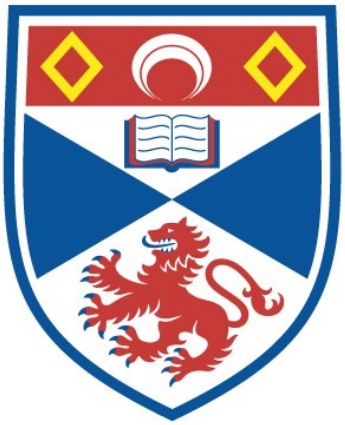
A STUDY OF MOLECULES INVOLVED IN THE REGULATION OF THE GROWTH OF HAEMATOPOIETIC CELLS AND HEART MUSCLE CELLS IN CULTURE

William Samuel Gilmore

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1986

UNIVERSITY OF ST. ANDREWS

FACULTY OF SCIENCE

A STUDY OF MOLECULES INVOLVED IN THE REGULATION OF THE GROWTH OF HAEMATOPOIETIC CELLS AND HEART MUSCLE CELLS IN CULTURE

A Thesis submitted to the
University of St. Andrews

by

WILLIAM SAMUEL GILMORE, B.Sc. (Hons., St. Andrews)

for the degree of

Doctor of Philosophy

September 1985

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

C O N T E N T S

ABSTRACT

A STUDY OF THE MOLECULES INVOLVED IN THE REGULATION OF THE GROWTH OF HAEMATOPOIETIC CELLS AND HEART MUSCLE CELLS IN CULTURE

WILLIAM SAMUEL GILMORE

The description of the molecular events responsible for the control of cell division and differentiation is, currently, one of the major goals of molecular and cellular biologists. Cell and tissue culture techniques have proved to be promising laboratory tools for the study of the regulators of cellular growth and differentiation. Most cells in culture require specific polypeptide growth factors which are supplied by the addition of a complex biological fluid such as serum or, in some instances, by the cells themselves. These growth factors usually act on their target cell via a membrane receptor to which they bind.The events which occur after the growth factor binds to the membrane receptor have not been fully described, but the phosphorylation of tyrosine residues in certain proteins has been observed.

A study was made of the polypeptide growth factors responsible for the growth and differentiation of haematopoietic cells *in vitro* . These growth factors, called colony - stimulating factors (C.S.F.'s) were prepared from human placental conditioned medium, giant cell tumour conditioned medium and pokeweed mitogen stimulated spleen conditioned medium. A C.S.F. from human placental conditioned medium was radioiodinated and the binding of the labelled growth factor to an anti-C.S.F. antiserum was studied. The binding studies indicated that a purer C.S.F. preparation and/or a more specific antiserum was necessary in order to establish a radioimmunoassay for C.S.F. The C.S.F.'s from giant cell tumour conditioned medium were purified by ultrafiltration, hydrophobic - interaction chromatography, gel filtration and thiolpropyl - sepharose 6B chromatography. Two peaks of biological activity were observed on gel filtration. One of these peaks gave an apparent MW of 63,000 and the other peak gave an apparent MW of 30,200. The C.S.F. from pokeweed mitogen stimulated spleen conditioned medium was labelled with peroxidase and the binding of the labelled-C.S.F. to bone marrow cell membranes studied. The labelled-C.S.F. bound to the membranes and the binding exhibited a linear relationship with membrane protein content. Also, a defined growth medium for chick embryonic heart cells was developed. These cells were observed to differentiate from primitive foetal cells into mature "adult-type" cells. The cells grew as a monolayer, had spontaneous activity and were seen to beat.

D E C L A R A T I O N

I declare that the research described in this thesis is my own work except where acknowledgement has been made.

The work was carried out in the Department of Biology, University of Ulster, at Jordanstown, and has not been submitted for any other degree.

W.S. Gilmore
September, 1985.

I hereby certify that William S. Gilmore has spent nine terms engaged in research work under my direction and has fulfilled the conditions of Ordinance General No. 12 and Resolution of the University Court 1967 No. 1 and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

G.D. Kemp B.Sc. Ph.D.

ACKNOWLEDGEMENTS

This study would not have been possible without the help and co-operation of many people. I would like to thank Professor W. Thompson, Department of Obstetrics and Gynaecology. The Queen's University of Belfast, for providing me with placentas from the patients in his care. The students and staff of the Department of Biology, University of Ulster at Jordanstown (formerly, The Ulster Polytechnic) were a constant source of help and stimulation. In particular I would like to thank Dr. S.W.B. Irwin , Dr. G McKerr and Mr I. Moran for their assistance in preparation and interpretation of the electron micrographs.

