainly hydrophobic.

4.3.A. Solutions

The following solutions were employed to prepare gels of different porosities using a modification of the method of Weber et al. (1972):

A) Chamber buffer:

0.1M Tris/HCl	12.1g
0.2% (W/V) SDS	2g

The solution was adjusted to pH 7.4 with HCl, and made up to 1 litre.

B) Gel Buffer:

0.2% (W/V) SDS	1g
0.1M Tris/HCl	6.05g
6M urea	180g

Again, this solution was titrated to pH 7.4 with HCl and made up to 500ml.

C) Solutions for sample preparation

1) 8M urea 24.024g

3% (W/V) SDS 1.5g

For non-reduced samples, made up to 50ml.

2) 8M urea

3% SDS

3% β-mercaptoethanol

This solution was used for reduced samples.

D) Acrylamide Stock solution

The quantities in grams of acrylamide and N,N′-methylene bisacrylamide used in 200ml. of Gel buffer are listed in the table below in order to make gels of different porosities:

Final % Acrylamide Concentration	Acrylamide (g.)	Bisacrylamide (g.)
3	5.82	0.18
4	7.69	0.31
5	9.50	0.50

E) Ammonium Persulphate Solution

See Table 2 below for quantities utilised. Solutions were freshly prepared each time electrophoresis was performed.

F) Staining Solution

Coomassie Brilliant Blue R250 (BDS)	2.5gm.
Methanol	454ml.
Distilled water	474ml.
Glacial acetic acid	72ml.

G) Destaining Solution

Acetic acid	75ml.
Distilled water	675ml.
Methanol	250ml.

4.3.B. Standard Solutions

Carbonic anhydrase (29000), ovalbumin (43000), bovine serum albumin (68000) and phosphorylase b (92000) were used as standards.

Approximately 2mg. carbonic anhydrase was dissolved in 1ml. of chamber buffer in a thin walled test-tube, and placed immediately in a boiling water bath for 2 min. The process was simultaneously repeated with the other three proteins. The proteins were stored at -20°C.

50µl of each of the standards was mixed in a thin walled test-tube with 50µl of chamber buffer. 250µl of 8M urea /3% SDS/3% β-mercaptoethanol was added to the mixture, and the test-tube immediately placed in a boiling water bath for 2min. The solution was stored in a small vial at -20°C.

Approximately 25µl of the standard solution was used per gel.

4.3.C. Sample preparation

1) Desalting (Neal and Florini, 1973)

A 2ml. column of Sephadex G-25 was made in 2ml. syringe, which was thoroughly washed with chamber buffer (~20ml.). Subsequently, the column was bench centrifuged (setting 7) for approximately 15 sec. About 200µl of the sample was applied to the column, particularly aliquots from the gel filtration column. The column was centrifuged again under the above conditions, and the sample was obtained in the chamber buffer. Sephadex G-25 was regenerated by washing the column with chamber buffer.

2) Preparation of reduced fraction

A 0.25ml. fraction of a sample was introduced into a thin-walled test-tube containing 250µl of 8M urea/3% SDS/ 3% β-mercaptoethanol, and the tube instantly placed in a boiling water bath for 2min. Either, the reduced preparation was used at the time or stored in the freezer in a vial, and used as required.

Table 2 showing polymerising composition of gel solution for different porosities of gels

	Final concentration of acrylamide solution (%)		
Solution	3	4	5
Acrylamide stock	19ml.	19ml.	19ml.
Ammonium Persulphate	1ml. (150mg/10ml.H_2O)	1ml. (135mg/10ml.H_2O)	1ml. (100mg/10ml.H_2O)
TEMED	30µl	27µl	25µl

4.3.D. Gel Preparation

Clean glass tubes (10cm. long, 4mm. internal diameter), which were sealed with parafilm at one end, were employed to prepare gels. The gel solution, which had been freshly prepared, was immediately transferred to gel tubes using a Pasteur pipette and filled to 1cm. from the top. Any trapped air-bubbles were released by gently tapping the tubes. Distilled water was carefully and immediately layered on top of the gel surface in each tube, thus preventing an air-gel meniscus and ensuring a flat gel surface. Approximately 30 min. later, the gel buffer replaced the distilled water once the gel had been polymerised. The other ends of the tubes were also capped with parafilm to prevent dehydration and the tubes were then stored in the refrigerator before use.

4.3.E. Electrophoretic procedures

For each gel, from a desalted sample, an aliquot of fixed volume in microlitres (according to the optical densities) was taken from a sample (that is, gel filtration, ion-exchange,etc.), and washed in equal volume of sample buffer (SDS/urea). This mixture was then added to one drop of glycerol and a single drop of bromophenol blue onto parafilm and thoroughly mixed. This process was repeated with other samples, up to a maximum of eight.

Before sample loading, the parafilm covering the bottom and top of the gel tubes was removed and the buffer layer

shaken from the gel surface which was rinsed with a fresh volume of buffer; the tubes were then placed in the electrophoretic tank. The lower chamber of the electrophoretic tank was filled with chamber buffer, and the samples were loaded onto the top of the gels with a micropipette. The chamber buffer was carefully layered over each sample volume, and the upper chamber was filled with buffer.

Electrophoresis was performed at 5mA per gel (constant current) at room temperature, and stopped when the tracker dye was approximately 1cm. from the bottom of the tube. The process usually lasted 3 hr. Subsequently, the gels were displaced from the electrophoresis tube by squirting water from a syringe between the gel and the tube at both ends. The centre of the bromophenol blue band that migrated from the top of the gel was marked with Indian ink.

4.3.F. Staining and Destaining

The gels were submerged in staining solution (Coomassie blue R-250 is many fold sensitive than other dyes) contained in test-tubes at room temperature for 1 hr. (Frazekas de St. Groth, 1963). Excess dye was washed off with distilled water.

The gels were placed in destaining solution, which was replaced periodically with fresh solution until the backgrounds were clear.

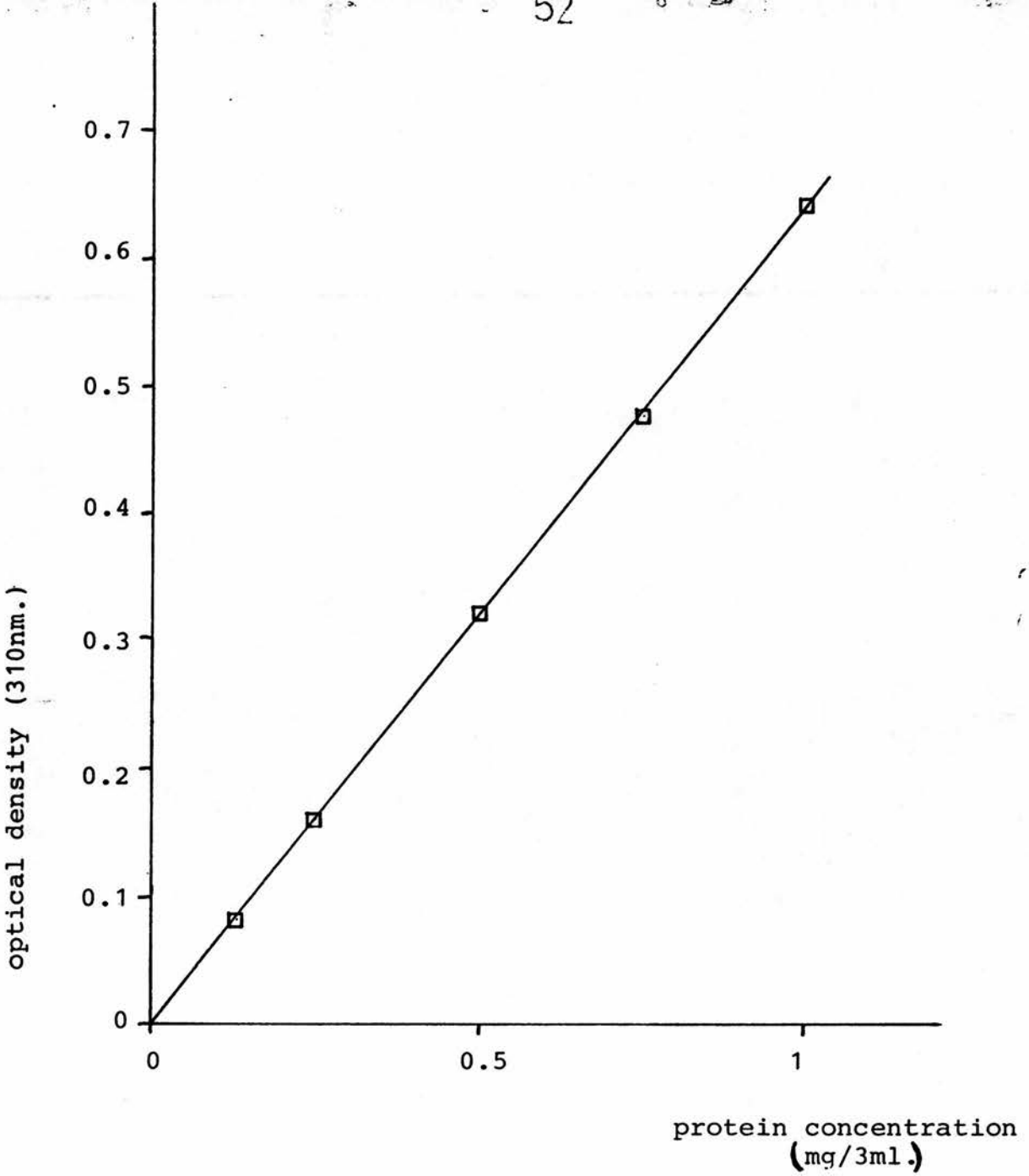


Figure 4

Standard curve for bovine serum albumin concentration

4.3.G. Molecular Weight calculations

Destained gels were photographed and scanned on a Vitatron densitometer (TLD 100 - Fisons). The distance of the protein band and the dye band migration from the top of the gel was measured and the values for mobility computed using the program SDS. Cal. Hence, the molecular weights were determined.

4.4 Analytical Ultracentrifugation

This method was used in the determination of molecular weights, and to test the purity of the fractions collected from gel filtration runs.

The pooled fraction, collected from ten gel filtration runs (see section 3.1.B.II), was lyophilised and dissolved in 1ml. of 0.01M potassium phosphate buffer supplemented with 0.1M KCl at pH 7.5, and dialysed overnight in 500ml. of the same buffer, and then for 3 days in 500ml. of fresh buffer.

Sedimentation coefficients were determined in a Beckman (Model E) analytical ultracentrifuge.

4.5 Protein determination

The protein content of the pooled fraction (from gel filtration studies) for ultracentrifugation was determined by the method described by Itzhaki and Gill (1964) with Bovine serum albumin as a standard. This is the micro-biuret method (see Figure 4).

4.6 Amino acid analysis

A sample from the pooled fraction from the ten gel filtration runs was hydrolysed for 24 hr. in 6N HCl at 110°C, dried by rotary evaporation over KOH in a desiccator, and analysed on a Jeol JLC-5AH amino acid analyzer.

4.7 Iso-electrofocusing

This was performed by Mr. Stuart Finlayson on an aliquot of the pooled fraction (see Results section 2.2).

Results

Results

		page
1.	The inhibition of cell-substrate adhesion by serum	56
1.1	Glass as substrate	56
1.2	Collagen as substrate	61
2.	The purification of serum proteins	66
2.1.A	Gel filtration (Run 1A)	66
2.1.B	Gel filtration (Run 1B)	66
2.2	Gel filtration (repeat of above two runs)	72
2.3	Molecular-exclusion chromatography (using a longer gel bed)	72
2.4	Amino acid analysis	77
2.5	Analytical techniques	77
2.6	Ammonium sulphate precipitation	88
2.7	Conditions for ion-exchange	88
2.8	Ion-exchange	95
2.9	Polyethylene glycol (PEG) fractionation	99
3.	The kinetics of lymphocyte adhesion	103

1. The inhibition of cell-substrate adhesion

That serum is an inhibitor of cell-substrate and intercellular adhesion has been indicated by several authors. The lymph node cell adhesion to glass and collagen substrates was assayed as described in materials and methods. The conditions for the assay had previously been established by Kellie (1980).

Mesenteric lymph nodes contain mainly lymphocytes (97%), and the remaining constituents are monocytes/macrophages and erythrocytes.

1.1 Glass

The effect of serially diluted calf serum was investigated on lymphocyte cell adhesion to glass. From the results (Figures 5-7 and Table 3) it can be concluded that the serum distinctly inhibits lymphocyte adhesion to glass (N.B. 10% serum concentration and above inhibits 80% of lymphocyte adhesion).

The percentage (%) cell adhesion to both glass and collagen is calculated as indicated below:

$$\%\ \text{adherent cells} = \frac{\text{No. of cells adhering to glass/collagen with serum}}{\text{No. of cells adhering to collagen/glass without serum}} \times 100$$

Percentage (%) inhibition is determined from:

$$\%\ \text{inhibition of cells} = 100 - \%\ \text{adherent cells}$$

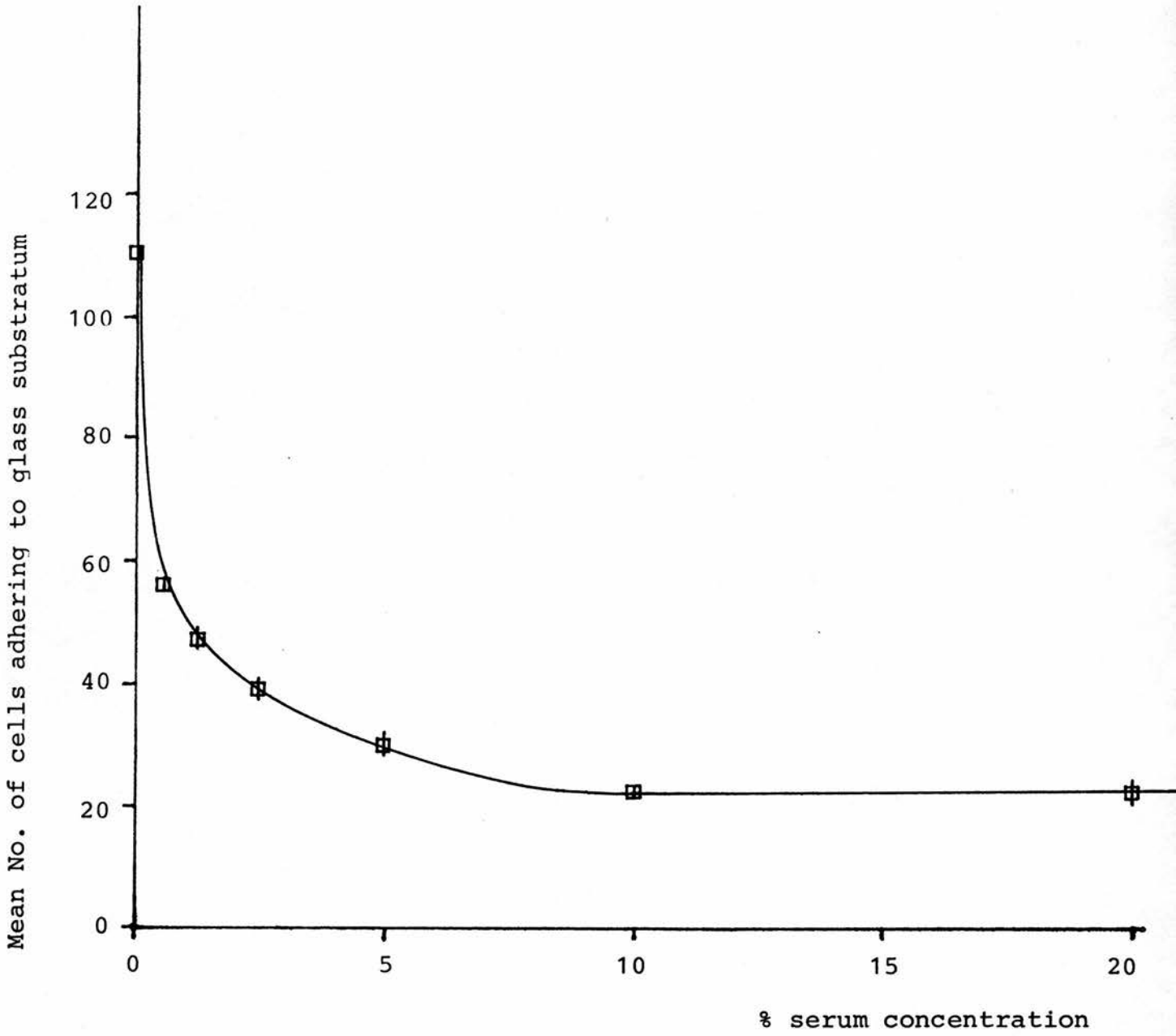


Figure 5

The effect of serum on lymphocyte adhesion to glass substratum

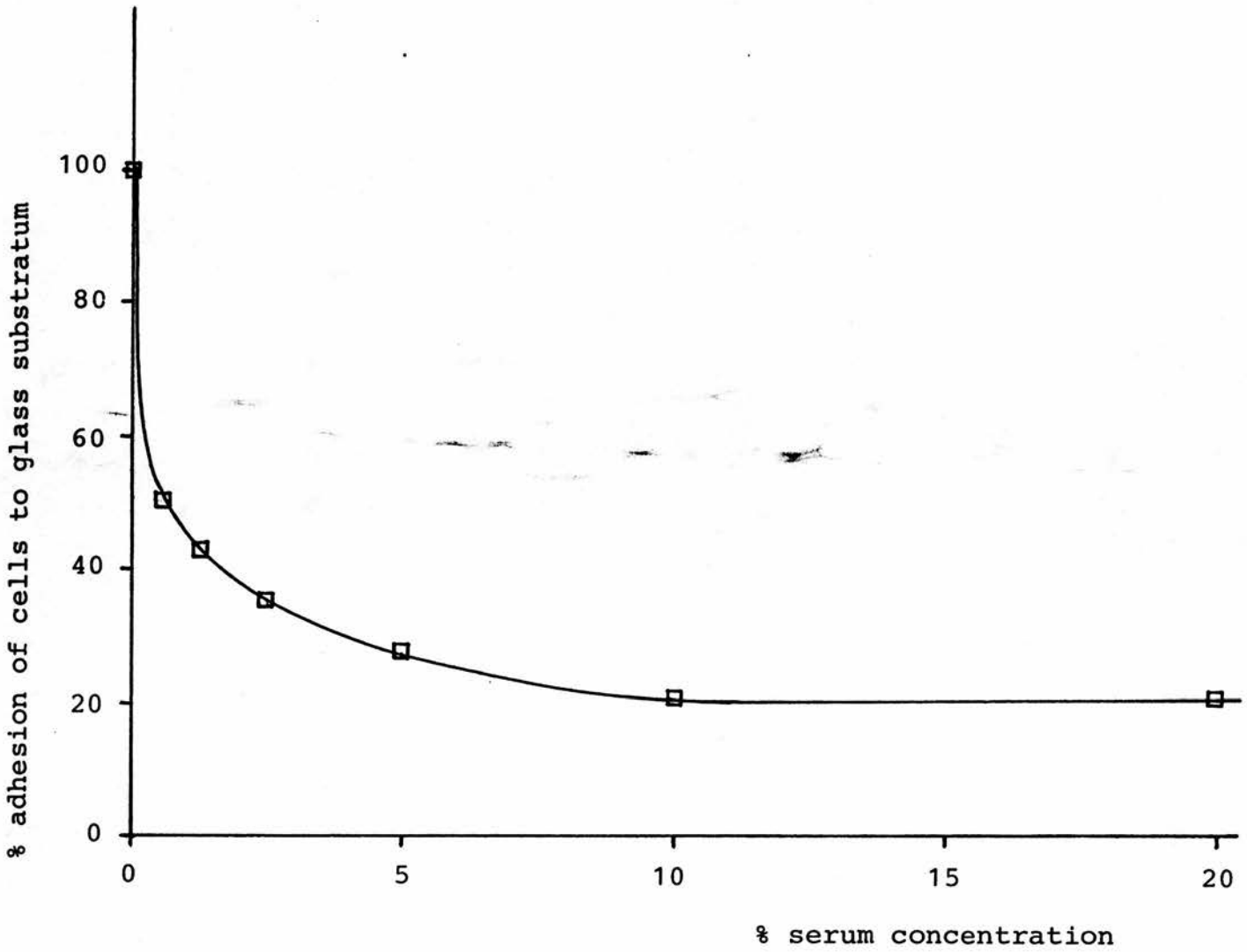


Figure 6

Effect of serum on lymphocyte adhesion to glass

Table 3

Influence of serum on lymph node cell adhesion to glass substratum

Serum Conc. (%)	Mean Number of cells adhering ± S.E.M.*	Mean % of cells adhering ± S.E.M.*	% Inhibition
0.0	110.55 ± 4.70	100	-
0.625	55.77 ± 1.67	50.45 ± 1.51	49.55
1.25	47.10 ± 1.86	42.61 ± 1.69	57.39
2.5	39.33 ± 1.98	35.58 ± 1.79	64.42
5.0	29.88 ± 1.78	27.03 ± 1.60	72.97
10.00	21.88 ± 1.09	19.80 ± 0.98	80.20
20.00	21.22 ± 2.24	19.20 ± 2.03	80.80

* n → 9

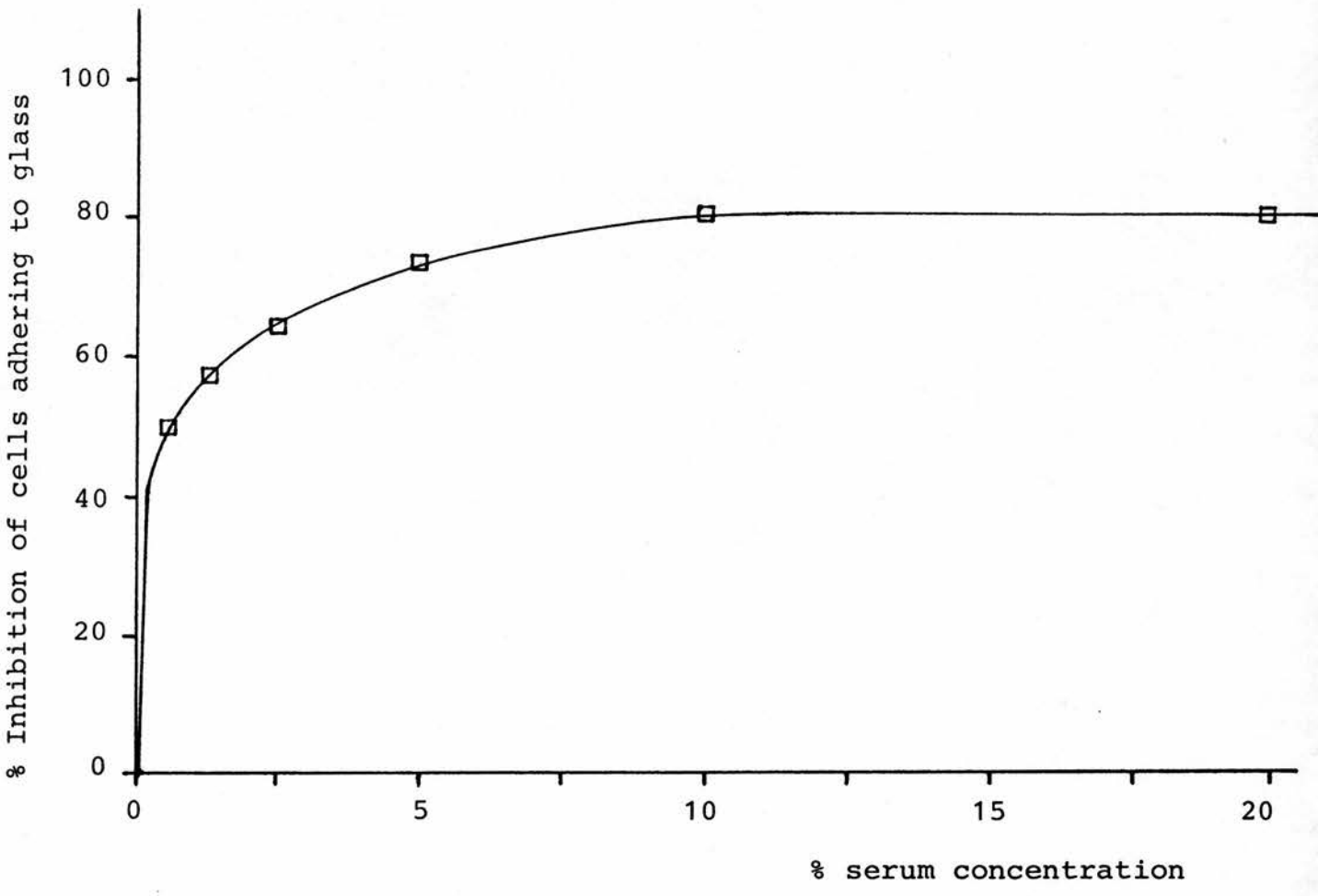


Figure 7

Effect of serum concentration on % lymphocyte inhibition to glass

1.2 Collagen

In contrast, the adherence of lymphocytes to collagen (both native and denatured) in the presence of low serum concentration (<10%) is promoted while at high serum concentrations a relative inhibitory effect is observed. Note however, that promotion of lymphocyte adhesion is more pronounced in the case of denatured collagen substrate (Figures 8 and 9, and Tables 4-5).