The work was supervised by Dr. G.D. Kemp, Department of Biochemistry and Microbiology, University of St. Andrews, and I am most grateful for his kindness and advice.

I would, particularly like to thank Dr. T.R.J. Lappin, Department of Haematology, Royal Victoria Hospital Belfast, for his constant help and encouragement.

Finally I thank my family whose time was taken in order that the work for this thesis be undertaken. I especially thank my wife, Kathleen, who typed this thesis.

ABBREVIATIONS

A	Absorbance
abl	Abelson leukaemia oncogene
B.F.U.- E.	Burst forming unit - erythroid
^{o}C	Degrees Celsius
cA.M.P.	Adenosine 3'5' monophosphate
C.F.U. - C.	Colony forming unit - culture
C.F.U. - E.	Colony forming unit - erythroid
C.F.U. - G,E.M.M.	Colony forming unit - granulocyte, erythrocyte, monocyte, megakaryocyte
C.F.U. - S.	Colony forming unit - spleen
cm	Centimetre
C.S.A.	Colony stimulating activity
C.S.F.	Colony stimulating factor
C.S.F. - 1	Colony stimulating factor - 1
c - onc	Cellular oncogene
Ci	Curie
DNA	Deoxyribonucleic acid
DEAE	Diethylaminoethyl
EDTA	Ethylenediaminetetraacetate
G.C.T.	Giant cell tumour
G.- C.S.F.	Granulocyte - colony stimulating factor
G.M.- C.S.F.	Granulocyte, monocyte - colony stimulating factor
g	Gramme
X g	gravitational field
hr	hour
H.P.C.M.	Human placental conditioned medium
K_{av}	Elution parameter (gel filtration)

pH	Minus logarithim of hydrogen ion concentration
pI	Isoelectric point
l	Litre
log	Logarithim
m	Metre
M	Molar
M.W.	Molecular weight
μ	Micro (10^{-6} X)
μg	Microgramme
μl	Microlitre
min	Minute
M. - C.S.F.	Monocyte - colony stimulating factor
p.	Page
pp.	Pages
/	Per
%	Per cent
r.p.m.	Revolutions per minute
Tris	2-amino-2-hydroxymethylpropane -1,3,-diol
V	Volt
v	Volume
V_e	Elution volume
V_o	Void volume
V_t	Total bed volume

TABLES INCLUDED IN THE TEXT

FIGURES INCLUDED IN THE TEXT

GENERAL INTRODUCTION

INTRODUCTION

One of the major challenges in biochemistry is the description of the molecular events responsible for the control of cell proliferation and differentiation. The first detailed description of the various stages of cell division was given by Schneider in 1873. In 1875 Strassburger published the first monograph on cell division and in 1882 Flemming named the complete process karyomitosis(= a threadlike metamorphosis of the nucleus).

We now know that mitosis is governed by other events which take place in the cell cycle.After mitosis (M-phase) the cells undergo a period of growth (G1-phase) followed by a period of DNA synthesis (S-phase). A second period of growth (G2-phase) in which the cells prepare for mitosis follows S-phase. The cells may remain for long periods of time in a resting phase (G0-phase) or they may enter G1-phase and cycle again. For a review see Paweletz and Little (1980).

The study of cell proliferation has been facilitated by the introduction of cell culture techniques (Paul 1972). The growth and maintenance of cells in culture requires the presence of nutrients, usually in the form of a defined tissue culture medium (Waymouth, 1972). Serum is required by most mammalian cell lines in culture. The components of serum necessary for the maintenance and growth of cells *in vitro* have not been fully identified

(Temin *et al*, 1972). However, serum has been replaced by hormones and transferrin to permit the growth of a rat pituitary cell line (Hayashi and Sato, 1976) and the growth of erythroid colonies *in vitro* has been demonstrated in conditions where the serum concentration has been lowered and selenite, transferrin, albumin and lecithin included in the tissue culture medium. (Guilbert and Iscove, 1976). Serum-free cell culture has been developed for a number of cell lines so permitting their growth in a defined medium (Barnes and Sato, 1980a; Barnes and Sato, 1980b).

In the present study serum-free cell culture was developed for the growth and differentiation of chick myocardial cells. The medium consisted of Leibowitz L-15 tissue culture medium with added transferrin, albumin, phosphatidyl choline, glucagon, insulin, somatotropin, sodium pyruvate and sodium selenite.

Macromolecules responsible for the growth and maintenance of cells in culture have been recognised for some time. These growth factors include; nerve growth factor, epidermal growth factor, fibroblast growth factor, multiplication stimulating activity, insulin-like growth factors, platelet-derived growth factor, interleukin-2, interleukin-3, B-cell growth factor, thrombopoietin, erythropoietin and colony - stimulating factor (Gospodarowicz and Moran, 1976; James and Bradshaw, 1984).

Nerve growth factor was first described in 1954 (Cohen et al). This protein stimulates nerve cells to divide and differentiate. Nerve growth factor is present in a large number of tissues but the richest source is the male mouse submaxillary gland. The molecule is present in the submaxillary gland as a complex protein of 130,000 molecular weight and a sub-unit structure of $\alpha_2 \beta \gamma_2$. The β sub-unit possesses all of the biological activity and is composed of two identical polypeptide chains of 13,259 molecular weight. It has been proposed that nerve growth factor binds to plasma membrane receptors and internalisation takes place. The internalised nerve growth factor is thought to be transported to the nucleus where a D.N.A. template is uncovered and gene activity directly influenced. For a review see Bradshaw (1978).

Epidermal Growth factor was isolated from the mouse submaxillary gland during the early studies on nerve growth factor (Cohen, 1959). Epidermal growth factor stimulates the proliferation of epidermal cells in culture. Epidermal growth factor is a polypeptide with a molecular weight of 6,100. Cells responsive to epidermal growth factor possess membrane receptors to which the factor binds. The epidermal growth factor is then internalised. The sequence of events which occur after internalisation of the growth factor is unclear. However epidermal growth factor stimulates the phosphorylation of tyrosine residues of membrane bound proteins in epidermal growth factor acitivated cells. (Ushiro and Cohen, 1980). For a review of epidermal growth

factor see Carpenter and Cohen (1979).

The first haematopoietic growth factor to be recognised was erythropoietin, the stimulator of erythropoiesis (Carnot and Deflandre, 1906) Other haematopoietic growth factors include; interleukin-3 which stimulates the early stem cells, interleukin-2 which stimulates the production of T-lymphocytes, B-cell growth factor, thrombopoietin which stimulates platelet production and the colony-stimulating factors which stimulate the growth of granulocytes, monocytes and eosinophils in culture (Table 1).

The haematopoietic system consists of cells which are continuously dividing and differentiating. These cells are readily accessible for study in the laboratory and haematopoiesis provides a model for the investigation of cell proliferation.Early descriptions of haematopoiesis relied on morphological data obtained by observing Romanowsky stained preparations by light microscopy. There are five main cell lineages; lymphoid, erythroid, myeloid, megakaryocytic and monocytic.In the normal adult each cell lineage consists of a morphologically defined set of cells found in the bone marrow.The earliest recogniseable cell in any cell lineage pathway is the blast. Given the appropriate stimulus the blast divides and differentiates to give rise to the mature functioning cell(Figure 1).

Haematopoietic cells are thought to arise from stem

cells which have the ability to divide and differentiate into cells of any series. Evidence for the existence of these pluripotent stem cells was first obtained in mice where radiation damaged chromosomes, used as markers, were recognised in all cell lineages including the lymphoid series (Barnes et al .,1952). Additional evidence was provided by the use of isoenzymes of glucose-6-phosphate dehydrogenase as markers.These studies indicated that erythocytes, granulocytes and lymphocytes had a common ancestor (Gandini and Gartler, 1969; Fialkow, 1979). The presence (Lala and Johnson, 1978) and the absence (Paige et al , 1979) of chromosomally marked progenitors of B-lymphocytes in splenic colonies indicated that separate stem cells existed for lymphoid and myeloid cells. The Philadelphia chromosome is present in the cells of the erythroid, granulocytic, monocytic, and megakaryocytic cells but is absent in the lymphoid cells of chronic granulocytic leukaemia. This finding, again, suggests that separate stem cells are present for lymphoid and myeloid cells (Figure 2).

The study of early cells in haematopoiesis has been facilitated by the introduction of clonal culture techniques. In 1961 Till and McCulloch reported the assay of haematopoietic stem cells by the mouse spleen colony technique. In this assay haematopoietic cells introduced into a lethally irradiated mouse gave rise to macrosocopic colonies on the spleen of the mouse. These colonies may be erythroid, granulocytic or megakaryocytic and have been shown to be derived from a single cell. This pluripotent

stem cell is known as a colony-forming unit-spleen (CFU-S).