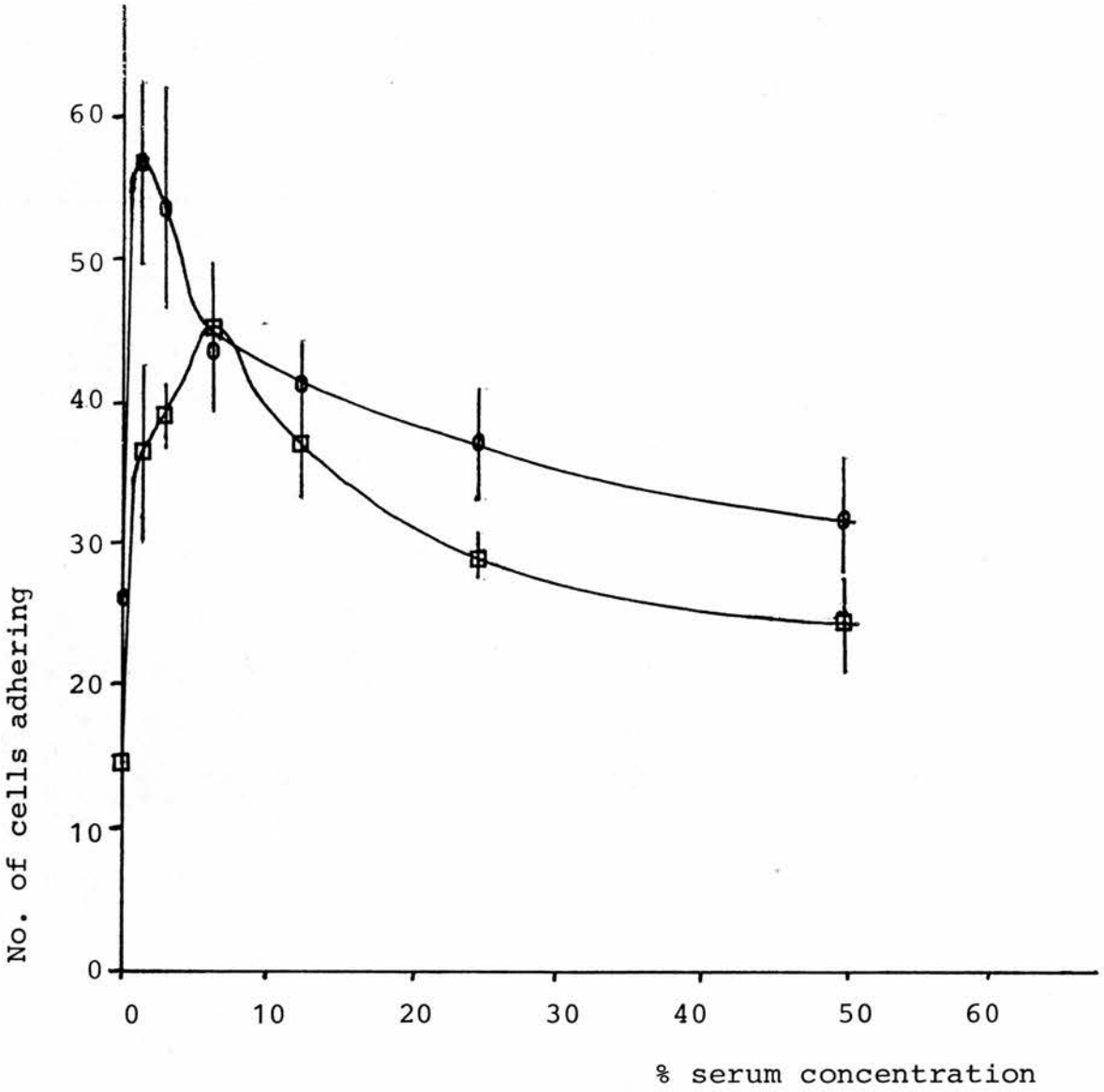


Figure 8

Comparative effect of serum on lymphocyte adhesion to native and denatured collagen substrate

o—o Native collagen

□—□ Denatured collagen

Table 4

Influence of serum on lymph node cell adhesion to collagen substrate (native)

Serum Conc. (%)	Mean number of adherent cells ± S.E.M.*	Mean % adherent cells ± S.E.M.*
0.00	26.66 ± 3.53	100
1.56	57.33 ± 5.40	215.05 ± 20.24
3.125	54.66 ± 7.91	205.05 ± 29.67
6.25	44.33 ± 4.38	166.29 ± 16.43
12.50	41.50 ± 3.39	155.66 ± 12.73
25.00	37.33 ± 3.66	140.04 ± 13.75
50.00	32.16 ± 4.14	120.65 ± 15.54

* $n \longrightarrow 6$

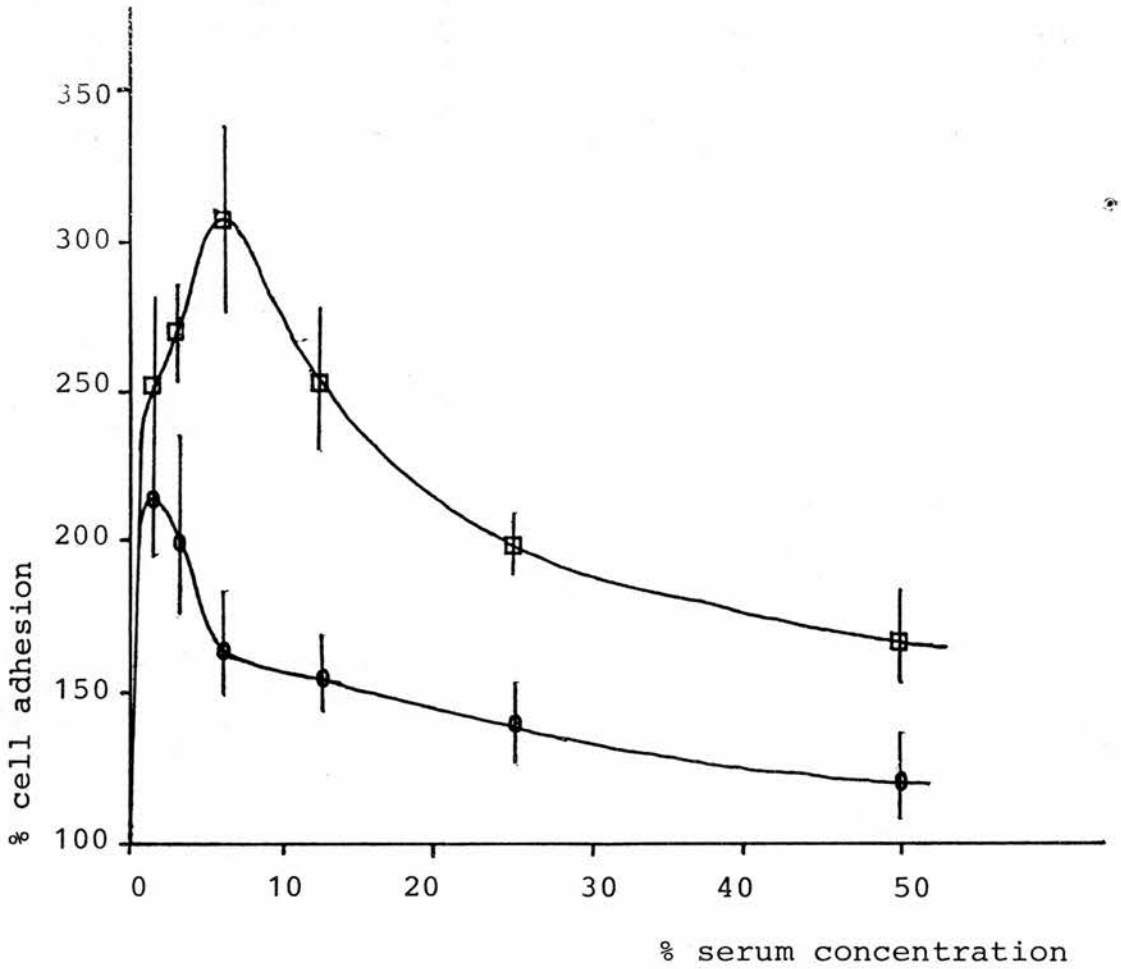


Figure 9

Comparative effect of serum on % lymphocyte adhesion to native and denatured collagen substrates

Denatured collagen

Native collagen

Table 5

Effect of serum on lymphocyte adhesion to denatured collagen

Serum Conc. (%)	Mean number of cells adhering ± S.E.M.*	Mean Percentage adherent cells ± S.E.M.*
0.00	14.83 ± 1.25	100
1.56	36.50 ± 6.32	246.12 ± 42.64
3.125	39.00 ± 2.55	262.98 ± 17.24
6.25	45.33 ± 4.72	305.69 ± 31.85
12.50	37.33 ± 3.78	251.74 ± 25.51
25.00	29.16 ± 1.72	196.67 ± 11.60
50.00	24.66 ± 2.35	166.33 ± 15.83

* n $\longrightarrow$ 6

2. The Purification of Serum Proteins

In the previous section, it was shown that lymphocyte adhesion to glass was inhibited by serum. The next step was to fractionate the serum, and isolate and purify the corresponding inhibition factors.

2.1.A. Gel Filtration (Run 1A)

Calf serum was separated on a Sepharose 4B column, and the fractions assayed for inhibitory effect (Table 6). Figure 10 illustrates the elution profile and inhibitory activity. From this diagram it can be seen that three areas of inhibitory activity were present. Molecular weights of the selected fractions are given in Table 7. The separation conditions were:

Sample	:	5ml. serum
Eluent	:	Dulbecco's PBS, pH 7.3
Flow rate	:	~30ml./hr (~7.5ml./fraction)
Detection	:	280 nm.
Temperature	:	Room temperature

2.1.B. Gel Filtration (Run 1B)

Fractions 14-21 from 2.1.A. above were pooled and concentrated to 6ml. by ultrafiltration, and reapplied to the Sepharose 4B column.

The optical density at 280nm. of the fractions was measured. The fractions were tested for their inhibitory

Table 6

Mean percentage adhesion and percentage inhibitory activity of separation of serum on a Sepharose 4B column (Run 1A)

Fraction Number	Mean % Adhesion ± S.E.M.* (after 50% dilution)	% Inhibition
14	90.55 ± 5.37	9.45
16	59.44 ± 3.34	40.56
18	30.44 ± 2.20	69.56
19	36.84 ± 2.65	63.16
20	24.00 ± 1.06	76.00
22	16.33 ± 1.10	83.67
24	33.53 ± 2.36	66.47
26	55.52 ± 4.04	44.48
28	44.68 ± 2.40	55.32
30	95.42 ± 3.86	4.58

* $n \longrightarrow 9$

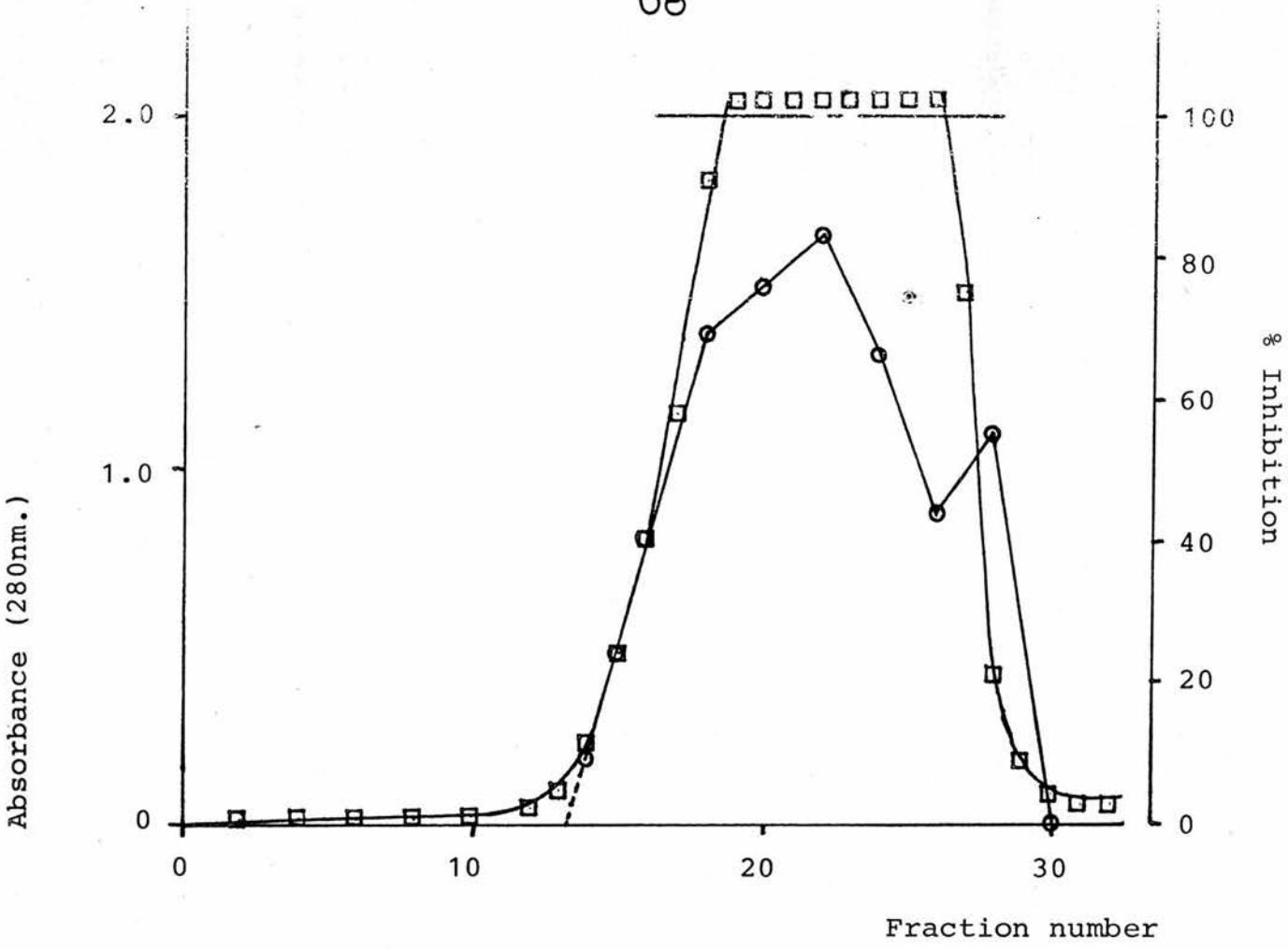


Figure 10

Separation of calf serum on Sepharose 4B (Run 1A) with percentage inhibitory activity of fractions tested

□—□ optical density (protein concentration)

o—o inhibitory activity

Table 7

Apparent molecular weights (4% acrylamide gel) of the fractions from Run 1A

Fractions	Molecular Weights
14	500000
16	470000
	420000
	300000
	130000
17	500000
	400000
	300000
	130000
22	220000
	130000
	80000
	40000
26	130000
	70000
	50000
	20000
28	50000

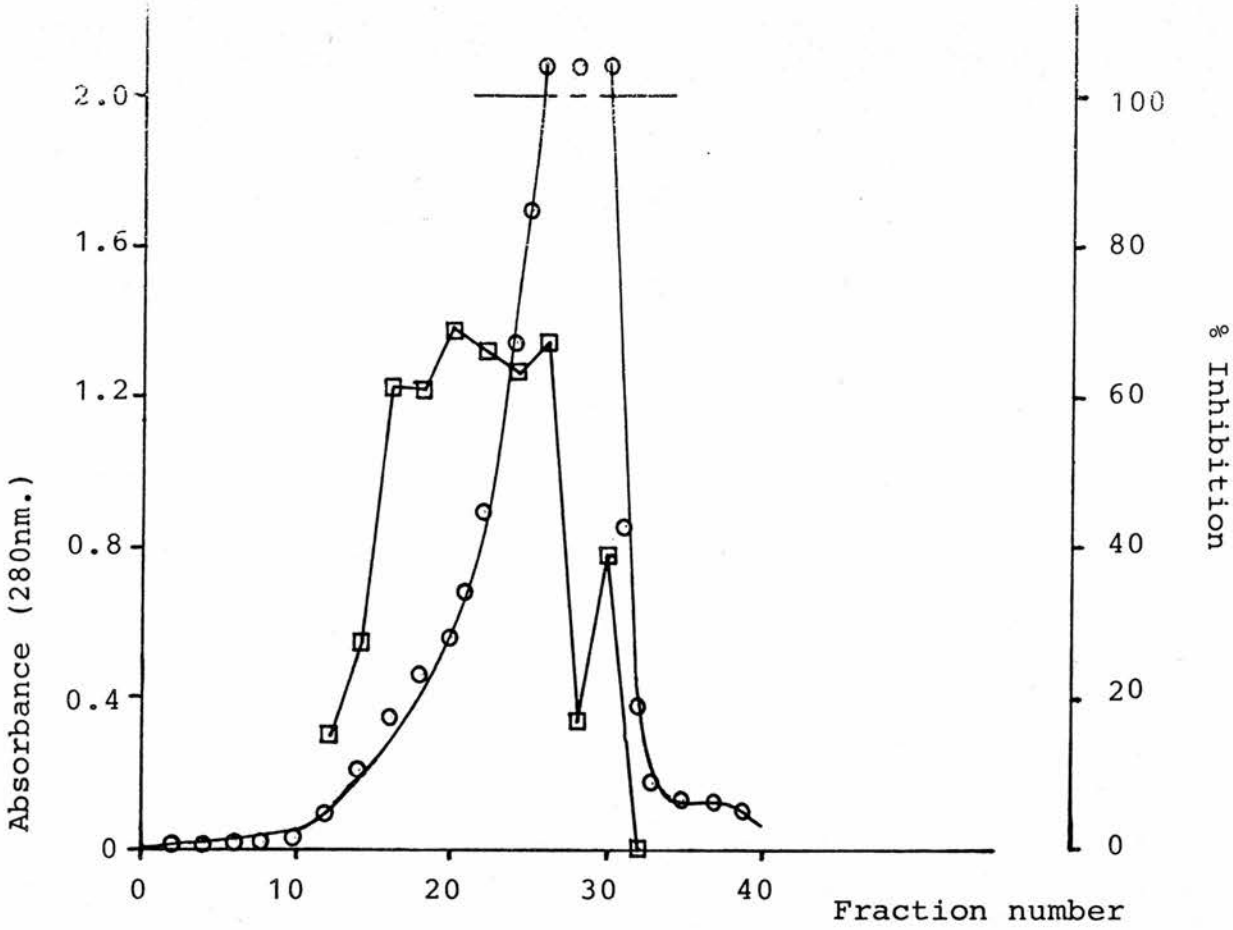


Figure 11

Elution profile of pooled fractions (14-21), from Run 1A, on Sepharose 4B (Run 1B) and percentage inhibition

o—o optical density (protein concentration)

□—□ inhibitory activity

Table 8

Inhibitory activity of the fractions collected from gel filtration Run 1B

Fraction Number	Mean % adhesion ± S.E.M.* (after 50% dilution)	% Inhibition
12	84.71 ± 5.02	15.29
14	72.42 ± 3.95	27.58
16	38.66 ± 1.24	61.34
18	38.97 ± 2.58	61.03
20	31.44 ± 0.97	68.56
22	34.11 ± 1.76	65.89
24	36.77 ± 1.47	63.23
26	32.50 ± 0.08	67.50
28	83.57 ± 9.66	16.43
30	62.56 ± 3.05	37.44
32	99.66 ± 4.62	0.34

* n ⟶ 9

effect on murine mesenteric lymph node cell adhesion (Table 8 and Figure 11). The inhibition profile reflects four peaks of activity. Polyacrylamide gel electrophoresis results were interesting, as these showed that single bands were obtained for fractions 12 and 14. Furthermore, the values for the molecular weights of these 2 fractions (Table 9a) were lower than the molecular weights for subsequent fractions - see Discussion. A densitometer scan of the 4% gel of unreduced protein from fraction 12 is given in Figure 12 and a photograph of the gel in Figure 13. Fractions 11 to 15 gave a single band on SDS - acrylamide electrophoresis. These fractions were pooled along with fractions collected from 2.2. Chromatographic conditions were as for 2.1.A.

2.2 Gel Permeation (Repeat of above Two Runs)

The above two runs (A and B) were repeated nine times with serum so as to collect enough protein for further study: ultracentrifugation, amino acid analysis, iso-electrofocusing. Only the fractions giving a single band on SDS - acrylamide electrophoresis in the second run from the ascending portion of the elution profile were collected and pooled.

2.3 Molecular-exclusion chromatography (using a longer gel bed)

Fractionation of serum on a longer Sepharose 4B gel bed (2.2 x 100cm) was investigated (Run 11A). The resolution of proteins in the fractions was improved; however, single bands

Table 9a

Apparent molecular weights (4% acrylamide gels) of the fractions collected from gel filtration Run 1B

Fractions	Molecular Weights
12	400000
14	400000
16	500000 450000 400000 260000
18	500000 400000 260000
20	500000 400000 260000
21	500000 400000 260000 115000
22	420000 400000 260000 125000
23	400000 250000 190000 150000
24	340000 240000 190000 140000 115000
25	400000 340000 240000 185000 140000 115000

/continued...

Table 9a/cont'd...

Fractions	Molecular Weights
26	400000
	340000
	240000
	185000
	140000
	115000
27	400000
	240000
	185000
	115000
	50000
28	115000
	50000
29	115000
	65000
	50000
30	115000
	50000
32	115000
	50000

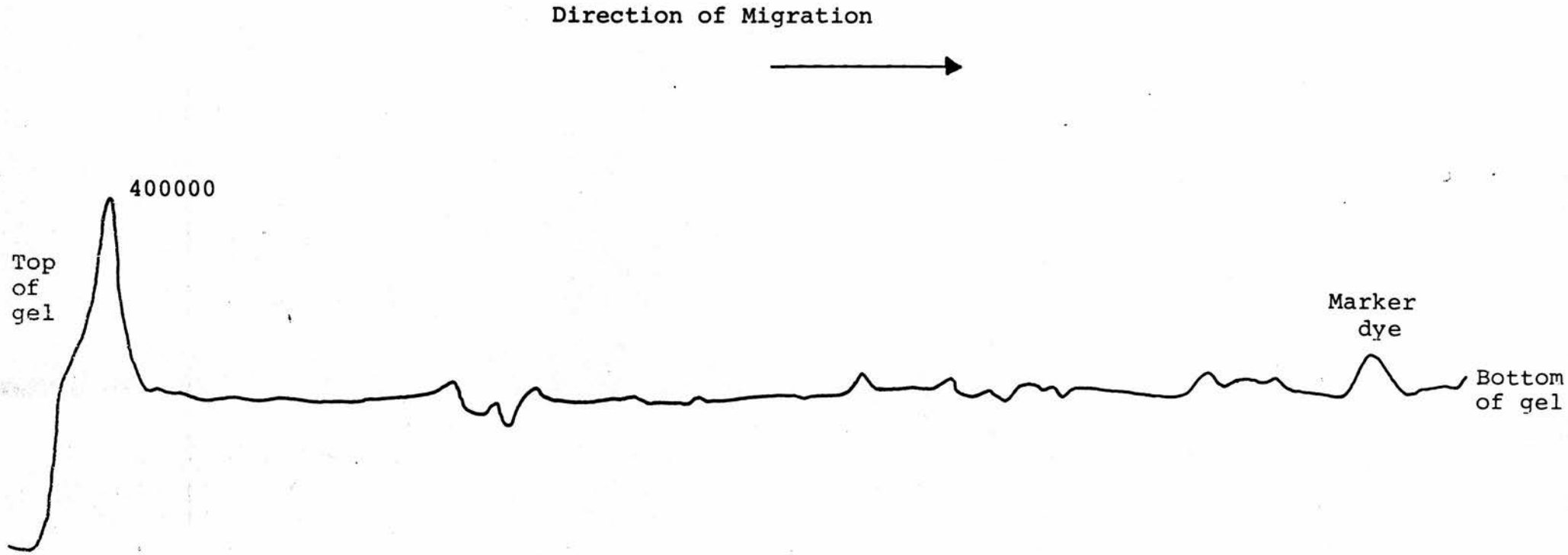
Table 9b

Apparent molecular weights of the reduced pooled fraction collected from gel filtration Runs 1-10

180000
140000
75000
65000
26000

Figure 12

Densitometer scan of protein from fraction 12 (Sepharose 4B separation - Run 1B), 4% acrylamide gel



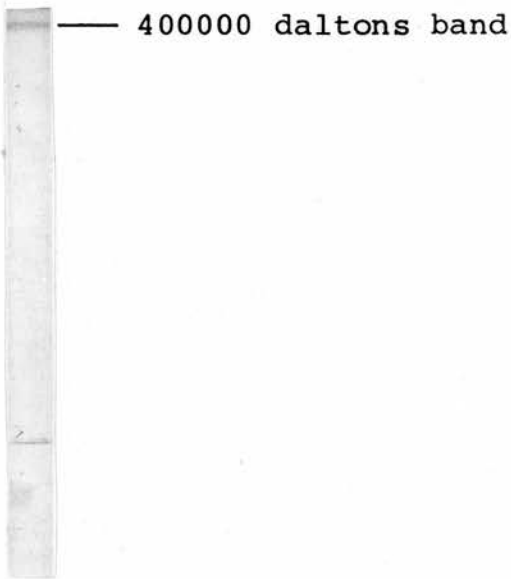


Figure 13

Fraction 12 (gel filtration Run 1B), 4% acrylamide gel

on SDS-PAGE electrophoresis were not obtained. Tables 10-11 and Figures 14-19.

From the first run, fractions 46-66 were pooled, concentrated and reapplied to the Sepharose 4B column (Run 11B).

The conditions for gel permeation were as follows (both runs):

Sample	:	10ml. serum
Eluent	:	Dulbecco's PBS, pH 7.3
Flow rates	:	3.2ml./20 min.
Detection	:	280 nm.
Temperature	:	Room temperature

2.4 Amino acid Analysis

The following general comments can be made on the amino acid analysis Table 12 (the fraction used for analysis was collected from Sepharose 4B runs 1-10, see section 2.2):

1) Uncharged polar side group : Glycine, Serine, Threonine, and the amount of tyrosine is significant.
2) The two acidic amino acids: Aspartic and glutamic acid.
3) Non polar side chains: Valine and Leucine.
4) The presence of cysteine residues is indicative of disulphide bonds.

2.5 Analytical Techniques

Figures 20 and 21 show the results of an ultracentrifuge equilibrium experiment (a sample from the pooled fraction from

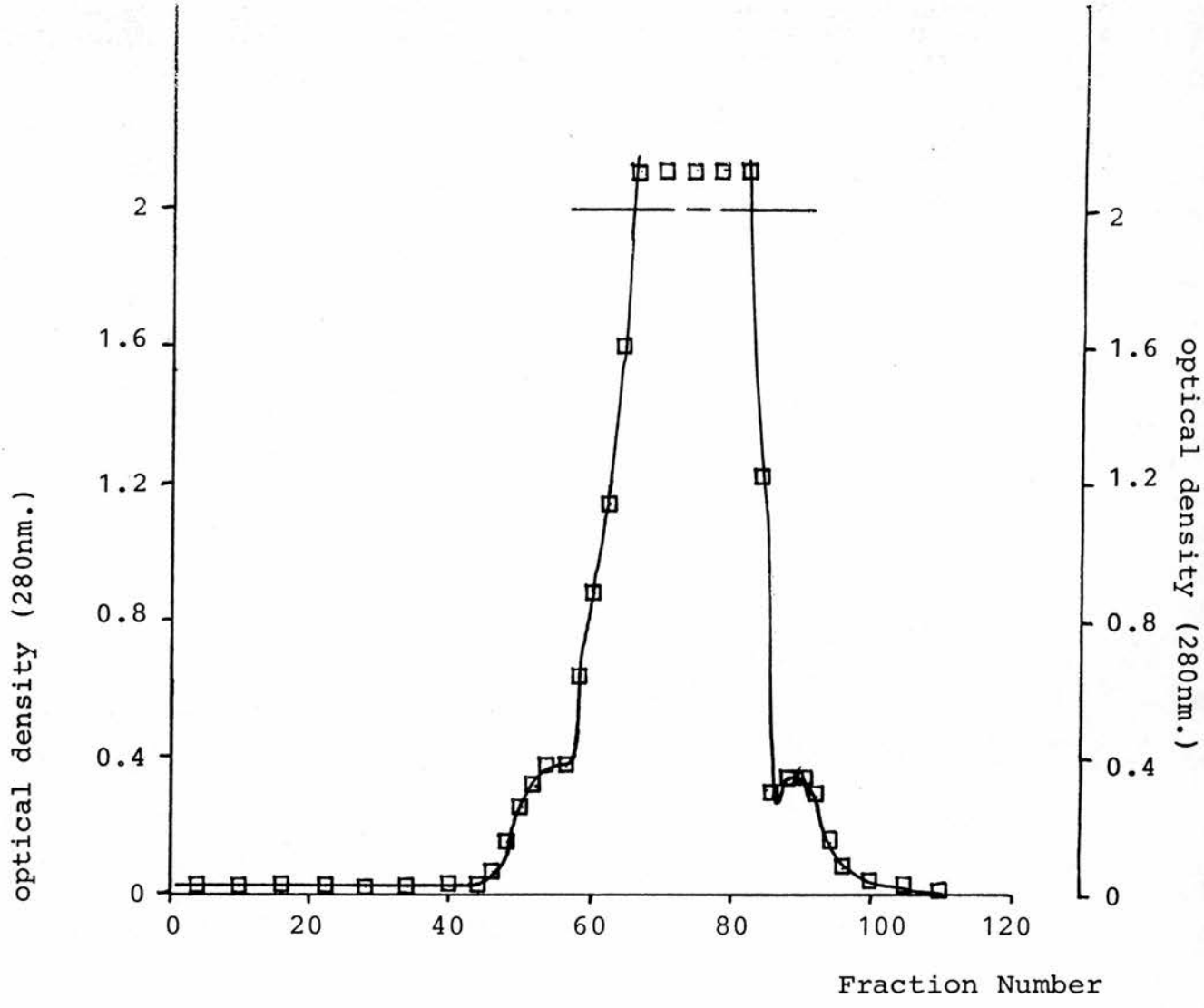


Figure 14

Elution diagram of serum on Sepharose 4B (Run 11A)

Table 10

Inhibitory activity of the fractions collected from the Sepharose 4B column (Run 11B)

Fraction Number	Mean % Adhesion ± S.E.M. (After 50% dilution)	n	% Inhibition
49	90.87 ± 3.71	3	09.13
51	86.32 ± 2.27	6	13.68
53	84.90 ± 7.71	6	15.10
55	82.23 ± 3.80	6	17.77
57	79.56 ± 7.99	6	20.44
59	71.86 ± 5.23	6	28.14
61	44.66 ± 6.53	6	55.34
63	55.03 ± 8.09	3	44.97
65	48.74 ± 2.08	6	51.26
67	47.49 ± 4.94	6	52.51
69	42.77 ± 5.92	6	57.23
71	29.72 ± 2.79	6	70.28
73	34.90 ± 2.96	6	65.10
75	57.86 ± 5.45	3	42.14
80	98.43 ± 4.25	6	01.57

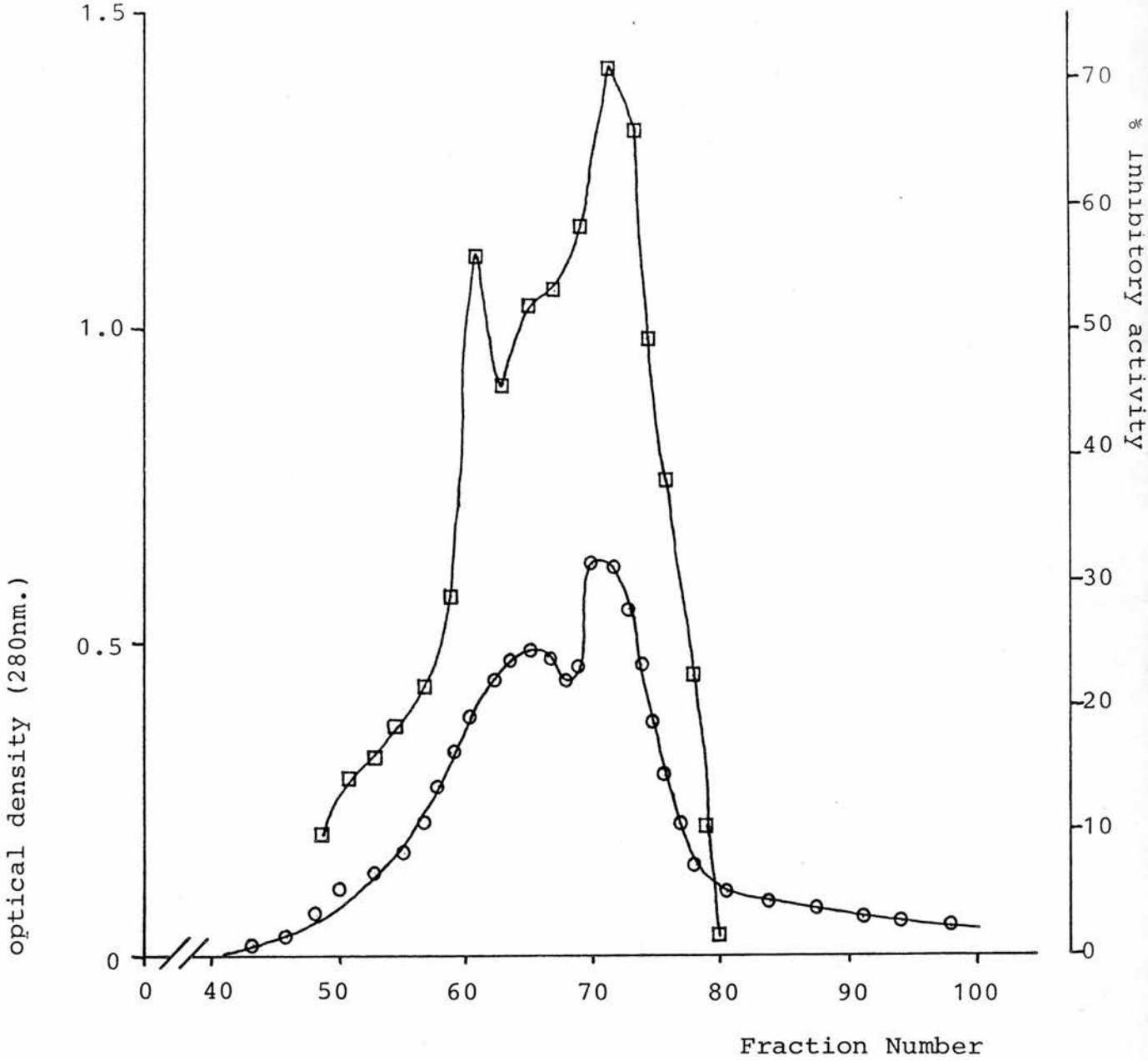


Figure 15

Fractionation of pooled fractions on Sepharose 4B (Run 11B) and percentage inhibitory activity

□—□ percentage inhibitory activity

o—o protein concentration

Table 11

Fractions collected from the longer bed of Sepharose 4B gel (Run 11B) with corresponding apparent molecular weights

Fractions	Molecular Weights
49	900000
51	900000 340000
53	900000 400000
49-53	1000000 400000
54-61	900000 560000 350000 320000
63	340000 270000 220000 190000 150000 125000
65	290000 220000 185000 140000 125000
67	290000 210000 170000 135000 115000
69	300000 275000 210000 170000 135000 115000

P.T.O.

Fractions	Molecular Weights
71	280000
	210000
	140000
	115000
73	300000
	275000
	210000
	150000
	115000
75	220000
	140000
	125000
Reduced pooled fraction 49-53	135000
	74000
	44000
	25000
Reduced pooled fraction 54-61	180000
	134000
	100000
	76000
	62000
	56000
	30000

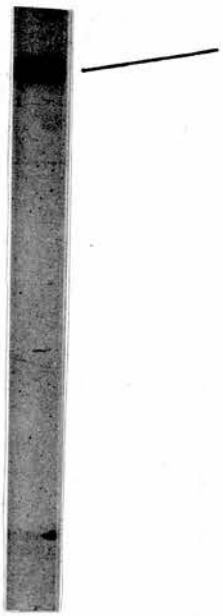


Figure 16

Fraction 49 (gel filtration Run 11B), 3% acrylamide gel

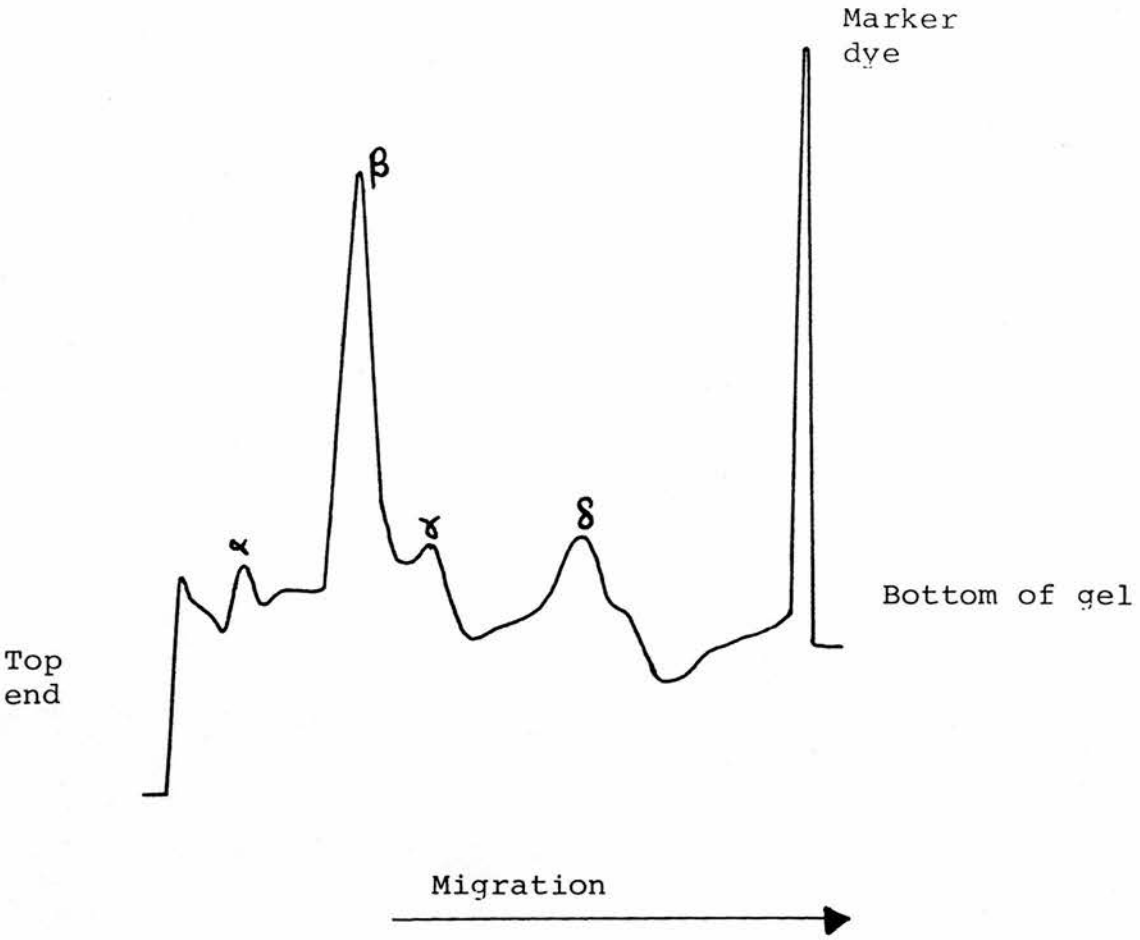


Figure 17

Densitometer scan of reduced pooled fraction (49-53), gel filtration Run 11B, 4% acrylamide gel

where,

α - 135000 daltons

β - 74000 daltons

γ - 44000 daltons

δ - 25000 daltons

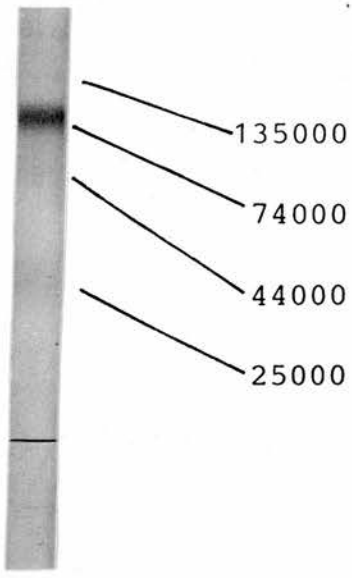


Figure 18a

Reduced pooled fraction 49-53, gel filtration Run 11B, 4% acrylamide gel

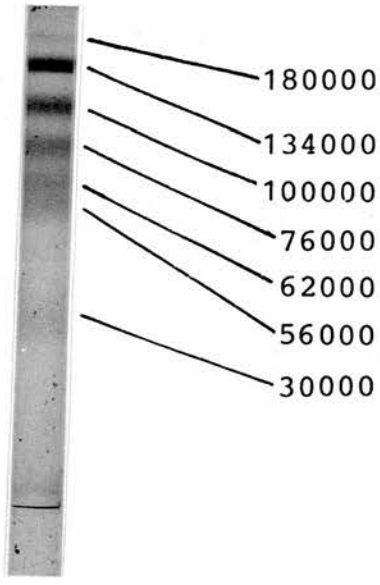


Figure 18b

Reduced pooled fraction 54-61, gel filtration Run 11B, 4% acrylamide gel

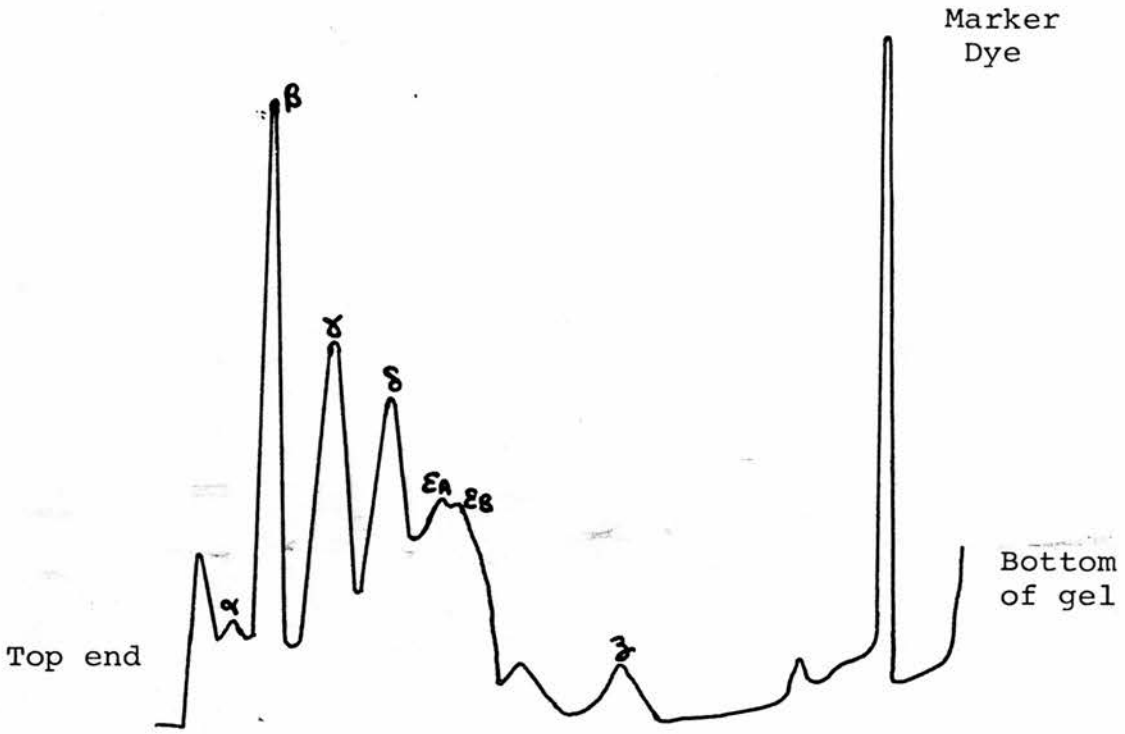


Figure 19

Densitometer scan of reduced pooled fraction (54-61), gel filtration Run 11B, 4% acrylamide gel

where,

α	180000
β	134000
γ	100000
δ	76000
ε_A	62000
ε_B	56000
ʒ	30000

Table 12

Constituent Amino Acids of the pooled fraction from gel filtration Runs 1-10

Amino Acid	Amount (Amino acids/1000 residues)
Cysteic Acid	23.75 (24)
Aspartic Acid	78.23 (78)
Threonine	76.73 (77)
Serine	116.02 (116)
Glutamic Acid	97.13 (97)
Proline	13.50 (14)
Glycine	72.28 (72)
Alanine	60.75 (61)
Valine	80.70 (81)
Methionine	3.79 (4)
Isoleucine	32.22 (32)
Leucine	83.26 (83)
Tyrosine	48.12 (48)
Phenylalanine	36.11 (36)
Lysine	41.76 (42)
Histidine	14.39 (15)
Ammonia	90.33 (90)
Arginine	29.84 (30)
	998.91 (1000)

gel filtration Runs 1-10 was used for this procedure, see section 2.2). The conditions were as follows:

The partial specific volume ($\bar{V}$) = 0.723, calculated from the method of Zamyatnin (1972).

Density of solution = 1.0091

operating speed = 4800rpm

Temperature = 295.20K

The plot of log ($Y-Y_o$) against concentration should have been a straight line; however, as this curves at the top, it reflects that the protein population is heterogeneous. This is confirmed by Figure 21, which demonstrates a heterogeneous population again.

The impurity of the sample is also revealed by the photograph for isoelectrofocusing (Figure 22), and by SDS-electrophoresis of a reduced aliquot (see Table 9b).

2.6 Ammonium sulphate precipitation

Serum was fractioned by ammonium sulphate precipitation (Table 13), and from this information it was concluded that the precipitate formed at 45% ammonium sulphate concentration possessed the inhibitory activity.

2.7 Conditions for Ion-Exchange

Preliminary Ion-exchange experiments imparted that 92% of the inhibition activity is adsorbed onto the exchanger at pH 3.5, and 58% of the inhibitor is adsorbed at

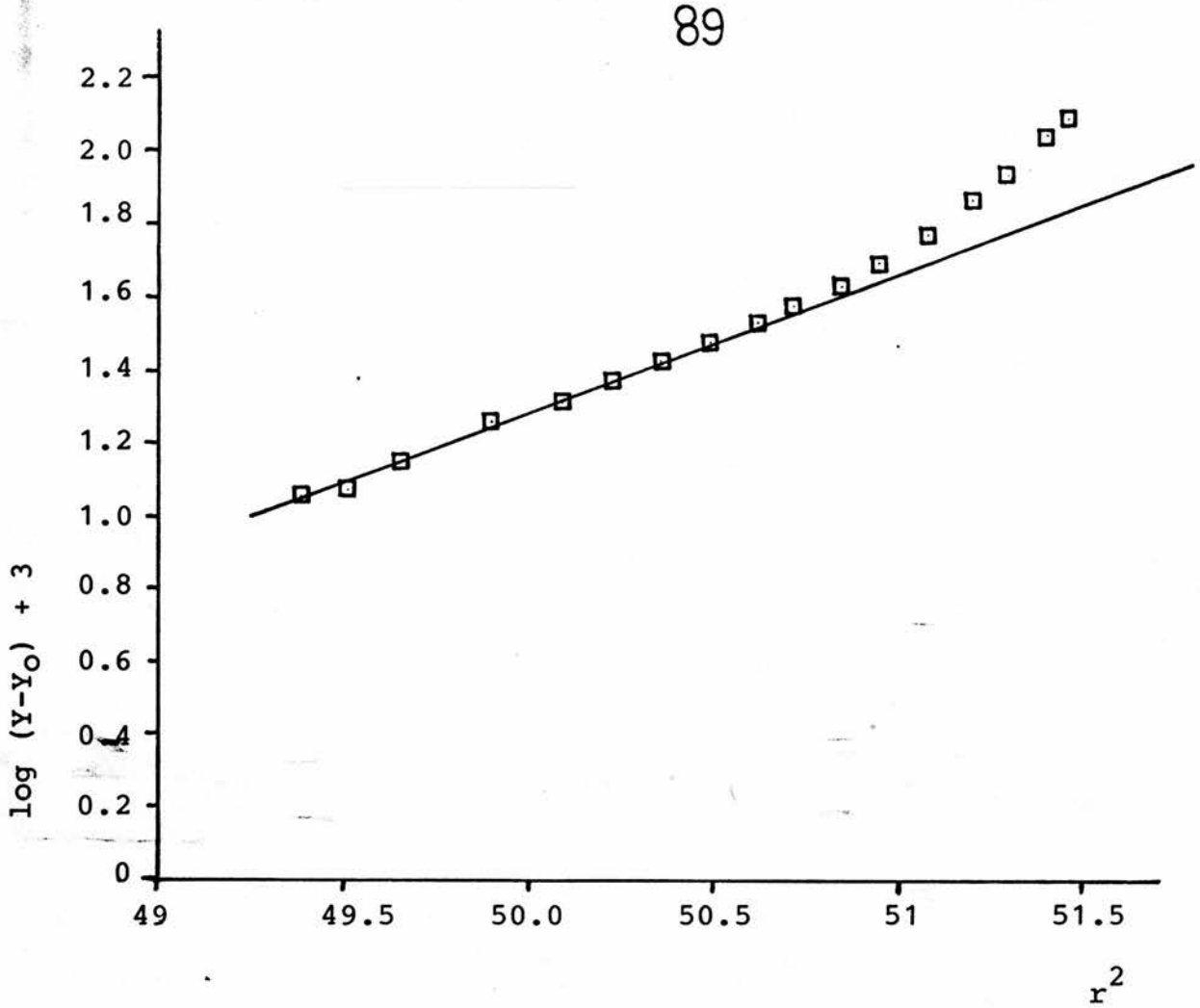


Figure 20

Log of fringe displacement ($y-y_o$) of the radial intervals against the square of the distance to the centre of rotation (r^2) of the radial intervals

Figure 21

Ultracentrifugation behaviour of pooled fraction collected from gel filtration Runs 1-10

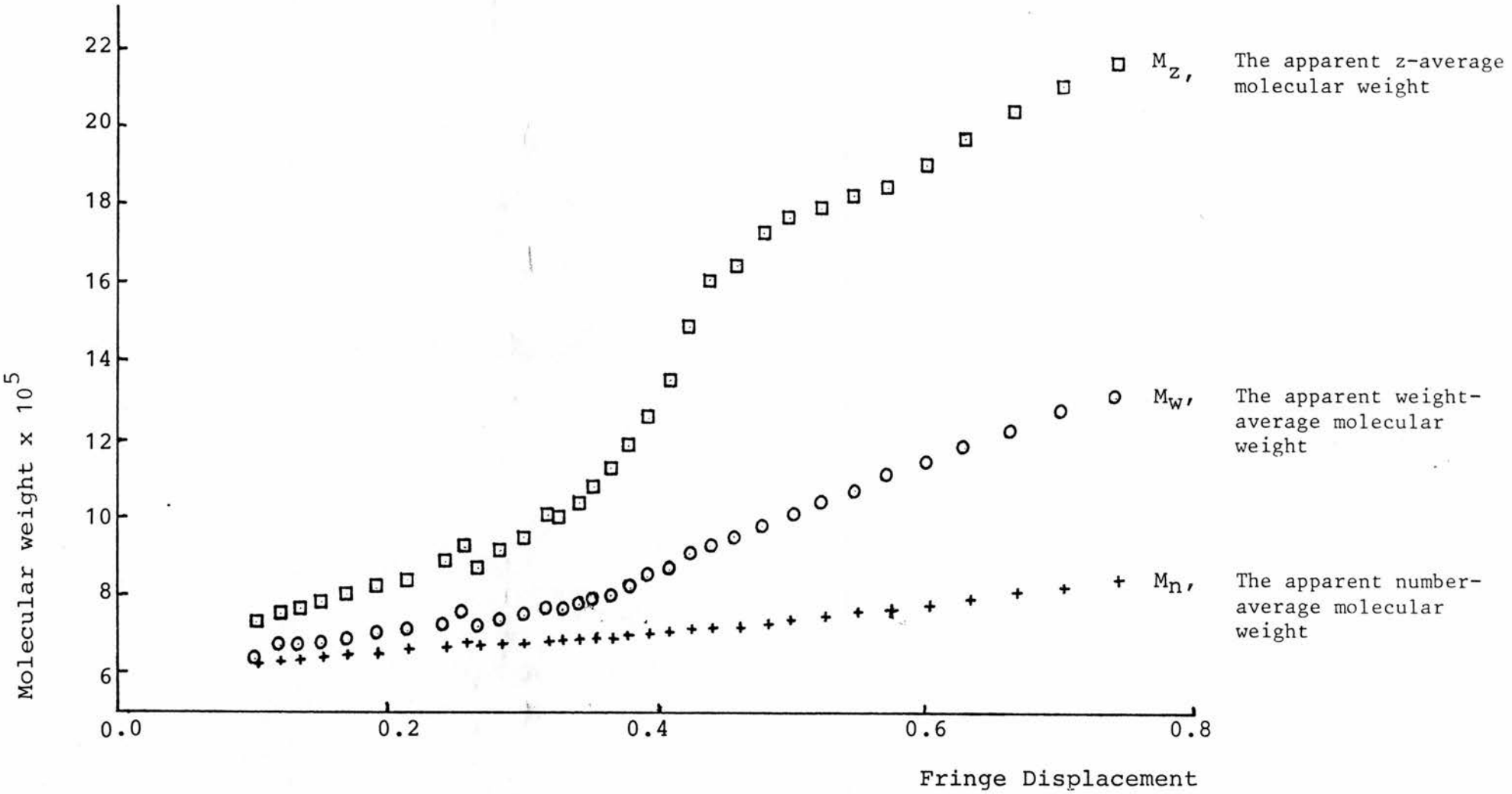


Figure 22

Electrofocusing (pH3.5-10) of a sample from the pooled fraction collected from gel filtration Runs 1-10

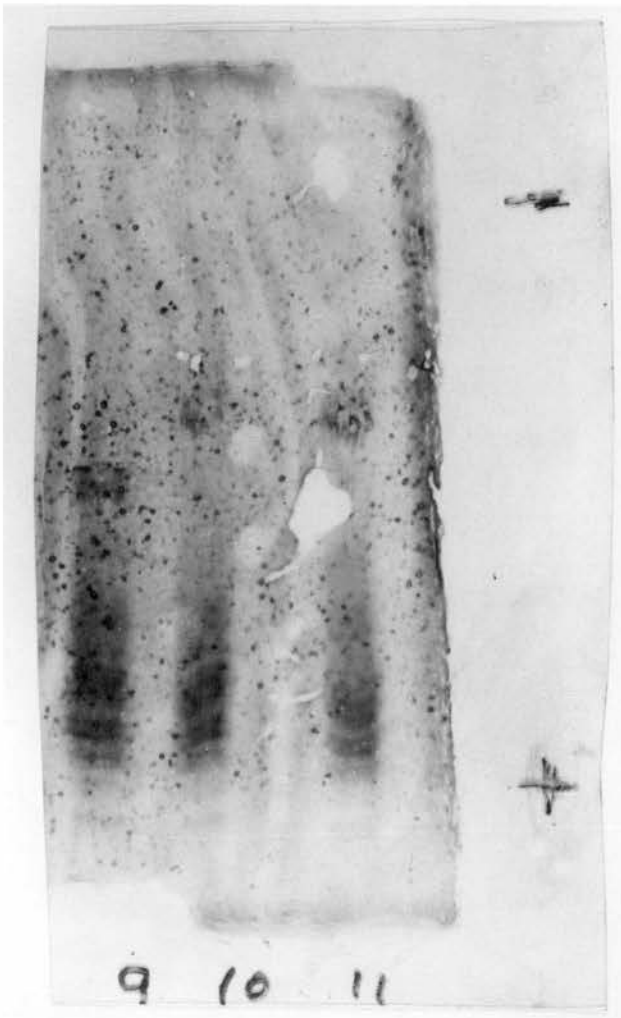

9 10 11

Table 13

Ammonium sulphate fractionation of serum and mean percentage adhesion with percentage (%) inhibition

Fractionation	Mean % Adhesion ± S.E.M.* (after 50% dilution)	% Inhibition
PBS	100	-
45% ppte.	39.89 ± 2.54	60.11
68% ppte.	125.15 ± 7.77	-
68% supernatant	119.46 ± 5.2	-

*n ⟶ 9

Table 14a

Preliminary Ion-Exchange Experiment

Adsorption Experiment

pH	Mean % Adhesion ± S.E.M.* (after 50% dilution)	% Inhibition
Buffer	100	-
3.5	92.72 ± 4.10	7.28
4.0	25.90 ± 2.11	74.10
4.5	24.47 ± 3.43	75.53
5.0	57.64 ± 3.09	42.36
5.5	26.76 ± 2.15	73.24
6.0	35.95 ± 4.87	64.05

Table 14b

Salt elution

pH (Salt concentration)	Mean % Adhesion ± S.E.M.* (after 50% dilution)	% Inhibition
3.5 (0.5M)	47.39 ± 2.55	52.61
3.5 (1M)	83.58 ± 3.56	16.42
5.0 (0.5M)	59.70 ± 4.88	40.30
5.0 (1.0M)	86.57 ± 3.78	13.43
Buffer	100	-

* n $\longrightarrow$ 6

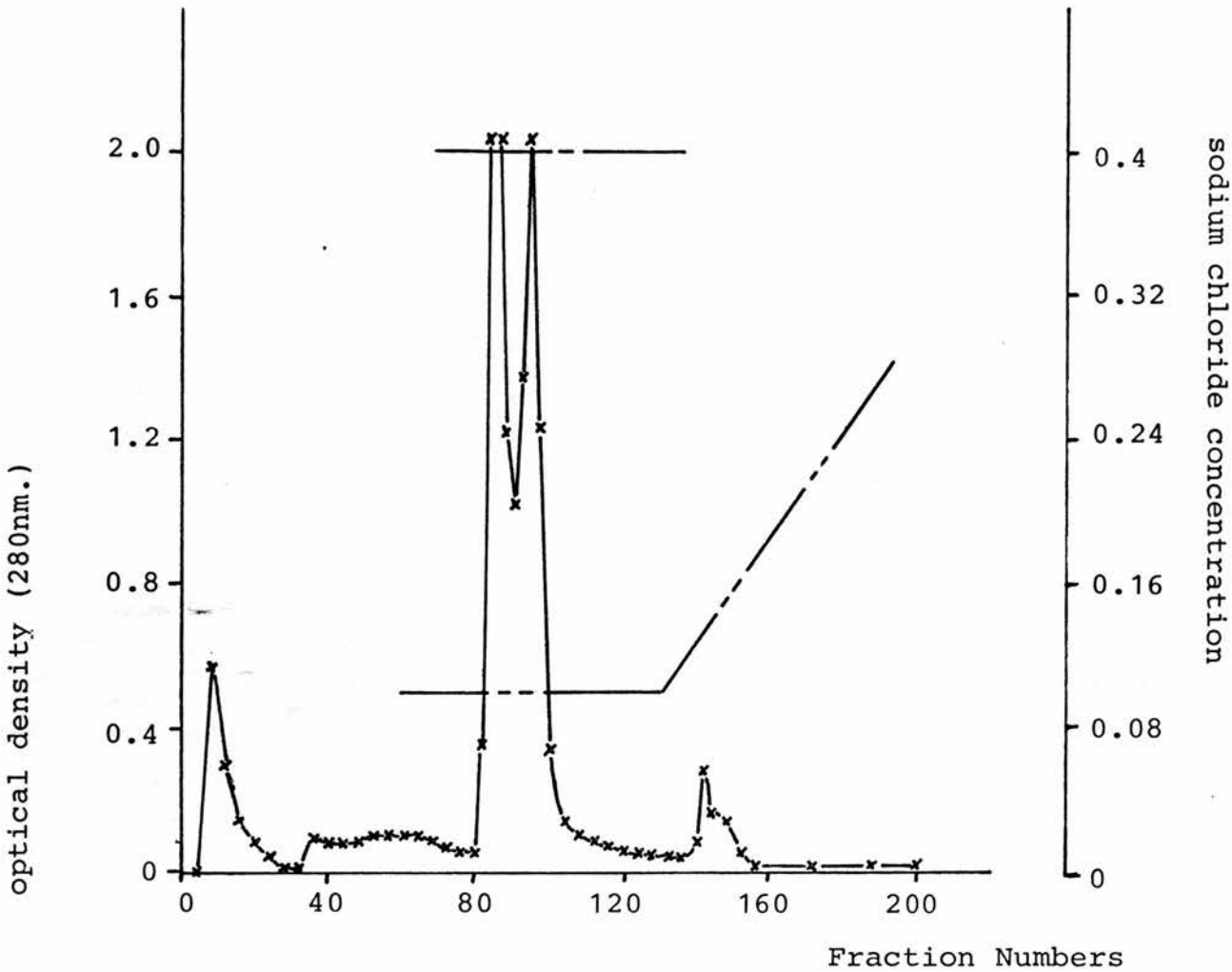


Figure 23

Elution pattern of ammonium sulphate fractionated serum on cation exchanger CM-cellulose 52

pH5 (Table 14a). However, only 40% of the inhibitor is eluted at a higher salt concentration after binding at pH5, and at pH 3.5 the amount of the inhibitor eluted is 52% (Table 14b). But, as α_2M is stable only in the range of pH5-8.4, it was decided that the latter conditions be used for Ion-Exchange Chromatography (see Section 2.8 below):

Bed dimensions : 1.6 x 30cm

Eluent : 0.05M sodium acetate buffer pH5, containing 0.1M NaCl, with a linear gradient of 0.1-0.5M NaCl.

Flow rate : 6.2ml./20 min.

Detection : 280nm.

Temperature : room temperature

2.8 Ion-Exchange

In the following experiments, the serum was initially fractioned with ammonium sulphate, and the precipitate obtained at 45% saturation was dissolved in water and dialysed in 0.05M sodium acetate buffer, pH5, at 4°C (see materials and methods). The dialysed solution was applied to a CM-cellulose cation exchanger column, and eluted sequentially with 0.05M sodium acetate, 0.05M sodium acetate containing 0.1M NaCl, and finally with a linear gradient of 0.1-0.5M NaCl.

The elution profile is shown in Figure 23, and the mean % adhesion activity with molecular weights for the pooled fractions, 139-153, are given in Table 15 and 16 respectively. A scan of the gel for the pooled fraction is shown in Figure 24.

Table 15

Adhesion activity of pooled fractions from Ion-Exchange column

Fractions	Mean % Adhesion ± S.E.M.* (after 50% dilution)	% Inhibition
PBS	100	-
6-23	116.89 ± 8.67	-
75-82	65.52 ± 2.89	34.48
83-87	95.40 ± 5.94	4.60
88-93	39.51 ± 2.70	60.49
94-106	48.99 ± 4.52	51.01
107-130	85.20 ± 6.64	14.80
139-153	46.04 ± 5.41	53.96

* n $\longrightarrow$ 6

Table 16

Apparent Molecular Weights for the pooled fraction 139-153

Fraction	Molecular Weight
139-153	400000
	110000
	94000
	80000

Figure 24

Densitometer scan of the pooled fraction 139-153, from the Ion-Exchange column, 3% acrylamide gel

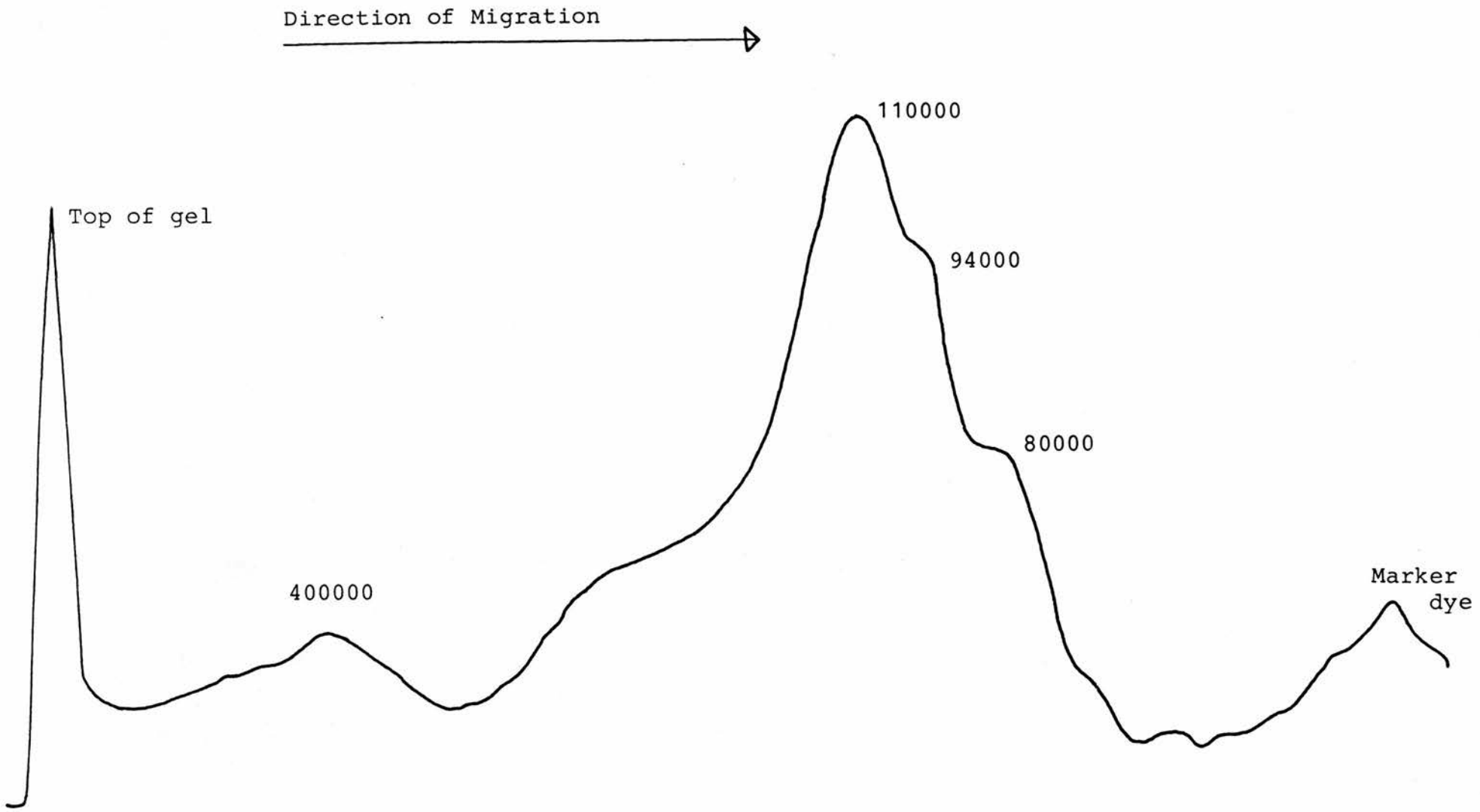


Table 17

Mean % adhesivity of lymphocytes in the presence of fractions separated on Sepharose 4B (Run 12)

Fractions	Mean % adhesion ± S.E.M.* (after 50% dilution)	% Inhibition
Buffer	100	-
9	90.76 ± 2.62	09.24
11	78.33 ± 2.72	21.67
13	54.24 ± 3.99	45.76
15	47.27 ± 2.14	52.73
17	50.00 ± 4.02	50.00
19	86.06 ± 12.65	13.94
22	107.27 ± 7.86	-

* n ⟶ 9

Table 18

Apparent molecular weight of fraction 13 collected from gel filtration Run 12

Fraction	Molecular Weight
13	100000

The pooled fraction (139-153) was concentrated and applied to a Sepharose 4B column (Run 12). Separation of this is shown in Figure 25, as well as the % inhibitory activity (see Table 17 for inhibition). The molecular weight for the contents of fraction 13 (Table 18) was 100000 daltons on SDS-PAGE, and the gel scan is shown in Figure 26. Conditions for chromatography were as indicated below using Sepharose 4B:

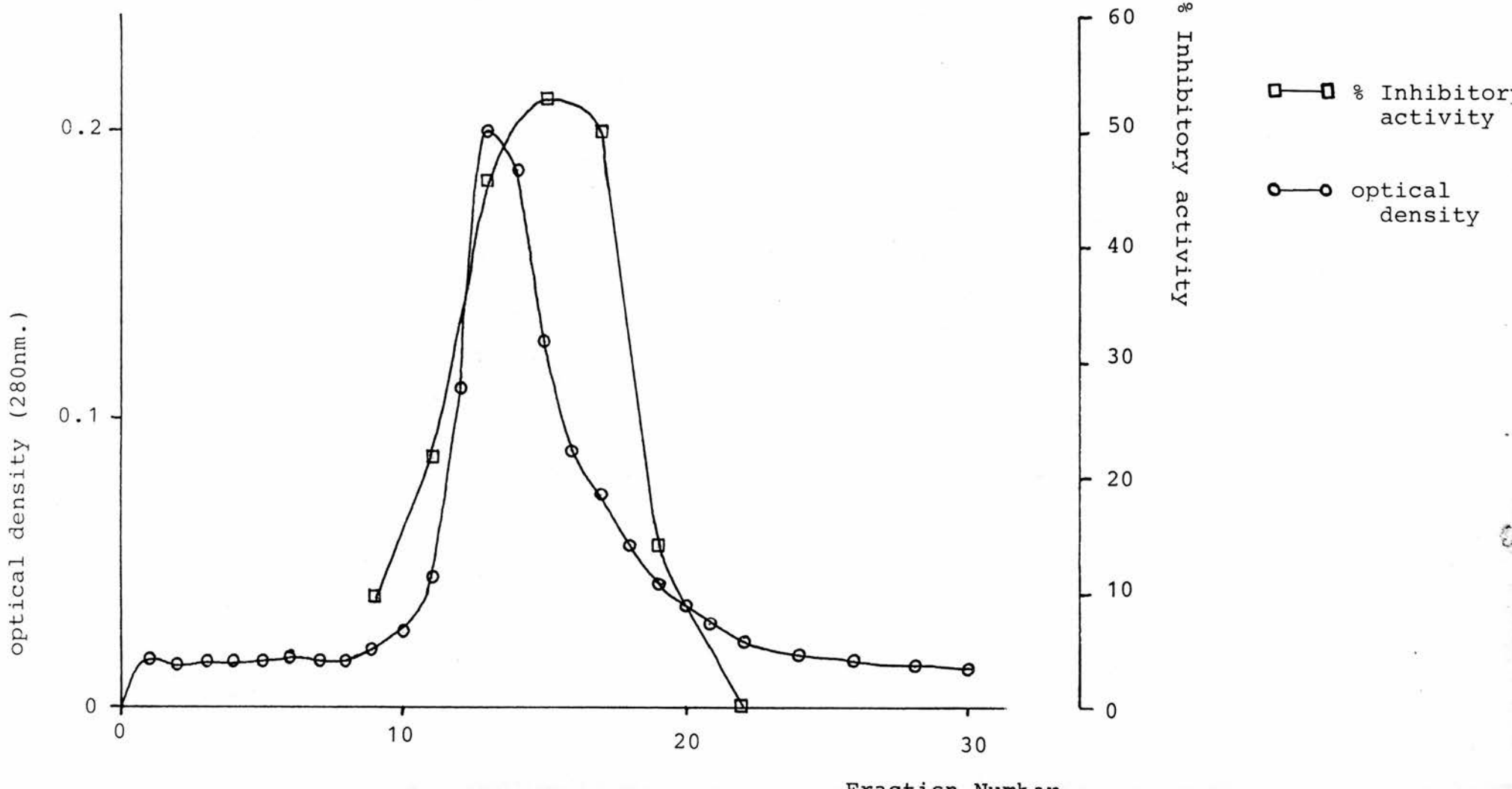
Bed dimensions	:	45 x 2.2cm
Eluent	:	Dulbecco's PBS, pH7.3
Flow rate	:	~6ml./20 min.
Detection	:	280nm.
Temperature	:	room temperature
Sample	:	5ml. of pooled fraction (139-153)

2.9 Polyethylene glycol (PEG) Fractionation

Serum was separated with PEG, and the precipitate obtained at 12.5% saturation was tested for inhibitory activity. The result is extremely promising (Table 19).

Figure 25

Gel filtration on a Sepharose 4B column (Run 12) of the pooled fraction 139-153 from the ion-exchange column, with the inhibitory activity



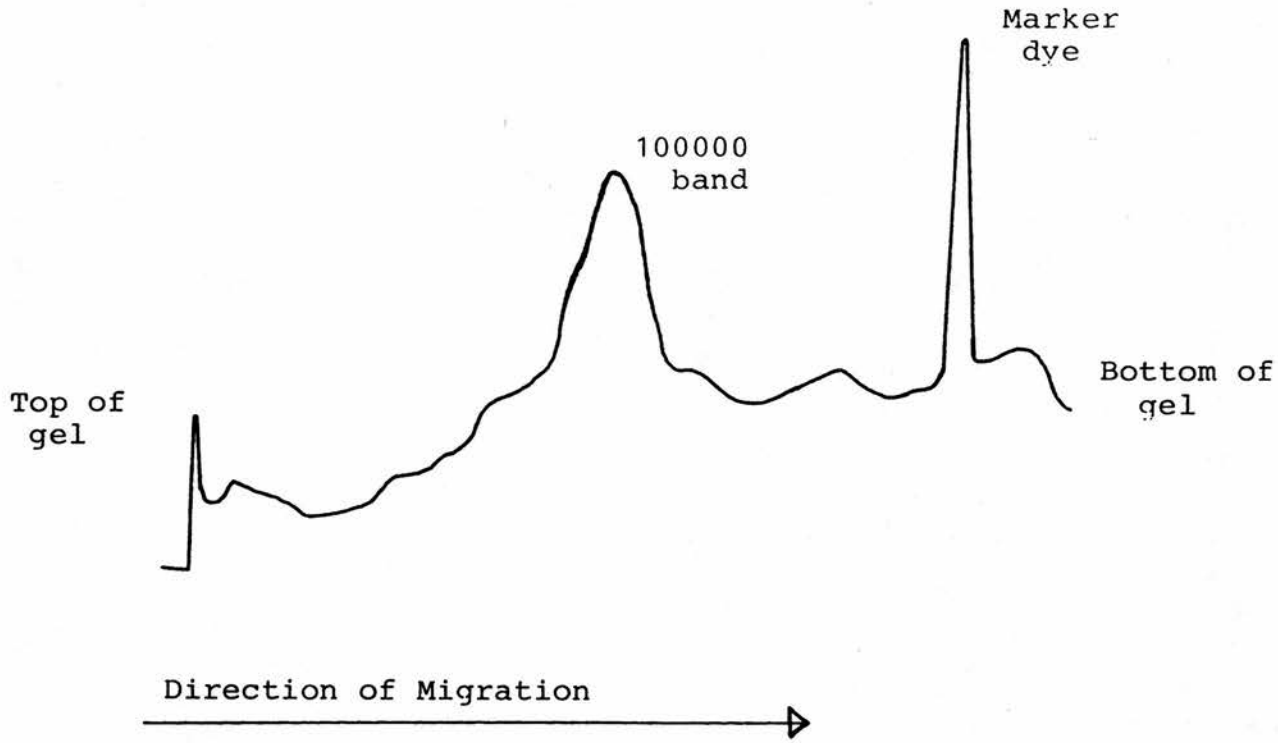


Figure 26

Densitometer scan of sample from fraction 13 (gel filtration Run 12), 4% acrylamide gel

Table 19

The effect of P.E.G. precipitation of serum on the adhesion of lymph node cells to glass

Fractionation	Mean % Adhesion ± S.E.M. (after 50% dilution)	n	% Inhibition
buffer	100	9	-
12.5% ppte.	6.61 ± 0.70	9	93.39

3. The kinetics of lymphocyte adhesion

The final objective of the present study was to establish a quantitative method for measuring the initial rate of adhesion of lymph node cells onto glass in the presence of serum. Firstly, the initial rates of adhesion were measured at different cell concentrations in the absence of serum. Figures 27 to 31, show time course plots of number of cells adhering to glass (Tables 20 and 21).

I may point out here that the process of settling (the time taken for the cells to reach the glass coverslip) was not taken into account and could affect the procedure.

The initial rate of adhesion is not linearly related to the cell concentration (Figure 32). This is similar to enzyme kinetics, that is lymphocyte adhesion may follow Michaelis-Menten kinetics. However, a Lineweaver-Burk plot is presented (double-reciprocal plot) of the data using rates calculated from the linear portion of the curves, a linear relationship is obtained (Figure 33).

The results obtained above were encouraging. It was therefore decided that a Dixon plot (Dixon, 1953) be drawn in order to see the type of inhibition obtained, that is, the initial rate of adhesion against inhibitor concentration (% serum concentration). Serum acts as an adhesion inhibitor of lymphocytes to glass. The time course of lymphocyte adhesion to glass was assayed in the presence of fixed concentration of serum at two different cell concentrations

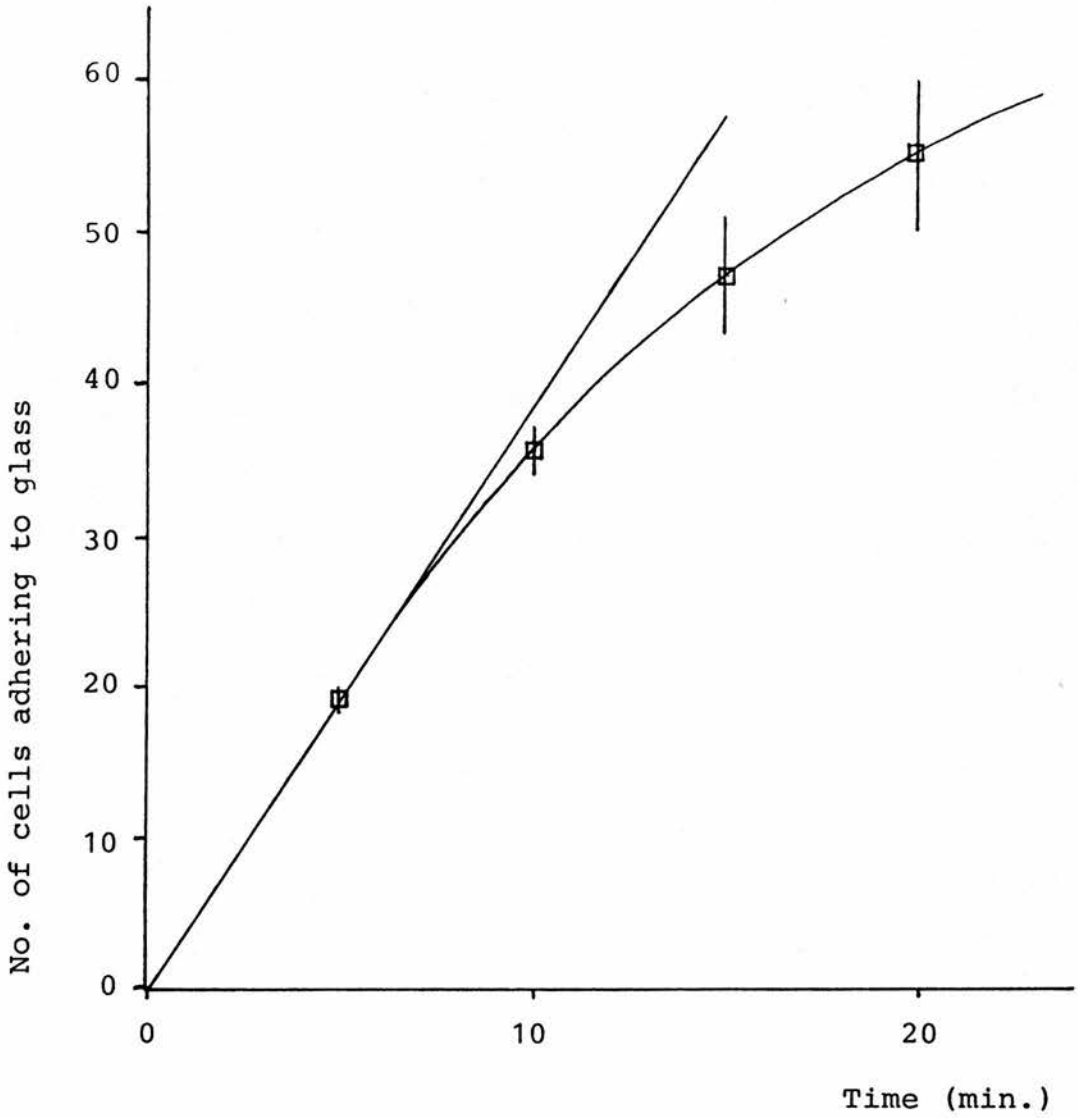


Figure 27

Dependence of lymphocyte adhesion to glass on time at a cell concentration of 1.6×10^6 cells/ml.

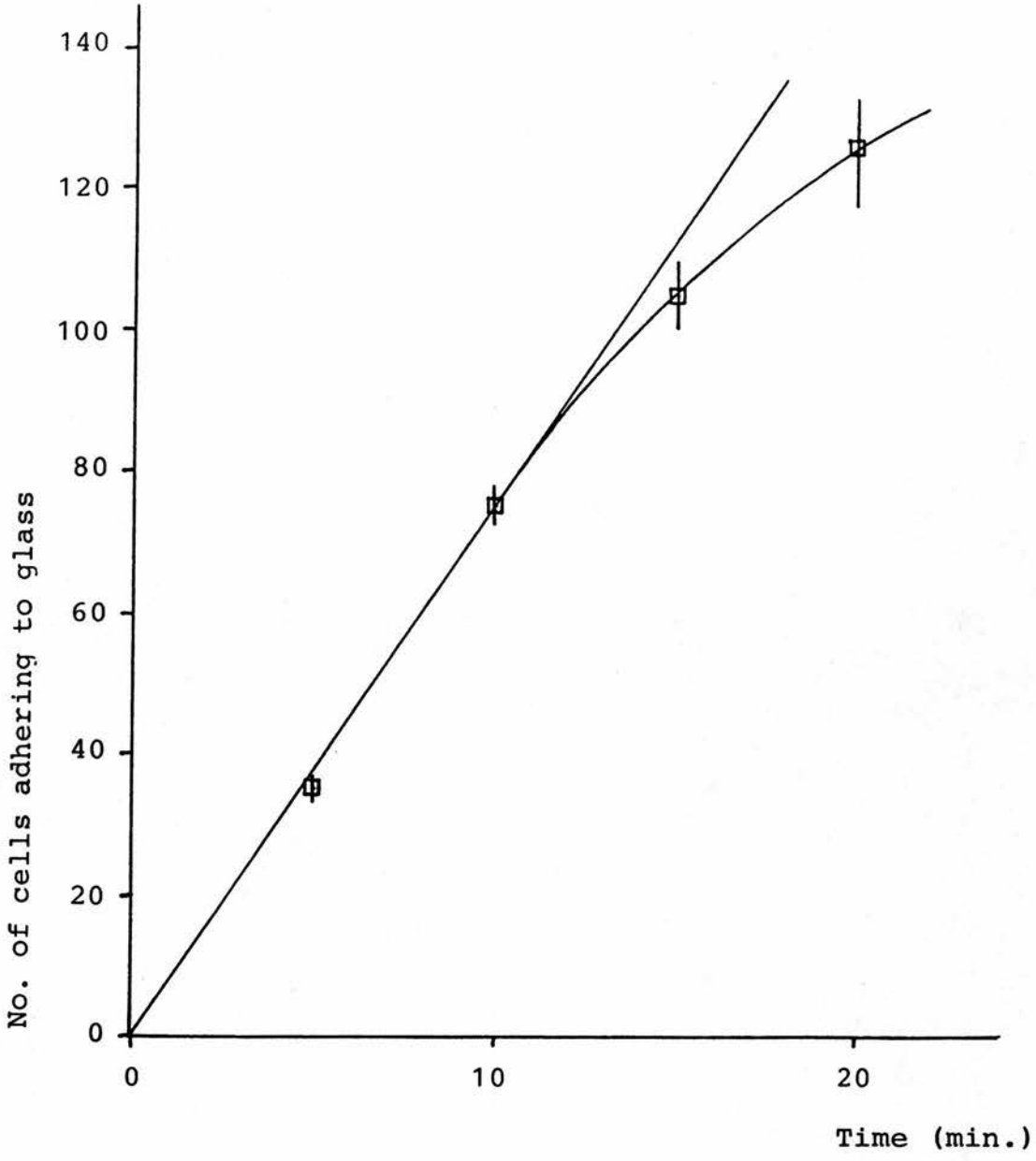


Figure 28

Time sequence of lymphocyte adhesion to glass at 3.6×10^6 cells/ml.

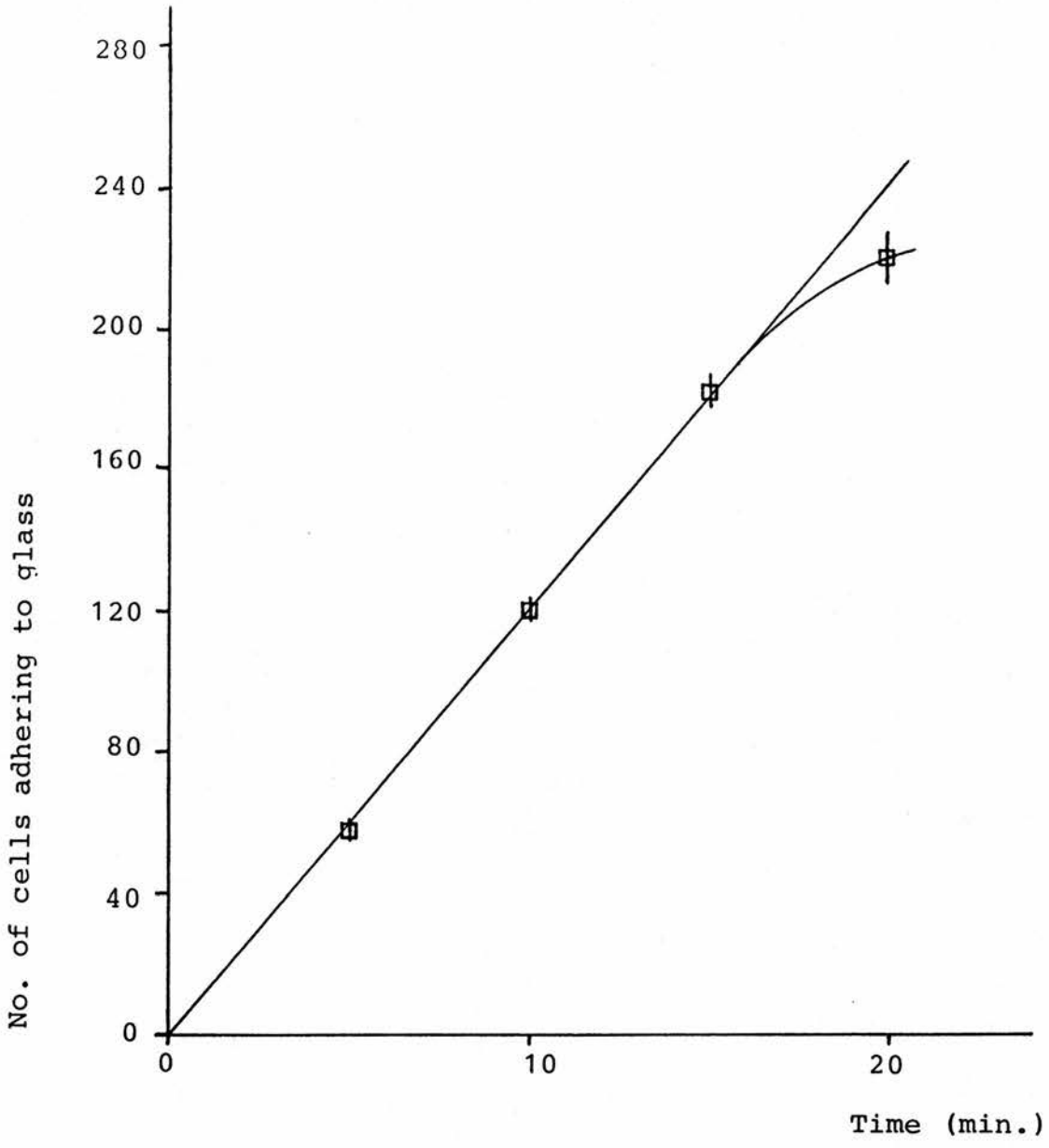


Figure 29

Time course of lymph node cell adhesion to glass at a cell concentration of 5 x 10^6 cells/ml.

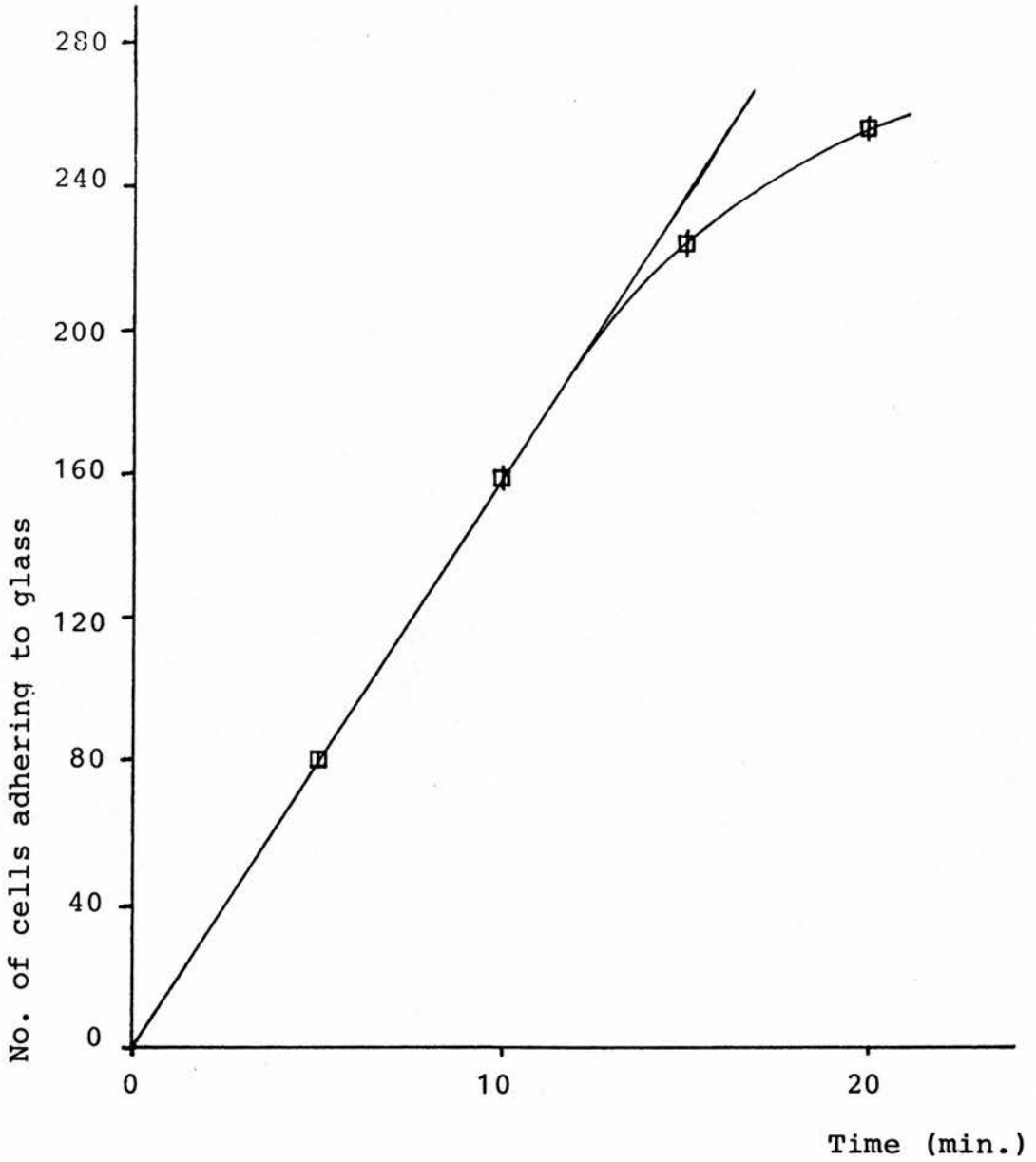


Figure 30

Rate of lymphocyte adhesion to glass at a cell concentration of 6.5×10^6 cells/ml.

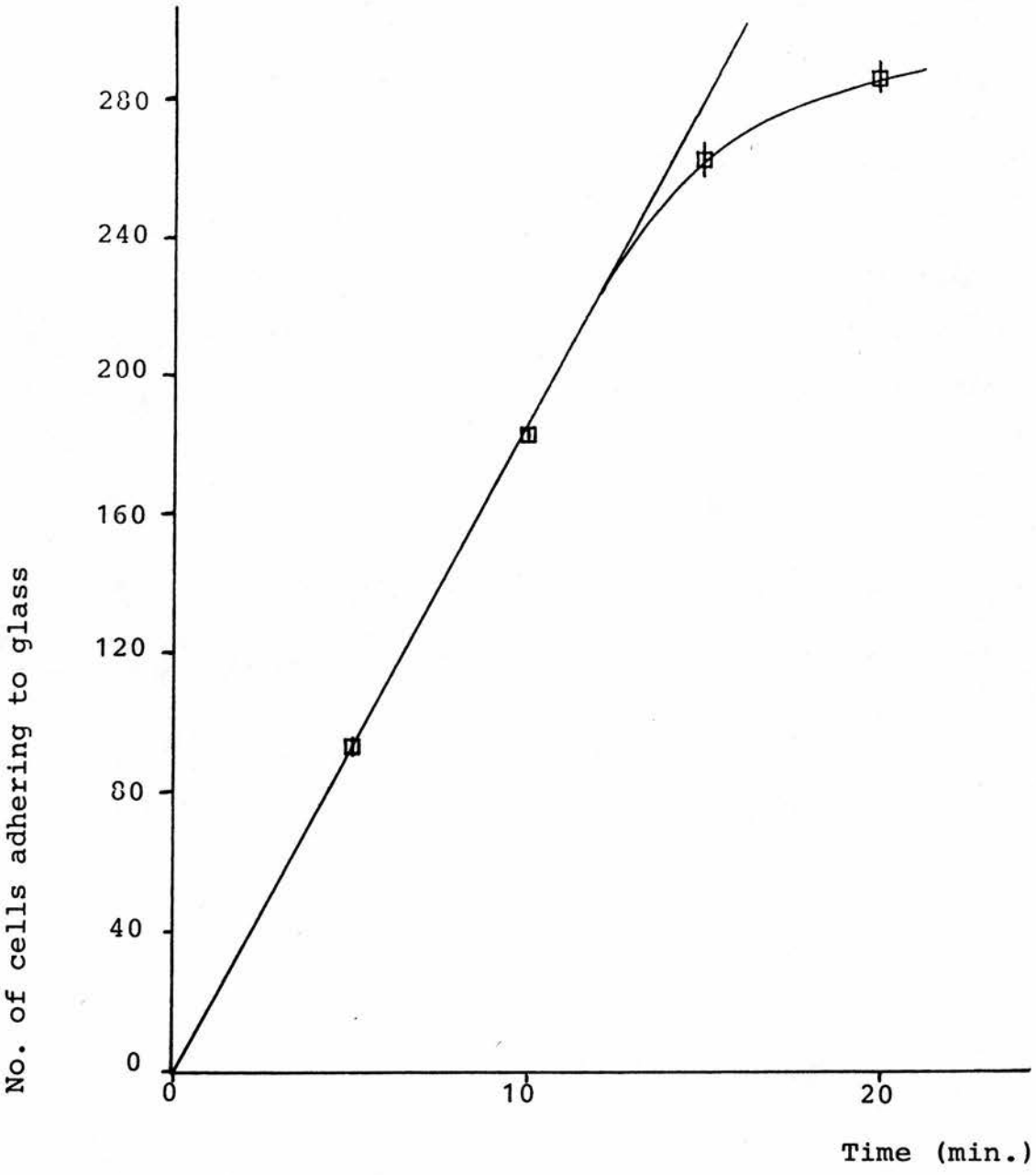


Figure 31

The time course of lymph node cell adhesion to glass at 8.6×10^6 cells/ml.

Table 20

Dependence of lymphocyte adhesion on time at different cell concentrations

Time (mins)	Mean number of adherent cells ± S.E.M.* at different cell concentrations ($x10^6$/ml.)				
	1.6	3.6	5	6.5	8.6
5	19.11±1.30	35.77±1.49	57.22±3.22	79.77±1.14	93.33±3.24
10	35.55±1.99	74.77±3.64	119.66±3.84	157.99±4.79	183.55±1.93
15	46.88±4.13	104.77±4.47	182.55±5.39	223.55±4.53	262.22±5.47
20	55.11±5.04	126.77±7.34	219.66±8.23	256.99±3.61	285.99±3.97

*n ⟶ 9

Table 21

Data collected from figures 27-31

C($x10^6$ cells/ml)	^{1}/C($x10^{-6}$ml/cell)	V(cells adhering/min)	^{1}/V(min/cell adhering)
1.6	0.625	3.80	0.263
3.6	0.277	7.55	0.132
5.0	0.200	12.075	0.0828
6.5	0.153	15.843	0.0631
8.6	0.116	18.57	0.0538

C ⟶ cell concentration

V ⟶ initial rate of cell adhesion

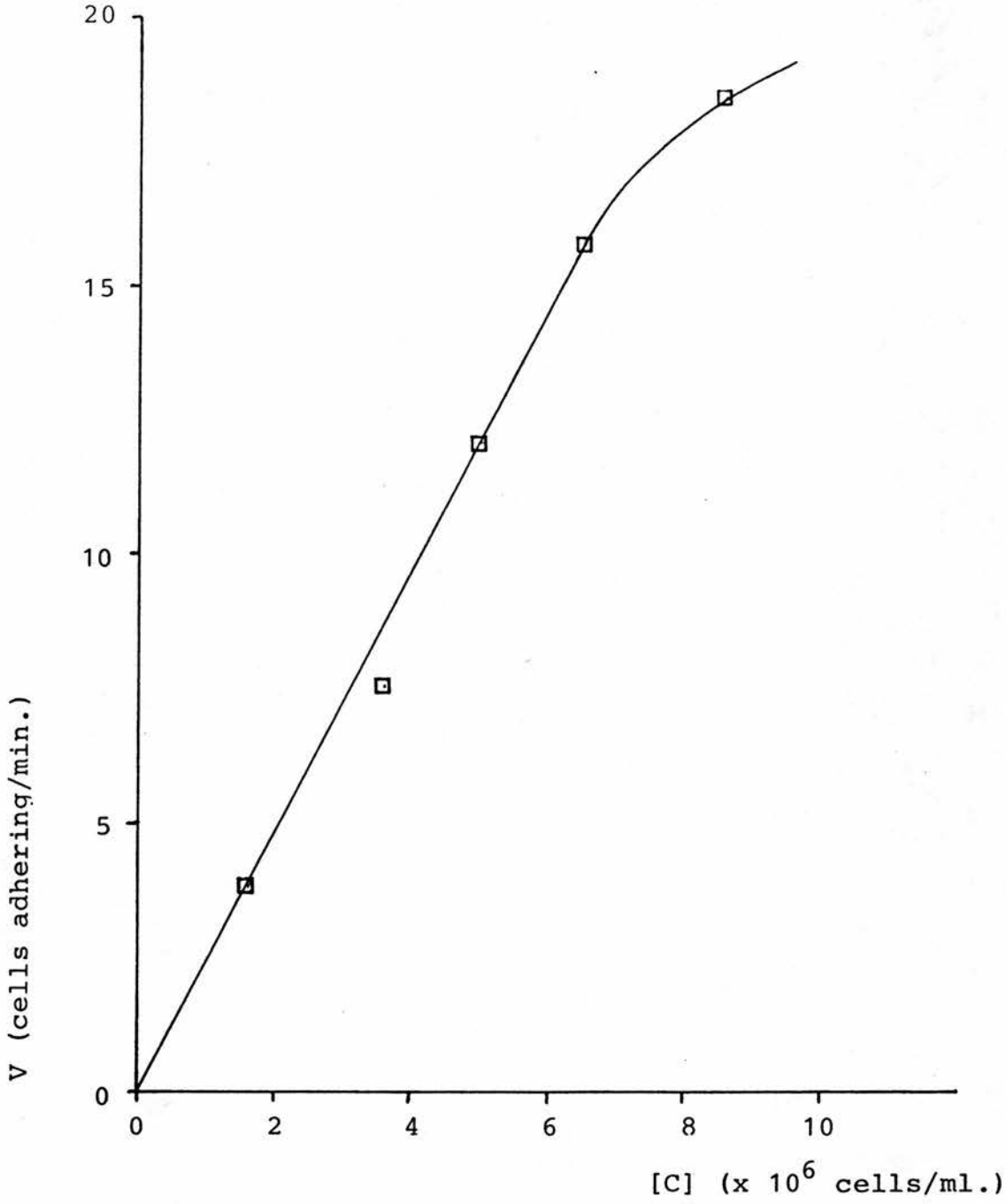


Figure 32

The relationship between the initial rate of adhesion (V) and lymph node cell concentration [C]

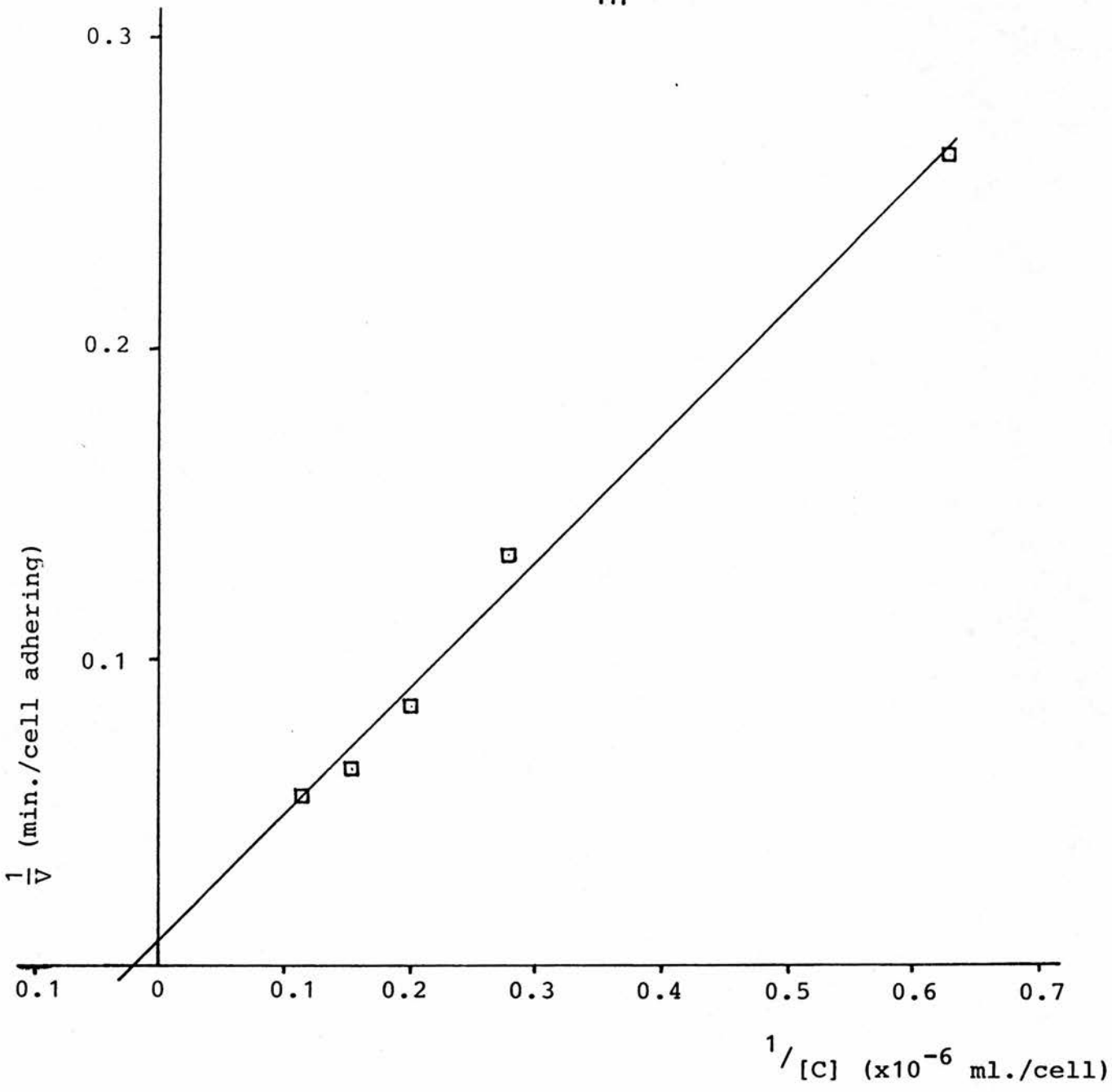


Figure 33

Lineweaver-Burk plot

By linear regression analysis:

$$y = 0.00435 + 0.41789\ (x)$$

The linear correlation coefficient is

0.9966

Table 22

Effect of serum on lymphocyte adhesion to glass with time at a cell concentration of 2.3×10^6 cells/ml.

% Serum	Time (Mins)	Mean number of adherent cells ± S.E.M.*
0.00	5	27.33 ± 3.84
	10	49.00 ± 3.78
	15	63.00 ± 6.65
	20	72.33 ± 2.96
0.625	5	21.33 ± 1.66
	10	28.00 ± 2.51
	15	38.33 ± 4.97
	20	42.66 ± 4.66
1.25	5	16.33 ± 1.20
	10	23.33 ± 0.88
	15	27.66 ± 2.33
	20	30.00 ± 1.73
2.5	5	9.33 ± 1.20
	10	14.66 ± 1.45
	15	18.33 ± 1.66
	20	20.66 ± 0.88
5	5	8.33 ± 0.33
	10	11.66 ± 1.45
	15	12.33 ± 1.20
	20	14.66 ± 1.66
10	5	5.66 ± 0.88
	10	8.00 ± 1.15
	15	9.00 ± 1.52
	20	10.66 ± 1.45

* n ⟶ 3

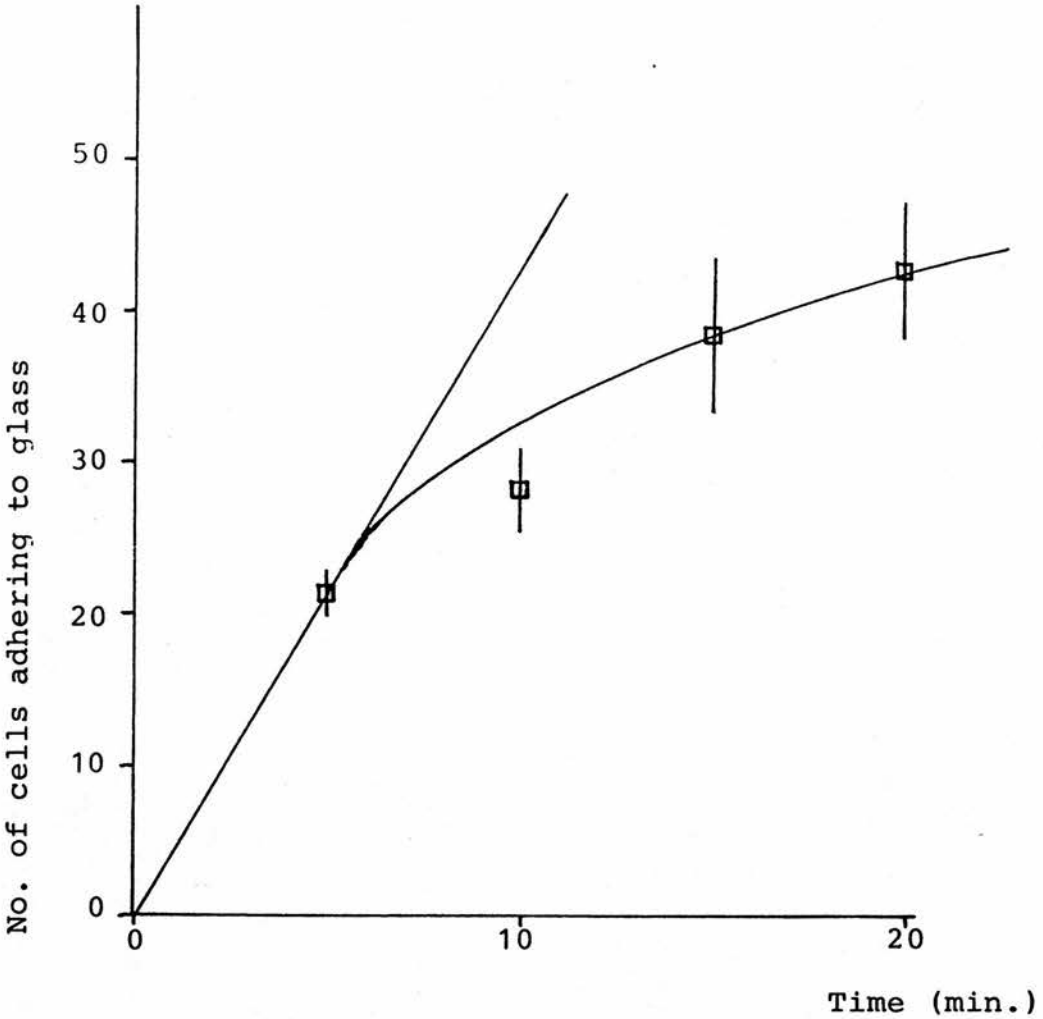


Figure 34

Influence of serum (0.625%) on the time sequence of lymphocyte adhesion to glass at 2.3 x 10^6 cells/ml.

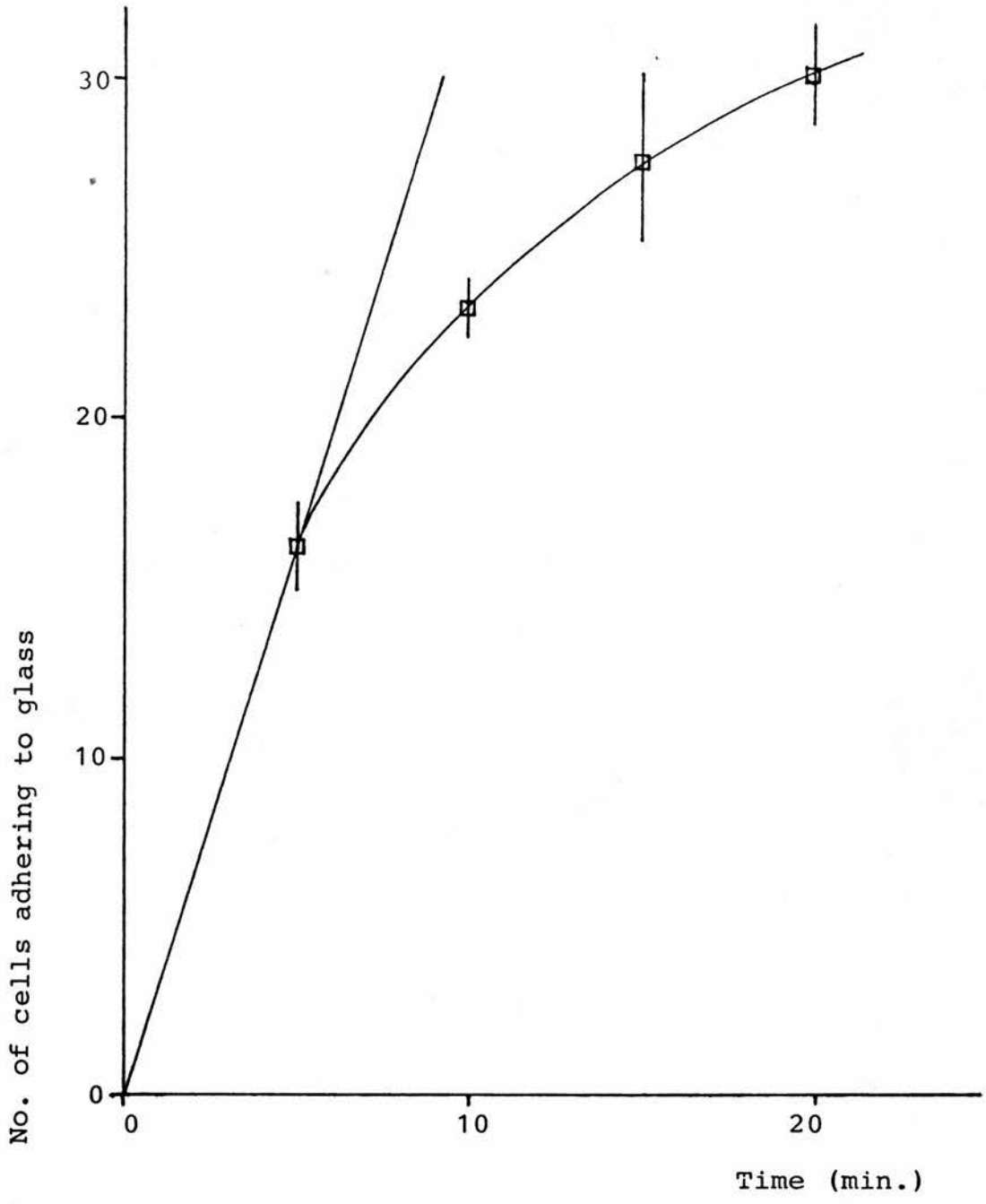


Figure 35

Influence of serum (1.25%) on the time sequence of lymphocyte adhesion to glass at 2.3 x 10^6 cells/ml.

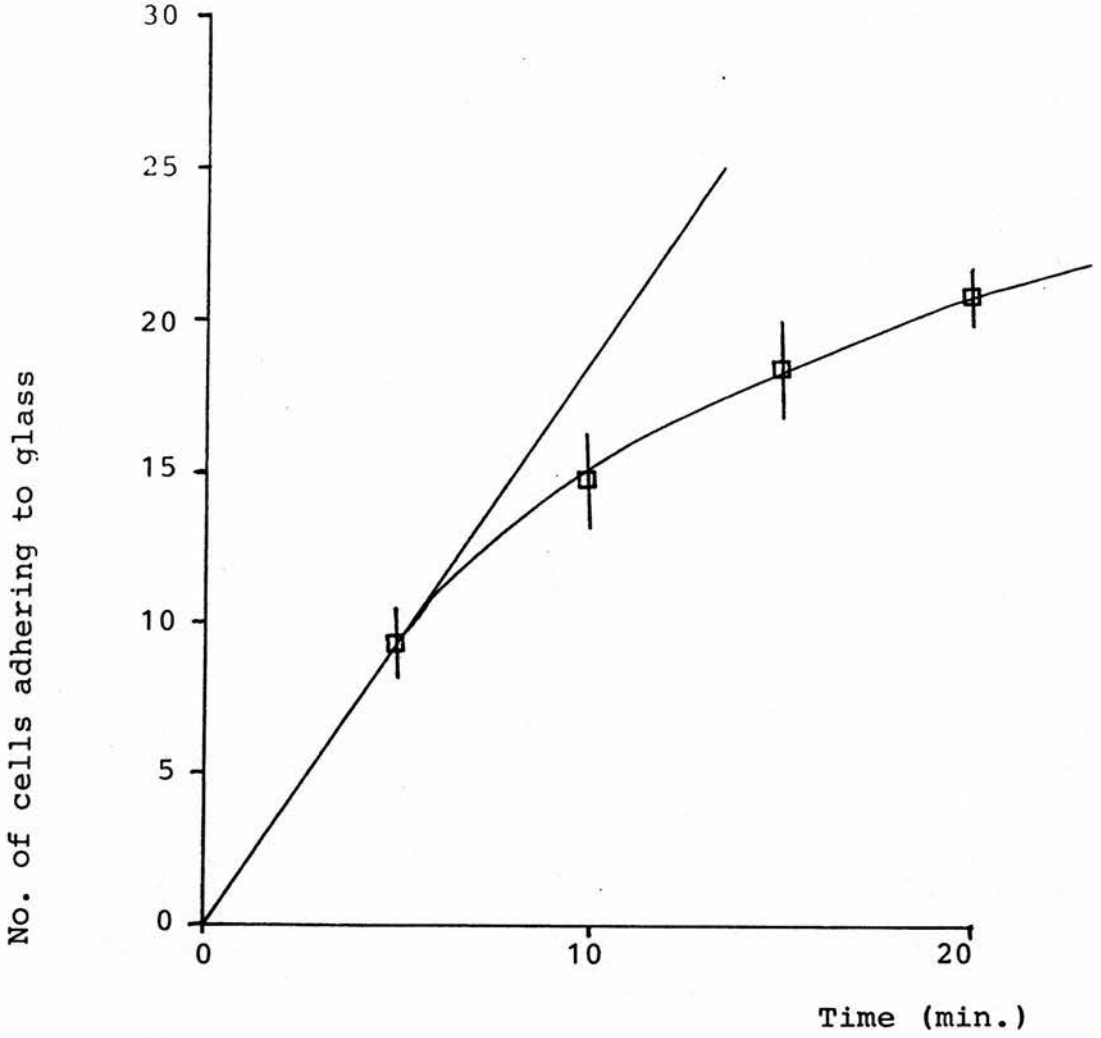


Figure 36

Influence of serum (2.5%) on the time sequence of lymphocyte adhesion to glass at 2.3 x 10^6 cells/ml.

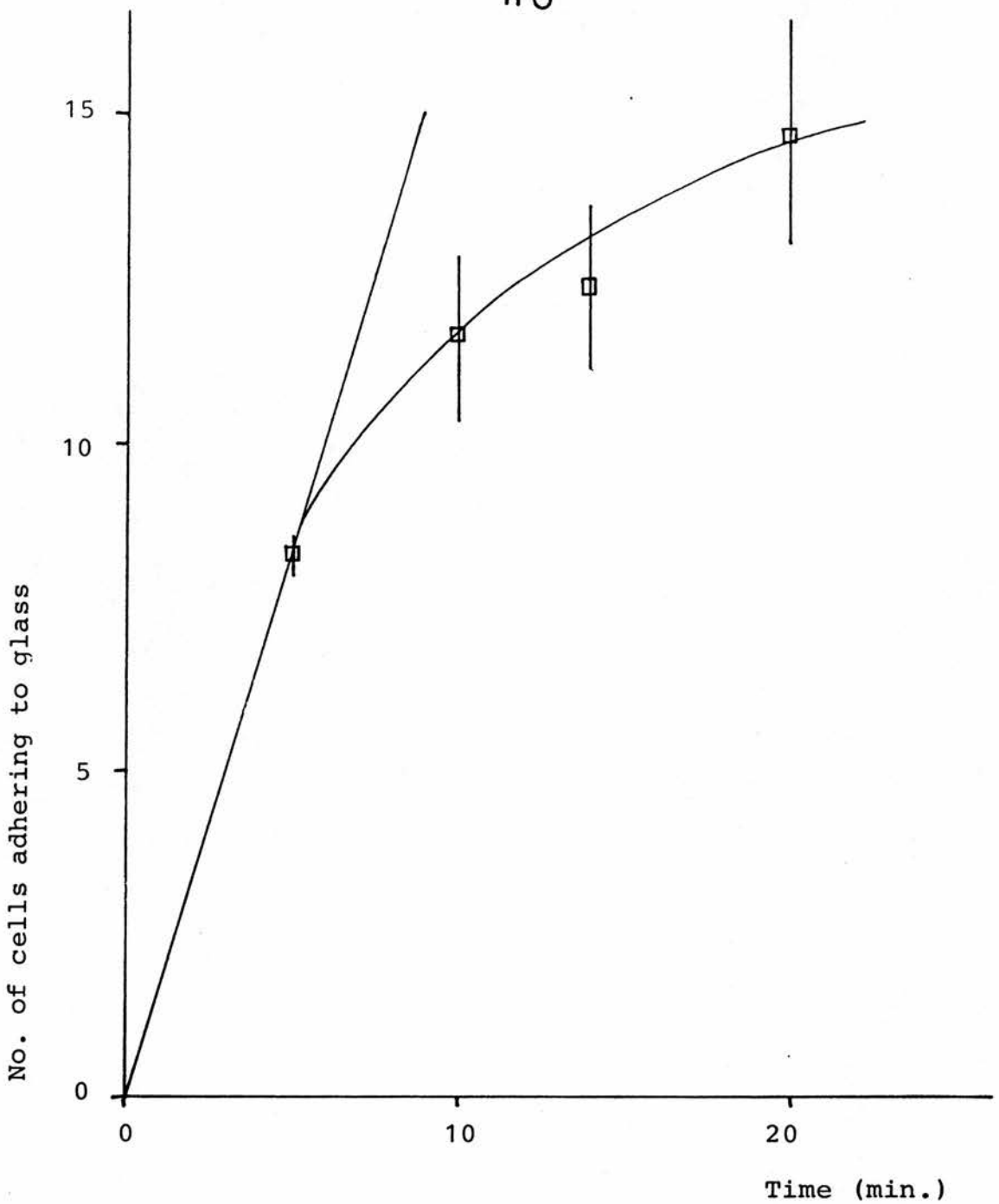


Figure 37

Influence of serum (5%) on the time sequence of lymphocyte adhesion to glass at 2.3 x 10^6 cells/ml.

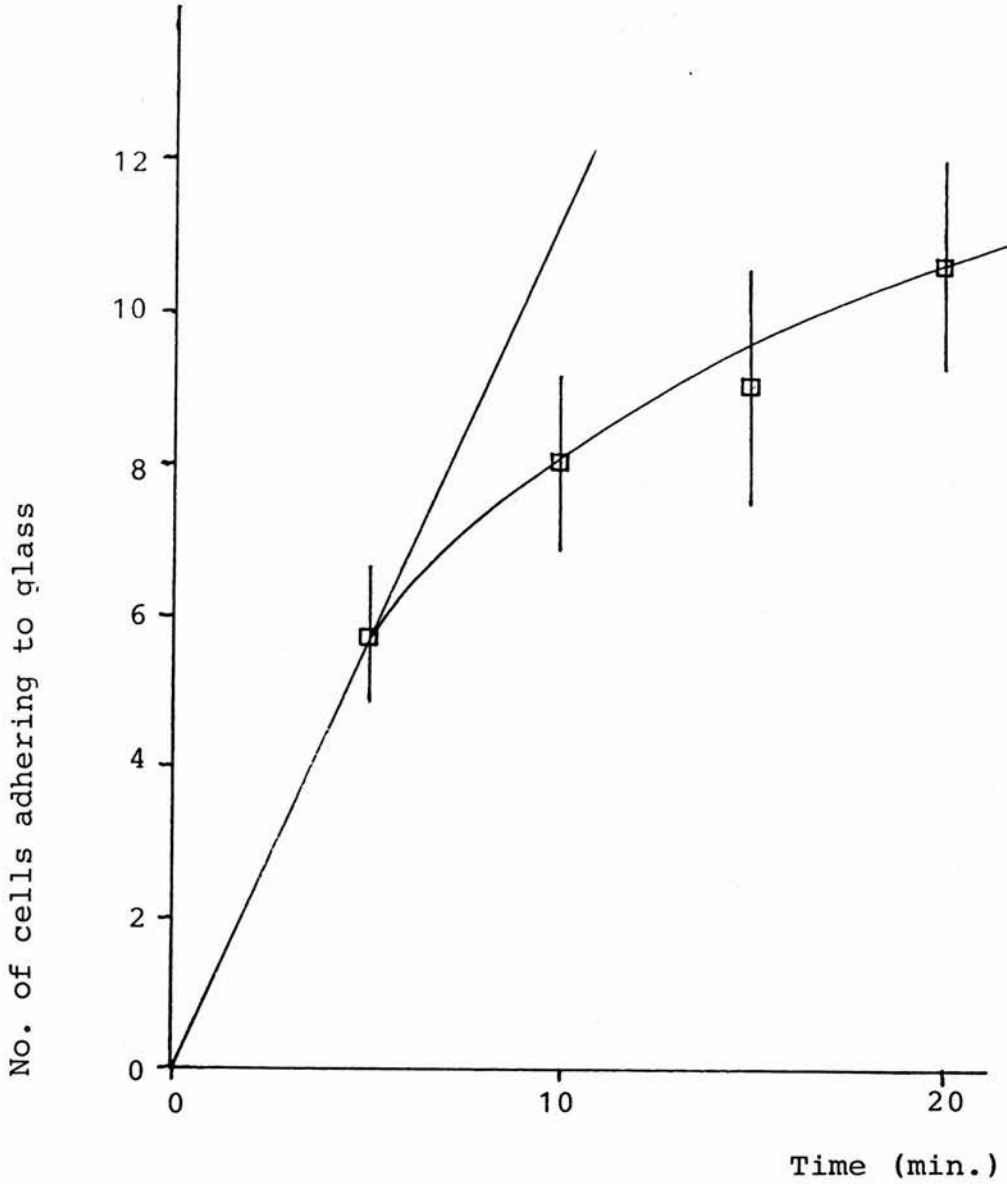


Figure 38

Influence of serum (10%) on the time sequence of lymphocyte adhesion to glass at 2.3×10^6 cells/ml.

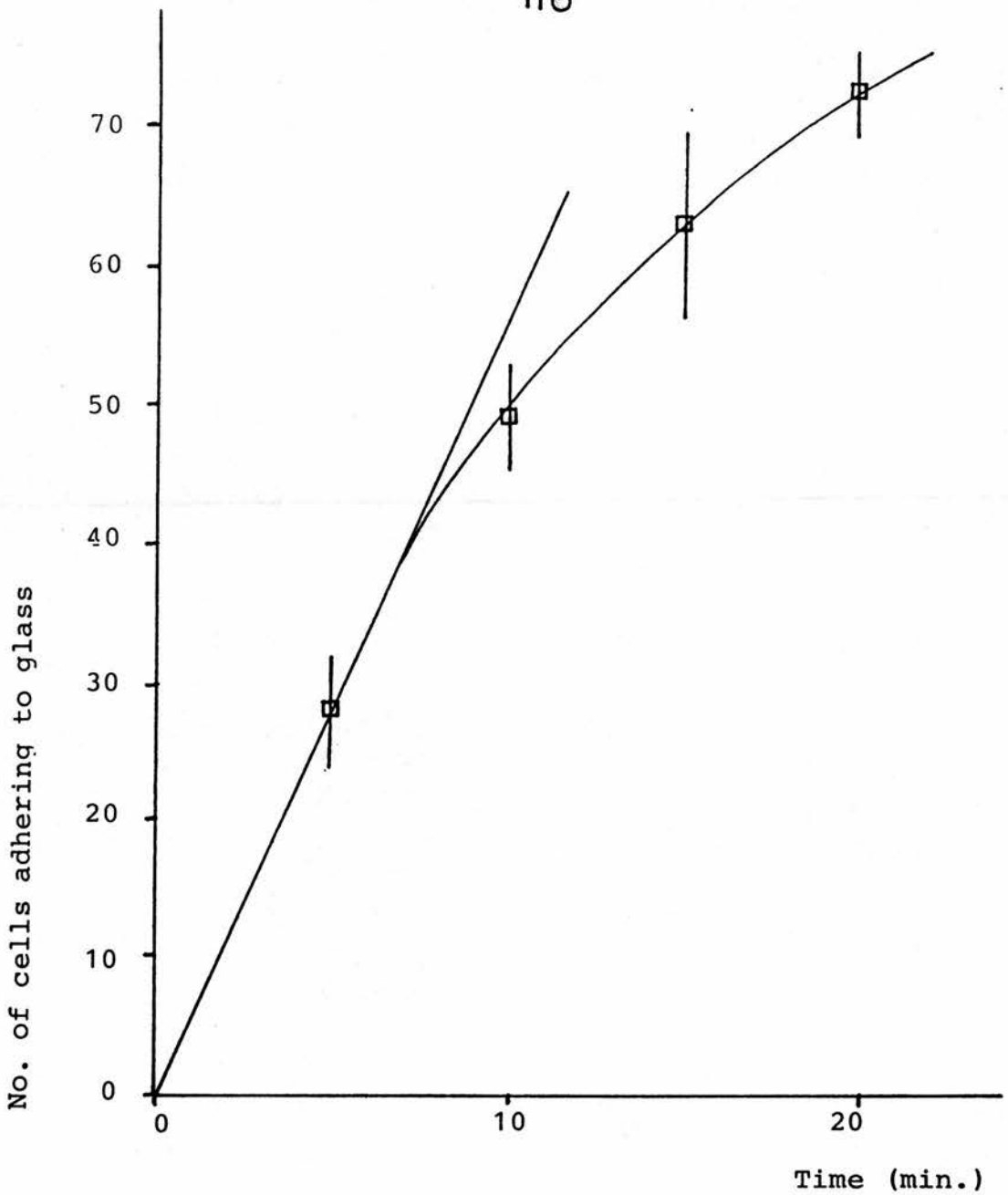


Figure 39

Rate of lymphocyte adhesion to glass at a cell concentration of 2.3×10^6 cells/ml.

Table 23

Effect of serum on lymphocyte adhesion to glass with time at a cell concentration of 3.5×10^6 cells/ml.

% Serum	Time (min)	Mean number of adherent cells ± S.E.M.*
0.00	5	39.33 ± 3.17
	10	70.00 ± 5.00
	15	98.33 ± 5.69
	20	111.66 ± 2.33
0.625	5	29.00 ± 3.60
	10	50.66 ± 1.45
	15	66.00 ± 1.15
	20	76.66 ± 2.96
1.25	5	24.33 ± 1.20
	10	35.66 ± 0.88
	15	46.33 ± 1.20
	20	55.66 ± 8.17
2.5	5	16.66 ± 1.20
	10	19.66 ± 2.40
	15	31.33 ± 2.40
	20	34.33 ± 2.90
5	5	14.33 ± 1.85
	10	15.00 ± 0.57
	15	23.00 ± 2.64
	20	25.66 ± 3.84
10	5	8.00 ± 0.57
	10	11.33 ± 1.85
	15	16.33 ± 1.76
	20	15.33 ± 2.22

* $n \longrightarrow 3$

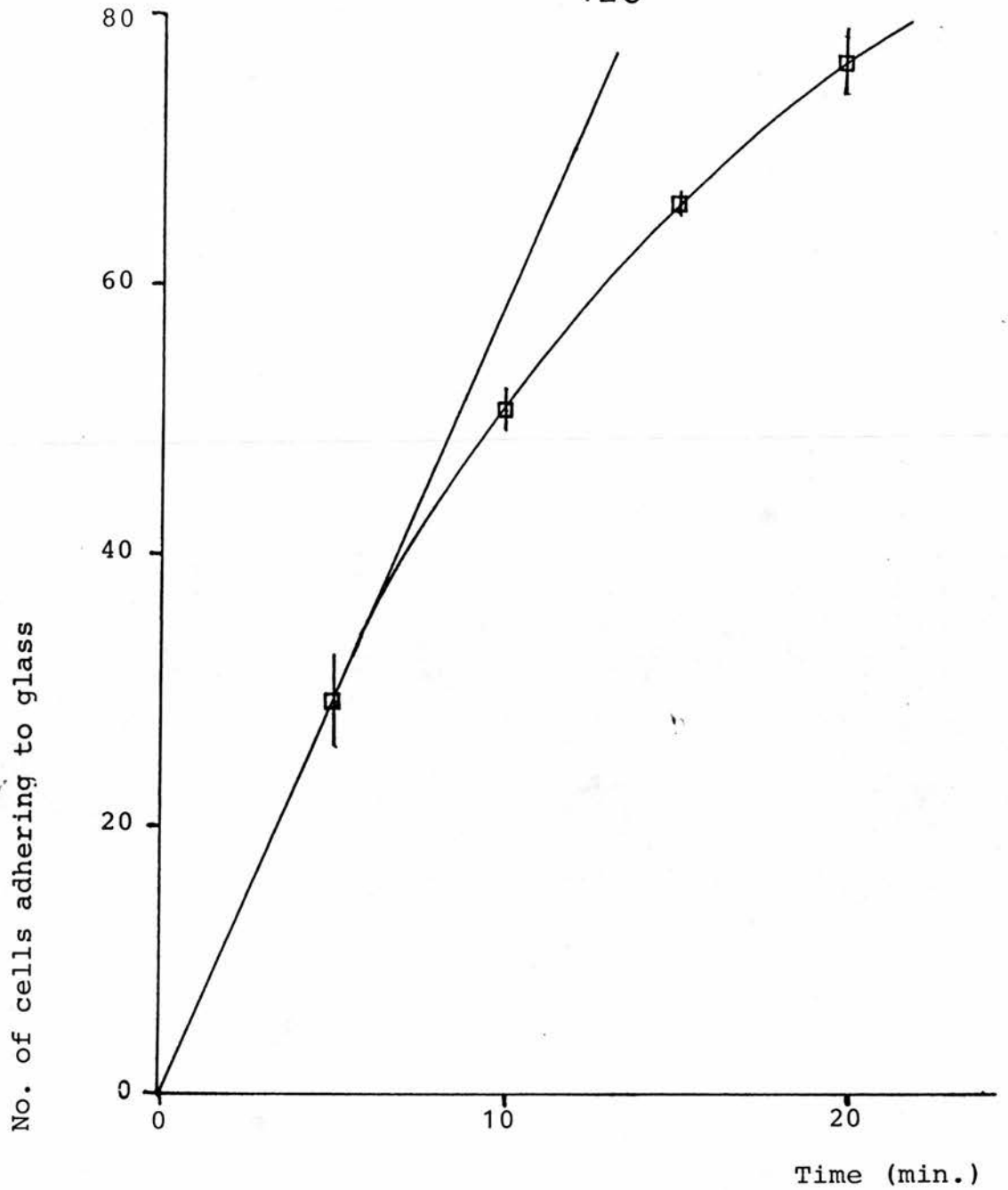


Figure 40

Effect of serum (0.625%) on time sequence of lymph node cell adhesion to glass at 3.5×10^6 cells/ml.

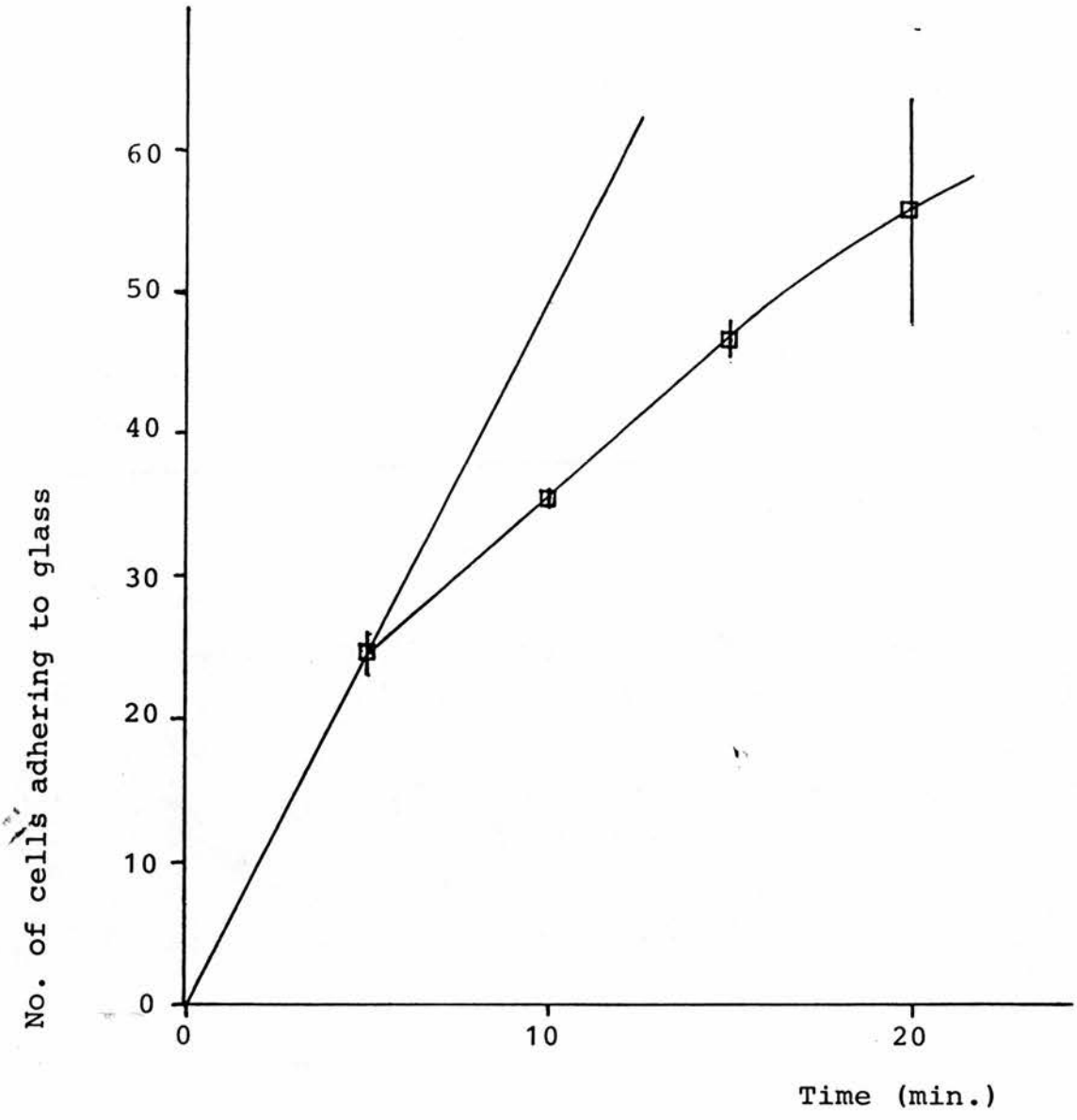


Figure 41

Effect of serum (1.25%) on the time course of lymphocyte adhesion to glass at 3.5×10^6 cells/ml.

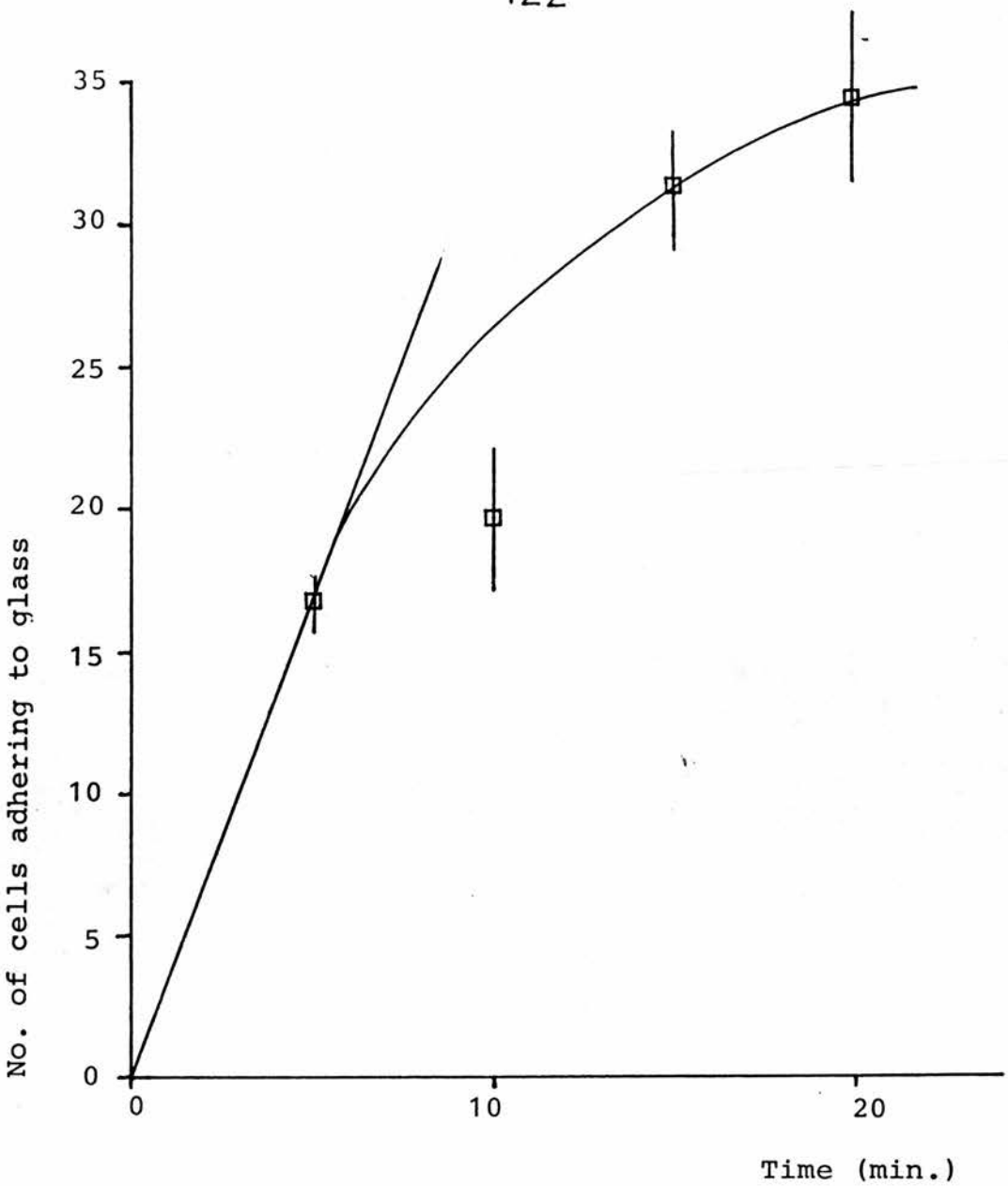


Figure 42

Effect of serum (2.5%) on the time course of lymphocyte adhesion to glass at 3.5×10^6 cells/ml.

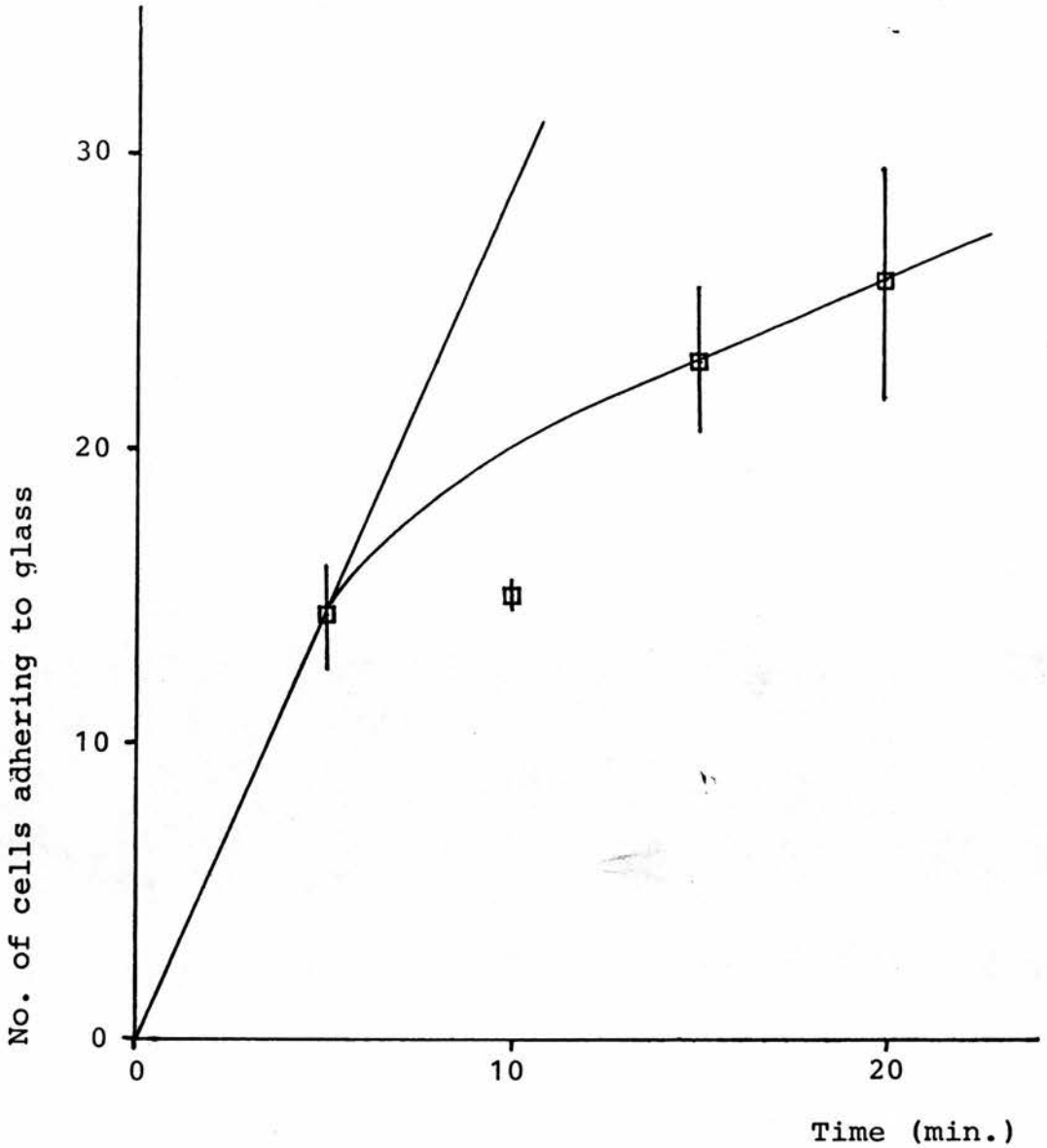


Figure 43

Effect of serum (5%) on the time course of lymphocyte adhesion to glass at 3.5×10^6 cells/ml.

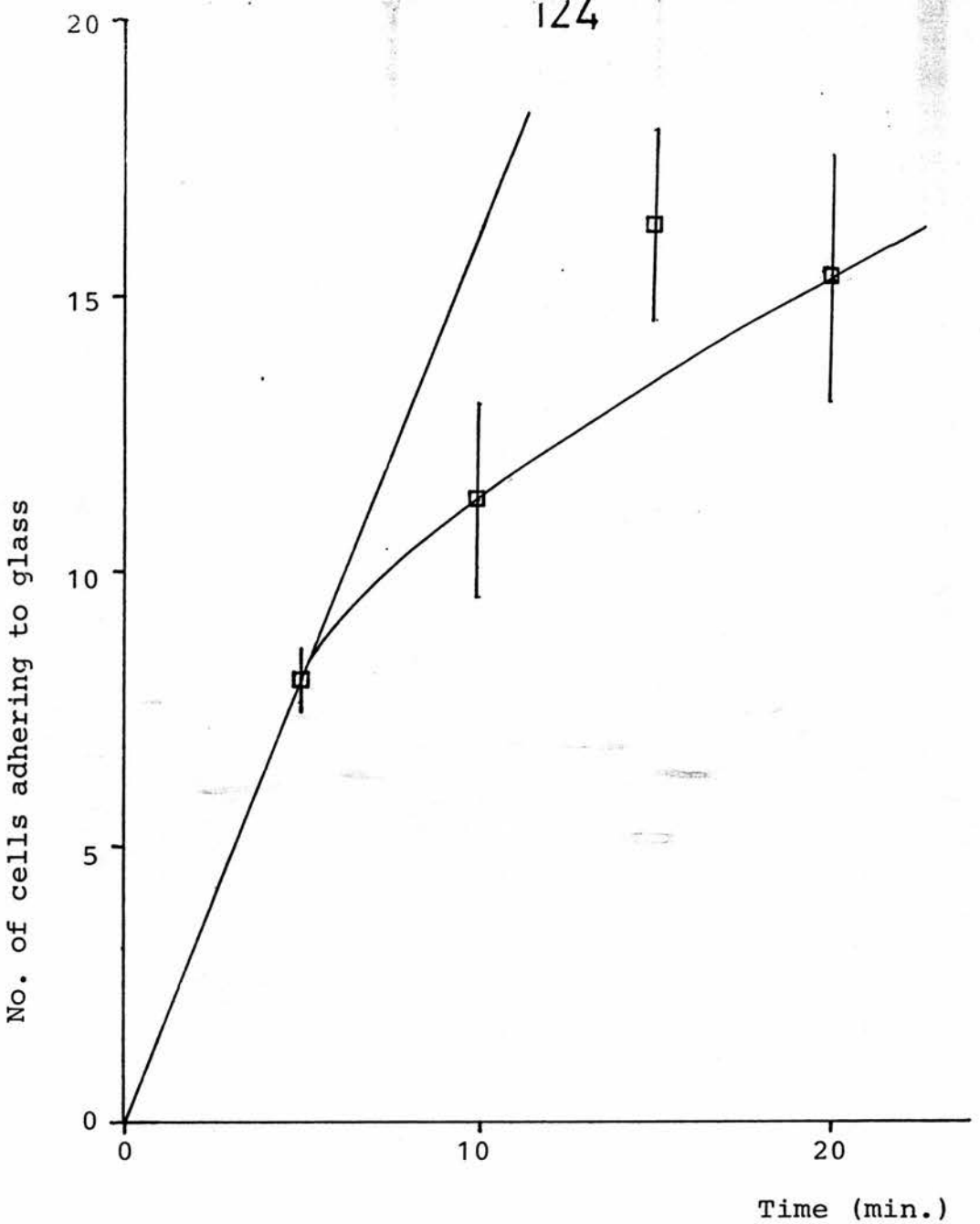


Figure 44

Effect of serum (10%) on the time course of lymphocyte adhesion to glass at 3.5×10^6 cells/ml.

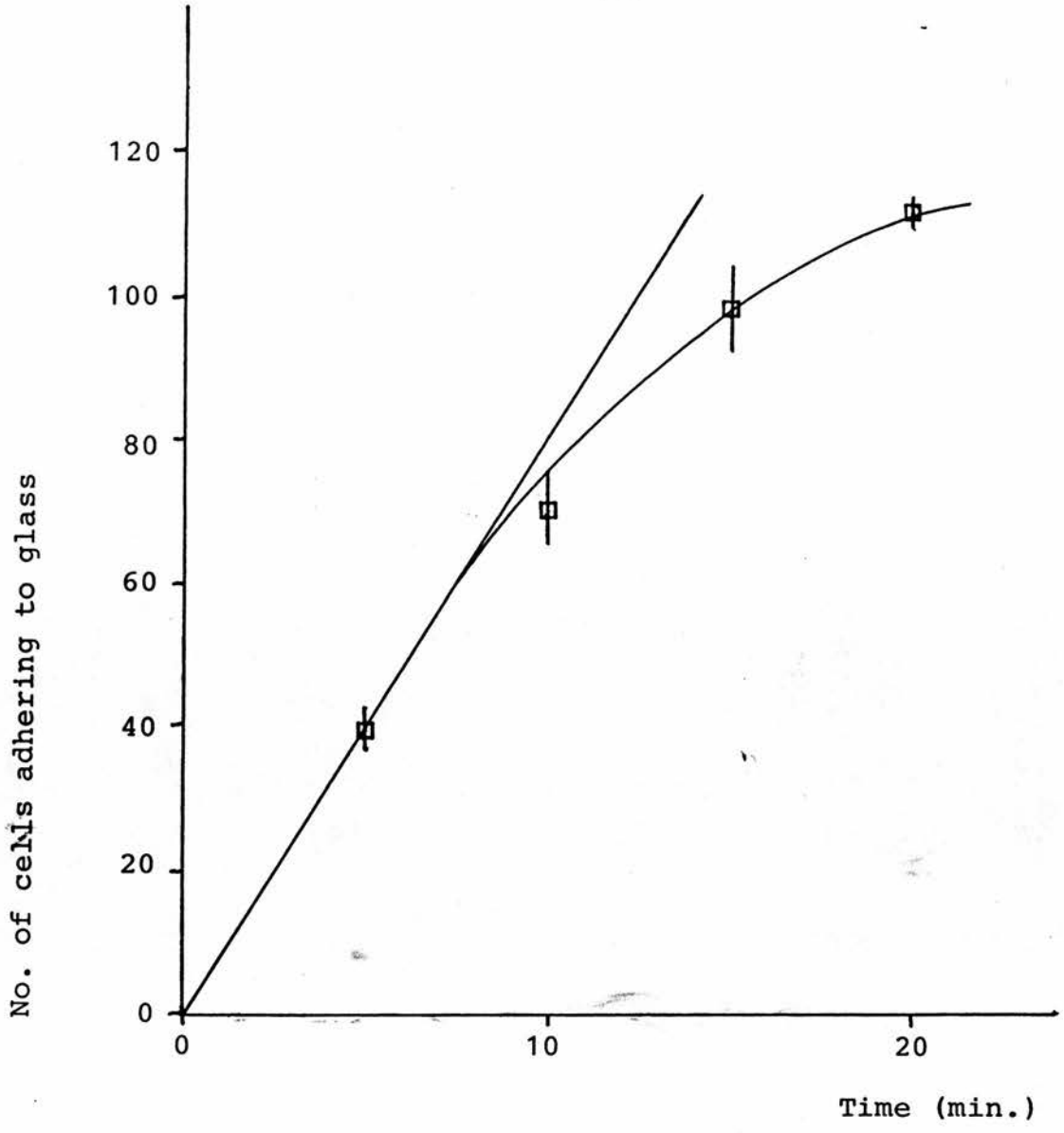


Figure 45

Rate of lymphocyte adhesion to glass at a cell concentration of 3.5×10^6 cells/ml.

Table 24

Data for the effect of % serum concentration (I) on the reciprocal of the initial rate of adhesion of lymphocytes to glass at two different cell concentrations collected from Figures 34-45

% serum concentration (I)	Reciprocal of the initial rate of adhesion at a cell concentration of	
	2.3×10^6 cells/ml. $^1/v$	3.5×10^6 cells/ml. $^1/v$
0.00	0.18	0.125
0.625	0.234	0.1725
1.25	0.306	0.2024
2.50	0.535	0.298
5.0	0.600	0.346
10.00	0.879	0.6235

(2.3×10^6 and 3.5×10^6 cells/ml.) - Figures 34-45, Tables 22-24. The Dixon plot is shown in Figure 46. The results are elaborated in the Discussion.

In order to determine the concentration of inhibitor in fractions 55 and 73 with respect to serum, the time course of lymphocyte adhesion to glass was plotted for both fractions (Table 25 and Figures 47-48). The values of initial rate of adhesion were calculated. These two values of initial rate of adhesion were used to find the corresponding values of the concentration of the inhibitor present in the fractions using the Dixon plot at the value of 3.6×10^6 cells/ml. (Table 26).

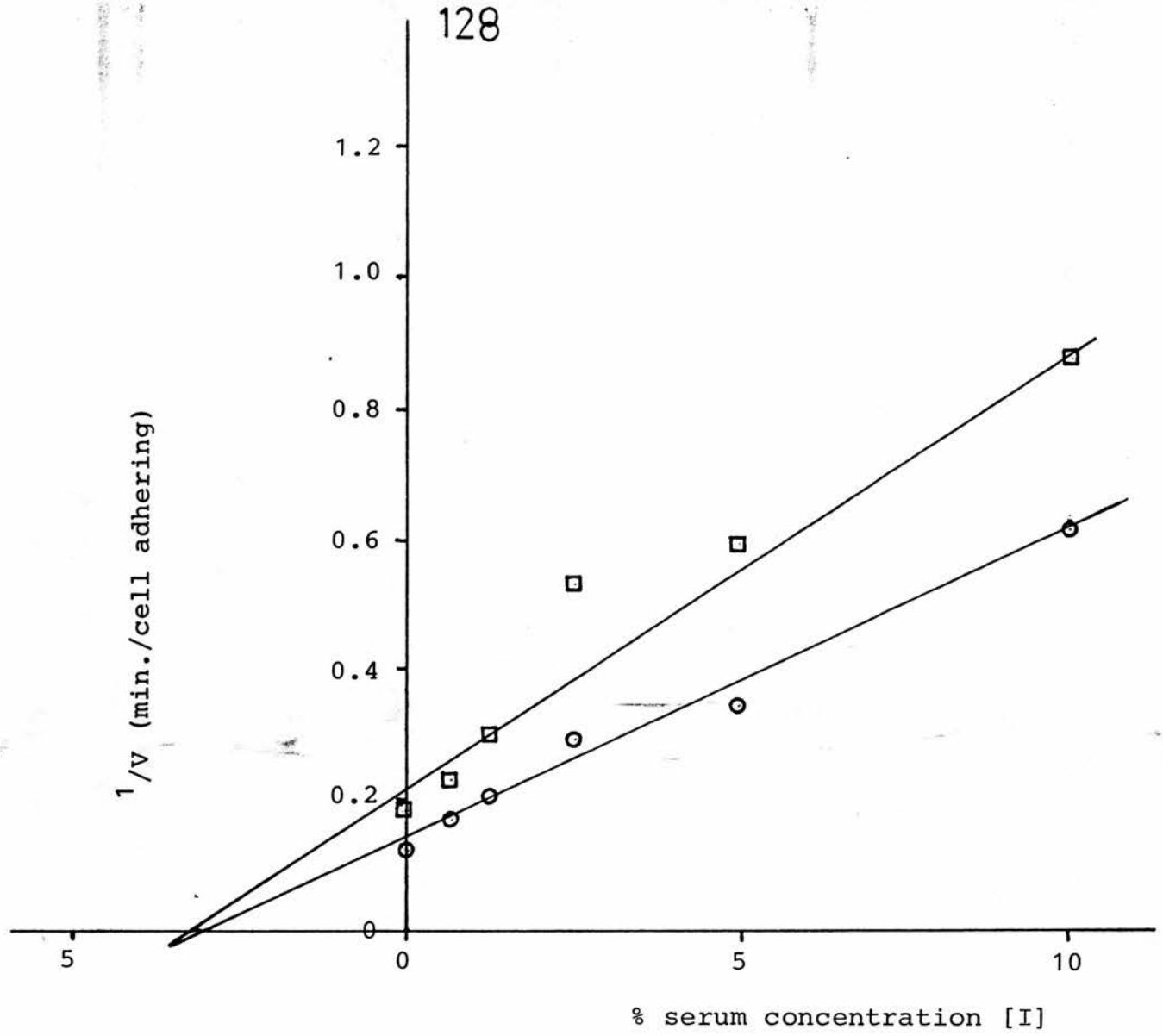


Figure 46

Dixon plot

For 2.3×10^6 cells/ml. (□—□),

by linear regression analysis

$y = 0.06834\ (x) + 0.2356$

and the linear correlation coefficient is

0.9647

For 3.5×10^6 cells/ml. (○—○),

by linear regression analysis

$y = 0.0476\ (x) + 0.1403$

and the linear correlation coefficient is

0.9912

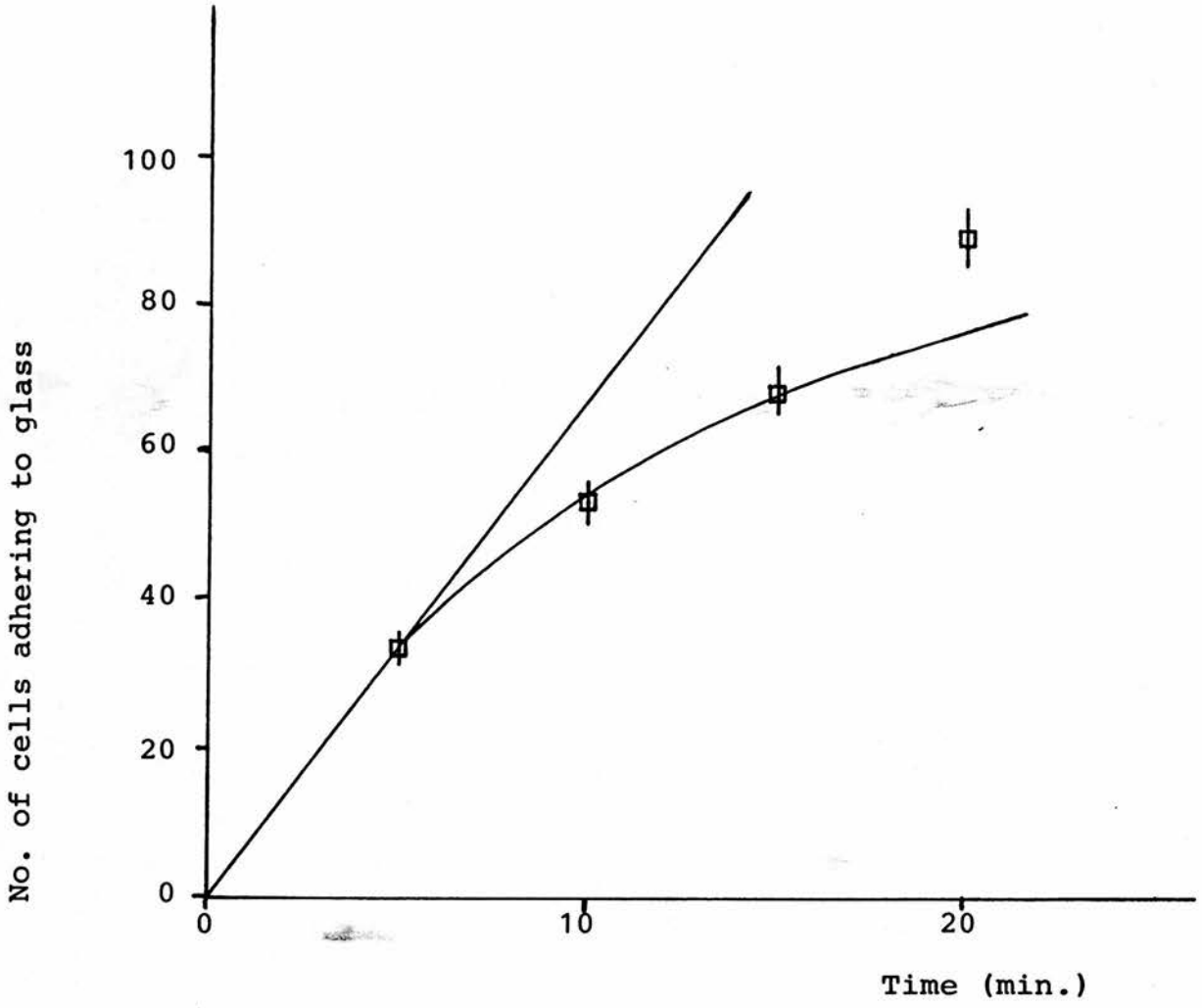


Figure 47

Effect of fraction 55 (from gel filtration Run 11B) on the rate of lymph node cell adhesion to glass

Table 25

Effect of fractions 55 and 73 (gel filtration Run 11B) on lymphocyte adhesion to glass with time at cell concentration of 3.6×10^6 cells/ml.

	Mean number of adherent cells ± S.E.M. At 50% dilution			
Time (Min.)	Fraction 55	n	Fraction 73	n
5	33.66 ± 2.73	6	23.30 ± 1.52	10
10	53.16 ± 2.54	6	28.50 ± 2.12	10
15	68.16 ± 3.54	6	41.60 ± 2.61	10
20	89.00 ± 3.77	6	42.10 ± 2.49	10

Table 26

Initial velocities (V) and the reciprocal of the initial velocities of fractions 55 and 73 (gel filtration Run 11B) from figures 47 and 48, with the corresponding inhibitor concentration (that is, % serum concentration) from the Dixon plot (Figure 46)

Fraction Number	V	$^1/_V$	% serum concentration
55	6.7	0.149	0.15
73	4.66	0.2145	1.5

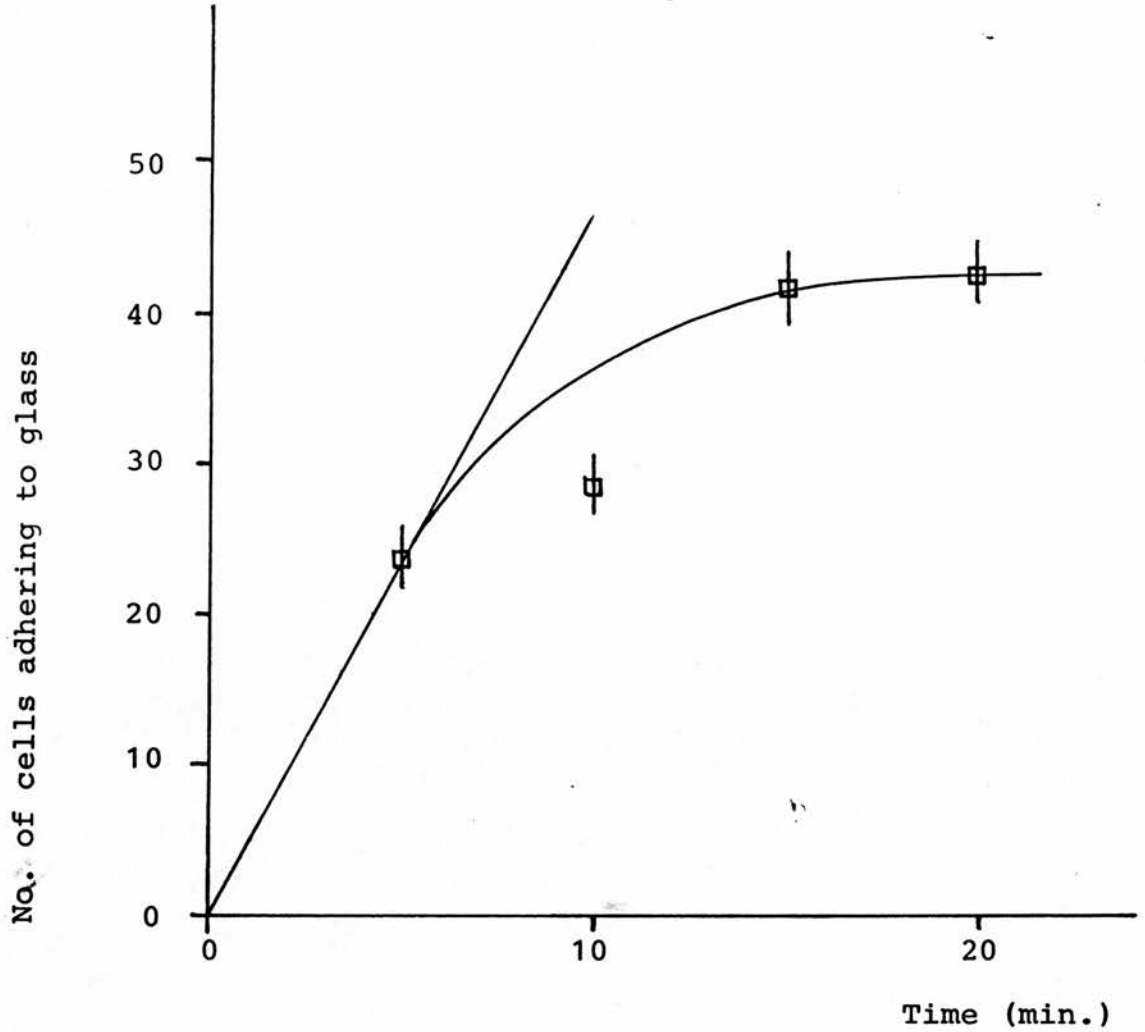


Figure 48

Effect of fraction 73 (from gel filtration Run 11B)

on the rate of lymphocyte adhesion to glass

Discussion

Discussion

		Page
1)	Effect of serum on lymphocyte adhesion to both biological and material surfaces.	133
2)	Purification of inhibitory factors.	139
3)	Kinetics of lymphocyte adhesion.	147

1. Effect of serum on lymphocyte adhesion to both biological and material surfaces

Glass as a substrate

The results obtained confirm the view that serum contains factors which inhibit lymphocyte adhesion to glass, as has already been pointed out by other authors (Musson and Henson, 1979; Kellie et al., 1980; Haston et al., 1982). Figure 5 illustrates that with increasing serum concentration, the number of adhering lymphocytes decline (that is, a "decay" type plot is obtained). Serum is very effective in its action and this is reflected by the fact that 0.6% of serum (V/V) reduces lymphocyte adhesion by almost 50%.

Serum appears to have a similar inhibitory effect on several other types of cells. The rate of adhesion of conjuctiva cells to glass was inhibited by serum (Taylor, 1961), and Witkowski and Brighton (1972) showed that serum inhibited the rate of spreading of a human diploid cell line. Harris (1973) observed that as the concentration of the serum was increased, there was a reciprocating decrease in the adhesion of 3T3 monolayers to glass. The number of HeLa cells adhering to plastic surface was reduced in the presence of serum than in the absence of serum (Schor and Court, 1979).

Inhibition of lymphocyte adhesion to glass could be the consequence of either or both of the two mechanisms below:

a) coating of the glass substrate by the serum inhibitory factors.

b) formation of a complex between the receptors on the lymphocytes and the serum factors.

A general expression for the curve in Figure 7 is given by the equation below, this describes the inhibition curve extremely well (the curve obtained from the equation fits within the experimental errors):

$$I = A\ (1 - e^{-c^{n}.\lambda})$$

where,

$I \longrightarrow$ % inhibition of cells

$c \longrightarrow$ % serum concentration

and A, n and λ are constants.

It is important to point out that lymphocytes are rather unusual cells in that most other cell types adhere and spread to serum components. Thus comparison between cell types is not valid.

Collagen as a substrate

Principally the data on lymphocyte adhesion to collagen substrate, Figure 8, in the presence of serum, show that adhesion is enhanced considerably at low serum concentrations, but begins to fall at higher concentrations for both native and heat denatured collagen; hence a relative inhibitory effect is observed. This inhibitory effect is possibly due to saturation of either or both the lymphocyte surface and

the substrate surface, consequently impeding cell-substrate interaction. In the present study, type I collagen was used. The maximum number of adherent cells for native collagen substrate was noted at 1.56% serum (V/V), and for denatured collagen-coated glass the maximum observed was at 6.25% serum. It is of significance that fewer lymphocytes adhered to denatured and native collagen than to glass, on the exclusion of serum from the assay medium. This suggests that lymphocyte adhesion to collagen occurs by a different mechanism, when compared to glass. Interestingly, Figure 9 reflects that in the presence of low serum concentrations, lymphocyte adhesion to denatured collagen is comparatively greater than native collagen.

Schor and Court (1979) have presented evidence that serum promotes to various degrees the cell (HeLa) attachment to different types of collagen films (native and denatured), and this has been confirmed in the present study. In addition, they found that cell attachment to gels of native collagen fibres was serum independent, as was the cell attachment to gels of native collagen treated with urea. They also have postulated that films of native collagen consist of a mixture of collagen fibres and randomly aggregated collagen monomers (tropocollagen), and observed no fibres of collagen in films of denatured collagen. This may explain the relatively lower enhancement of lymphocyte adhesion to films of native collagen at low serum concentrations compared with films of denatured collagen (Figure 9).

Recently, Haston et al (1982) have stated that serum had no effect on either attachment to or locomotion of the lymphocytes in collagen gels. However, Schor et al (1983) have indicated that "lymphocyte migration into the collagen gel matrix is not dependent upon the presence of serum, although serum does appear to have a measurable stimulatory effect".

An increase of cellular adhesion to various substrates by serum has previously been proposed by several authors (Klebe, 1974; Pearlstein, 1976; Grinnell, 1976). In many instances, it has become clear that fibronectin is an important adhesion factor. However, Wayman (1981) has indicated that fibronectin plays only a minor role in promoting lymphocyte adhesion to native and denatured collagen. Brown and Lackie (1981) have demonstrated that fibronectin does not affect adhesion to collagen-layered glass which in itself provides a substratum of very low adhesiveness for polymorphonuclear leucocytes (PMNs), but fibronectin reduces the adhesion of neutrophil granulocytes (PMNs) to gelatin-layered glass.

The physiological significance of the results of the present study, relating to the adhesion of lymphocytes to both forms of collagen substrates, may lie in the fact that the comparatively low adhesiveness provided by collagen-coated substratum for lymphocytes in the presence of serum is important in facilitating migration of lymphocytes through different tissues of various adhesive properties (types of collagens

are presumably of consequence) during inflammation. Moreover, an explanation for the observation of the enhanced adhesion of lymphocytes to denatured collagen is the possibility that damaged tissue cells at the inflammatory focus may release proteinases, and these may denature the collagen of the extracellular matrix. Coincidentally, Brown (1982) has indicated that high cellular adhesiveness would tend to immobilize cells and this effect may operate at an inflammatory locus as in the case of denatured collagen.

The reason why less cells may adhere to collagen substrata than to glass, may be that more contact sites are available for the case of glass and that these are minute spots of firm, specialised adhesions, but, in the case of collagen there may be fewer contact sites and furthermore the interaction may be diffusely spread out (comparatively large surface area between these spots). Sugimoto (1981) has reported on two types of contacts for cell-substrate adhesion:

1) Close approach (less than 10nm.) of the cell surface to the substrate.
2) Diffuse contacts, cell plasma membrane being 80nm. away from the substrate, less strong but more extensive than the area of close approach.

It would be interesting to investigate the strength of lymphocyte-collagen interaction.

Little is known about the receptors on the cell surface involved with cell attachment to collagen. However, it is plausible that cell adhesion to denatured collagen occurs by the ligand-receptor mechanism (Grinnell, 1978). That is, there is inter action between a cell-surface receptor for serum adhesion promoting factor, and that this factor is absorbed on the collagen surface.

In the present study, two-dimensional (2-D) collagen substrata was used. In future studies, the following should be considered:

a) it would be interesting to observe lymphocyte adhesion to 3-D matrices, in the presence of serum.

b) 3-D collagen types I and III matrices should be utilised, both as a single type of collagen substratrum, and as a mixture of both.

c) more complex macromolecular matrices prepared by the stepwise addition of other extracellular matrix components to the collagen gel, and the gel covered by a monolayer of endothelial cells. Hence, approaching the physiological environment of the cells.

d) determine initial rate of adhesion to collagen for different cell concentrations, and compare with enzyme kinetics.

2. Purification of Inhibitory Factors

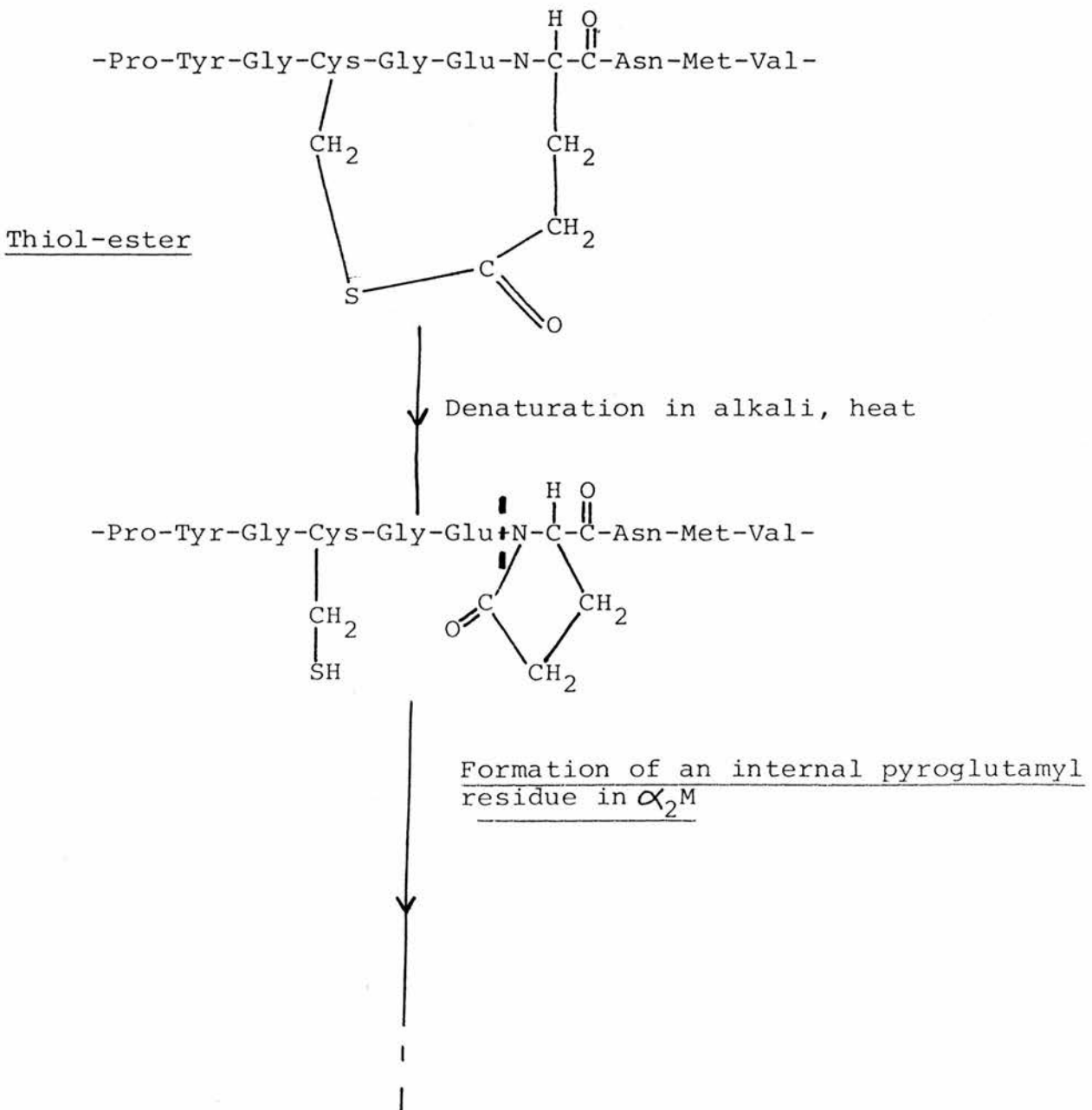
Serum was fractionated on a Sepharose 4B column; three areas of inhibitory activity were observed (Figure 10). Pooled fraction (14-21) from Run 1A (see results section 2.1.A.) was separated on a bed of Sepharose 4B. This revealed two regions of inhibitory activity (Figure 11), a broad band of activity from fractions 16-26 (culminating in 3 peaks), and a peak at fraction 30. This clearly reflects that inhibition is not a non-specific effect of protein, but can be associated with a small number of particular proteins.

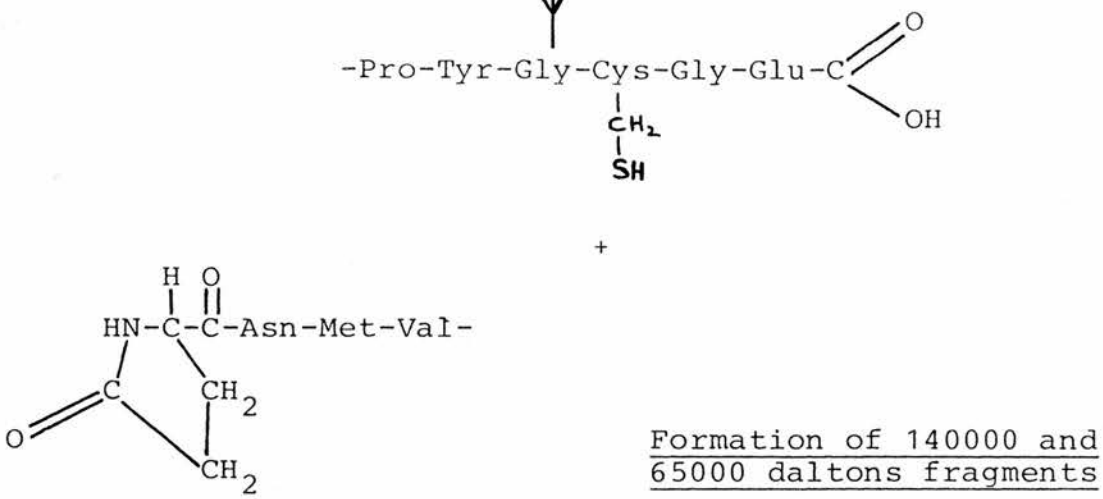
Ensuing SDS-polyacrylamide gel electrophoresis on aliquots of fractions from Run 1B (see results section 2.1.B), showed a single major band with molecular weight in the region of 400000 daltons for fractions 12 and 14 (Figure 12 and Table 9a). Apparently, this is lower than some of the molecular weights for subsequent fractions. However, this is consistent with the view that the minimal covalent subunit of α_2M is the half-molecule or the dimer. The molecular weight of α_2M is 725000 (Harpel, 1973; Hall and Roberts, 1978), and is a tetramer. The two dimers are held together by non-covalent bands, and each dimer is covalently bonded by disulphide bonds.

The reduced aliquot of the pooled fraction collected from gel filtration Runs 1-10 (see results section 2.2) gave 2 major bands of molecular weights 140000 and 75000 daltons, with 2 minor bands at 65000 and 26000, and a faint band at 180000 daltons (Table 9b). The 180000 band is perhaps the monomer of the oligomeric

α_2M molecule of about 725000, and the molecular weights at 140000 and 65000 daltons correspond to the reduced α_2M in the presence of β-mercaptoethanol and SDS (Barrett et al., 1979), and the other 2 bands (75000 and 26000 daltons) relate to reduced Immunoglobulin M (IgM).

Cleavage of the α_2M monomers into two fragments of molecular weight 140000 and 65000 occurs at the α-amino group of a particular Glx-residue (occurs one-third of the way from one end). The sequence shown below is common to all four subunits of α_2M as a reaction γ-glutamyl-thiol-ester (Sottrup-Jensen et al., 1980):





Formation of 140000 and 65000 daltons fragments

The thiol-ester in α_2M is so labile that treatment with denaturants leads to its cleavage even at room temperature. Incubation of α_2M in buffers at pH<4.5 or pH>9.5 also makes the thiol groups appear rapidly.

These results are in agreement with the estimations of Table 11, but the 100000 band obtained for the reduced pooled fraction 54-61 is interesting (see below).

Kellie (1980) has reported that the inhibitory factor is not a γ-globulin, hence IgM can be discounted. Therefore, it is apparent that α_2M is perhaps the high molecular weight inhibitor. This would be consistent with the findings of Kellie (1980), who suggests a molecular weight value of greater than 300000 daltons for the adhesion-inhibition factor, after fractionation by Amicon ultrafiltration.

The above findings are augmented by the fact that the precipitate of PEG fractionation (Table 19) which contained inhibitory activity, would be expected to contain α_2M (Virca et al., 1978; Hao et al., 1980).

Concurrent with the above, Aplin and Hughes (1981) have observed that α_2M failed to mediate cell adhesion when covalently attached to glass coverslips, and point out that α_2M interacts with Baby hamster kidney (BHK) cells and is taken up into the cells by accelerated (receptor-mediated) adsorptive endocytosis. Interestingly, the uptake of complexes of proteinase with serum α_2M by macrophages has been demonstrated in vitro (Debanne et al., 1975; Kaplan and Nielson, 1979). Also, other authors have presented evidence for the specific uptake of α_2M through receptors located on the surface of fibroblasts (Van Leuven et al., 1979; Willingham et al., 1979). According to Willingham and his co-authors, vacant α_2M receptors are spread on the surfaces of fibroblasts, and following the formation of α_2M-receptor complexes, they collect together in coated regions in the plasma membrane and are later internalized.

Work done prior to this study by other authors, has implicated various adhesion inhibitors. Aggregation of chick embryo cells was found to be inhibited by serum in the cold (Curtis and Greaves, 1965), and it was later identified as phospholipase A_2 by Curtis and colleages (1975). However, Kellie (1980) discovered that phospholipase A_2 had no effect on the adhesion of lymphocytes to glass at 37°C. Nordling (1967) studying the effect of substrate adhesion of HeLa cells in the presence of serum, found that γ-and β-lipoprotein fractions (after ethanol precipitation) were responsible for

the adhesion-inhibition of HeLa cells by serum. In the present study, the β-lipoprotein was present in the precipitate retained during PEG fractionation (Hao et al., 1980).

Sequential ammonium sulphate precipitation, fractionation on a CM-cellulose cation exchanger and gel filtration, and subsequent electrophoresis on SDS polyacrylamide gels revealed a molecular weight of about 100000 daltons (Table 18), which is consistent with the estimation of molecular weight of 100000 daltons by Kellie et al. (1980).

Furthermore, polyacrylamide gel electrophoresis data on fractions collected from the Sepharose 4B column (Figure 11 and Table 9a), show the molecular weight of approximately 100000 daltons to be well spread over a range of fractions (24-32). This agrees with the value for molecular weight obtained from the ion-exchange studies mentioned above. Also, a range of molecular weight values from 240000 and extending to 260000 daltons is found in the same broad band of inhibition activity. Moreover, a peak of inhibitory activity at fraction 30 (molecular weights - 115000 and 50000) was observed (Figure 11) and a peak at fraction 28 revealing a molecular weight of 50000 daltons (Table 7 and Figure 10). The report by Costello et al. (1979) that alpha-1-acid glycoprotein (molecular weight - 44100 and contains approximately 45% carbohydrate including 12% sialic acid) has the ability to inhibit platelet aggregation induced by ADP and adrenaline, may be interesting in this context.

Assuming that α_2M is one of the adhesion inhibitors, then ammonium sulphate fractionation would not be a good purification step as ammonium ion inactivites α_2M.

The immunosuppressive activity of α_2M has been proposed by several authors (Kamrin, 1959; Mannick and Schmid, 1967; Cooperband et al., 1976; Veitch et al., 1980; Barta, 1983), and Mowbray (1963) has indicated that it inhibits antibody production. The results of the present study are perhaps related to the above view; in that, if α_2M is the adhesion inhibitor of lymphocytes, then the interaction between α_2M and lymphocytes may arise in the following 3 ways:

1) α_2M may coat the cell surface thus blocking the receptors for adhesion, and obstructing receptors for antigen recognition (taking immunosuppressive activity into account).
2) if α_2M is an adhesion inhibitor. This could imply that proteolysis is involved in the adhesion process. Inhibition might then be caused by inhibition of some enzyme. However, this idea can be discounted, as it has been observed that lymphocyte and PMNs migration into collagen gels is not dependent on proteolytic digestion of the collagen fibres (Brown, 1982; Schor et al., 1983)
3) the interaction of α_2M with the antigen recognising receptors; this may cause conformational changes on the lymphocyte surface and lead to adhesion inhibition.

These results are consonant with those of Musson and Henson (1979), after fractionation on Sephacryl G-200. They found 3 major areas of activity corresponding to molecular weights of 230000, 90000 and 14000.

Recently, Sharma and colleagues (1981) have reported that albumin-IgG complexes inhibit human platelet adhesion to glass and constitute 12-16% of the total protein in serum. These complexes are present in the plasma as well, and appear to be a normal component of the blood. Albumin (68000) alone had a slight inhibitory effect and IgG (160000) alone or in a mixture with albumin was not inhibitory. Similarly, Kellie (1980) has found albumin to have a small inhibitory effect on the substrate adhesiveness of lymphocytes. He also adds that fibronectin had no discernible effect on cell adhesion at concentrations equivalent to that found in 10% serum. This complies with the findings of Wayman (1981). However, Brown and Lackie (1981) have shown that fibronectin inhibits the adhesion of neutrophil granulocytes to clean glass, HSA- and BSA-coated glass, and to gelatin-coated glass.

Suggestions for further work:

1) Purify α_2M by the method described by Binnie (1981), and assay for inhibitory activity to confirm that this is one of the inhibitory factors.
2) Purify the 100000 molecular weight factor in significant quantities to determine its molecular weight by ultracentrifugation, its amino acid content, and ascertain its other characteristics.

3) Purify the albumin-IgG complex to observe its effect on lymphocyte adhesion to glass.

4) Perform binding studies to discover the number of receptors on lymphocyte surface, and subsequently find the composition of the receptors.

5) Attempt to locate the domain of the cell attachment site on the inhibitor molecule.

6) Find the number of moles of zinc/mole of α_2M; the binding site of zinc within the α_2M molecule; effect of zinc, if any, on the lymphocyte adhesion to glass.

3. Kinetics of Lymphocyte Adhesion

Initial rates of lymphocyte adhesion to a glass substrate were measured at different lymphocyte concentrations. The results show that the initial rate was not directly proportional to the cell concentration (Figure 32). Hence, the data suggest that the cell-substrate adhesion process is a Michaelis-Menten type reaction, and could be analysed as shown:

$$C + G \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (CG)^* \xrightarrow{k_3} CG$$

where,

C ⟶ cell concentration

G ⟶ adhesion binding sites (number of sites is constant)

(CG)* ⟶ unstable intermediate complex

CG ⟶ adhered stable complex, which is measured in adhesion assay

k_1, k_2 and k_3 ⟶ rate constant of each step.

This is a two step process of recognition and binding, and is therefore in disagreement with Kellie (1980) who suggests that adhesion is instantaneous. Theoretically, the two steps are measurable.

Consequently, the velocity of adhesion can be calculated from:

$$V = \frac{Vmax\ [C]}{Km + [C]} \qquad (1)$$

where,

[C] ⟶ concentration of lymphocytes

Vmax ⟶ maximum velocity

Km ⟶ apparent Michaelis constant, $(k_2 + k_3)/k_1$

Km is expressed as the cell concentration at which the reaction rate is half of its maximal value, and it represents the reciprocal of the affinity between cells and receptors on the surface of glass coverslips in rapid equilibrium conditions. The results obtained in the experiment do not contradict the assumption of rapid equilibrium in this reaction.

If the number of adhesive sites is n_o, the maximum velocity (Vmax):

$$Vmax = k_3\ n_o$$

A decrease in Vmax could be attributed to a decrease in k_3 and/or n_o.

Manipulation of (1) gives (by reciprocating):

$$1/V = (Km/Vmax)\ \ 1/[C] + \frac{1}{Vmax}$$

A plot of $^{1}/V$ versus $^{1}/[C]$ (Lineweaver-Burk) yields a straight line with a slope of $Km/Vmax$ and an intercept of $^{1}/Vmax$ (Figure 33).

Other authors have also provided evidence that cell-substrate adhesion could be analysed by applying Michaelis-Menten type kinetics (Cohen et al., 1981; Ueda et al., 1981.)

As factors in serum inhibit adhesion of lymphocytes to unnatural surfaces, the initial velocity of lymph node cell adhesion was examined in the presence of variable percentage concentrations of serum, at two different cell concentrations. Adhesion of lymphocytes (initial velocities) was also investigated for 50% dilutions of two fractions collected from gel filtration column (Table 25).

The Dixon plot reflects that inhibition is of the non-competitive type (Figure 46). That is, Vmax increases with increasing cell concentration, but the apparent Km does not change.

This indicates that inhibition by serum factors is not due to the decrease of affinity between cells and substrate (glass), but that it may be due to a decrease in the ability of cells to adhere to glass in a stable state (CG). Accordingly, the inhibitor may be reacting at a site on the cell surface different to the place of interaction between the cells and the coverslips. This may lead to a sufficient alteration of the conformation of the cell surface to prevent the normal adhesion of cells to the glass substrate.

The plot obtained with a cell concentration of 3.6×10^6 cells/ml. (Figure 46) was used as a standard straight line, to determine the amount of inhibitor present in a given fraction, relative to the amount present in the serum. The initial velocities were determined, and the corresponding serum concentration read from the abscissa. The method appears to have been successful in defining an activity unit. Therefore, <u>One Activity Unit could be defined as The Initial Velocity of Adhesion Due To 1% of Serum Concentration (V/V) At The Expressed Cell Concentration.</u>

Furthermore, for a given fraction at a specified cell concentration, from a standard graph of percentage (%) adhesion of cells to glass against % serum concentration at the specified cell concentration (such as Figure 6), the value of % adherent cells to glass could be read off from the value of % serum concentration obtained from Dixon plot for the initial velocity at the specified cell concentration.

It will be interesting to determine the initial velocities of lymphocyte aggregation at different cell concentrations, both in the absence and presence of serum. Also, an Arrhenius plot of log. of initial rate of adhesion against reciprocal of temperature, could be used to calculate activation energy (E) and activation of enthalpy ($\Delta H^{\neq}$).

Finally, the kinetic treatment in this section assumes that the cell concentration throughout the diffusion chamber

is uniform, in the duration of an assay. In practice this may not be the case, as the cell concentration in the lower part of the chamber will increase with time due to settling, at a rate proportional to Stokes' Law, but, this effect will be comparatively small during the measurement of initial velocities (that is, at time zero), and in theory will have no effect. However, to ensure that the influence of the process of settling is negligible, it would be realistic to introduce, in future studies, some form of agitation to the chambers during the incubation period of the assay.

Thus, both adhesion and adhesion-inhibition of cells is part of the homeostatic process. The mechanisms underlying the two operations should be further determined, and the factors responsible for adhesion and inhibition isolated. This would assist in an insight into and the clarifying of many pathological disorders and the normal functioning of the organism.

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