The culture of granulocyte and macrophage colonies in the semi-solid agar technique was described independently by two groups of workers (Ichikawa *et al*, 1966, Bradley and Metcalfe, 1966). In this system, stem cells, called colony-forming units-culture (CFU-C), give rise to microscopic colonies of 50 or more granulocytic and/or macrophage cells. A growth factor, called colony-stimulating factor (C.S.F.) by Bradley and Metcalf (1966) and macrophage granulocyte inducer (M.G.I.) or mashran gm by Ichikawa *et al*. (1966), stimulates the growth and differentiation of these colonies. The most widely used name for this factor is colony-stimulating factor or C.S.F. Colony-stimulating activity or C.S.A. is a term generally reserved for impure material which stimulates the formation of colonies. C.S.F. is a glycoprotien of molecular weight 20,000-80,000 present in urine, serum and media conditioned by a number of cell types. Two murine C.S.F's, with different biological and physico-chemical properties, have been extensivly purified. One of these factors termed GM-CSF has been isolated from mouse lung-conditioned medium and stimulates the formation of granulocyte and/or macrophage colonies in vitro (Burgess et al., 1977a). The other factor, isolated from L-cell conditioned medium and termed M-CSF, stimulates the production of macrophage colonies in vitro (Stanley and Heard, 1977).

One of the best characterised C.S.A.'s acting on human bone morrow cells is that obtained from human placental conditioned medium (Burgess et al, 1977b ; Nicola et al ., 1978). Human placental conditioned medium (H.P.C.M.) contains at least three different colony stimulating factors separable by phenyl-sepharose hydrophobic interaction chromatography. One of these factors, designated EO-CSF, stimulates the formation of eosinophil colonies, a second, designated GM-CSFα, stimulates the formation of granulocyte and macrophage colonies by day 14 of culture, and a third, designated GM-CSFβ, stimulates the formation of granulocyte and macrophage colonies by day 7 of culture. GM-CSFβ is completely separated from EO-CSF and GM-CSFα by phenyl-sepharose hydrophobic interaction chromatography (Nicola et al., 1979).

Many sources of C.S.F. have been described.Recently, Abboud et al ., (1981) described colony stimulating factors present in giant cell tumour conditioned medium (G.C.T.). They reported that this source may contain C.S.F.'s similar to those found in human placental conditioned medium. The sources of a selection of colony stimulating factors are listed in Table 2.

Colony-stimulating factor is assayed by the semi-solid agar culture technique (Metcalf, 1977). This bioassay has been difficult to standardise since no suitable stable standard is currently available. The results of one laboratory are often obtained using different assay

conditions from those of another laboratory.

In the present study GM-CSFβ from human placental conditioned medium has been purified by calcium phosphate gel absorption, ultrafiltration, phenyl-sepharose chromatography and gel filtration on Sephacryl S-200 superfine to yield material with a specific activity of 7240 colonies/mg. In order to investigate the feasability of estabilishing a radioimmunoassay for this C.S.F. the purified material was radioiodinated and the binding of this radioiodinated GM-CSFβ to an antiserum raised against a less pure preparation has been studied. A radioimmunoassay could provide a more convenient and more specific means of measuring GM-CSFβ levels in biological fluids.

Since Abboud et al., 1981 reported that giant cell tumour conditioned medium contained C.S.F.'s similar to those found in human placental conditioned medium, colony stimulating factors were isolated from G.C.T. in the present study. C.S.F. was purified from giant cell conditioned medium by ultrafiltration, phenyl-sepharose chromatography, gel filtration and thiolpropyl-sepharose chromatography to give a preparation with a specific activity of 4000 colonies/mg. A minor peak which appeared to have high specific activity was resolved on gel filtration. However the insensitivity of the protein assays used in this study made determination of specific activity, of the material in this peak, impossible.

In order to study the binding of C.S.F. to target cell membranes, C.S.F. from pokeweed mitogen stimulated spleen conditioned medium was labelled with horseradish peroxidase. The labelled growth factor was observed to bind isolated haematopoietic cell membranes and the binding exhibited a linear relationship with the membrane protein content.

An imbalance in regulator control can lead to, or permit, the establishment of cancer (Furth, 1953) . In order to clarify the role of haematopoietic growth factors and their cell receptors in leukaemia two lines of investigation may be employed. First, plasma levels of GM-CSF could be measured using the radioimmunoassay and secondly, the binding of radioiodinated GM-CSF to leukaemic cells could be studied. These studies could estabilish if leukaemia is due to an upset in regulator levels or to abnormal receptors for haematopoietic growth factors.

Figure 1: A diagram showing cell lineage pathways in haematopoiesis.

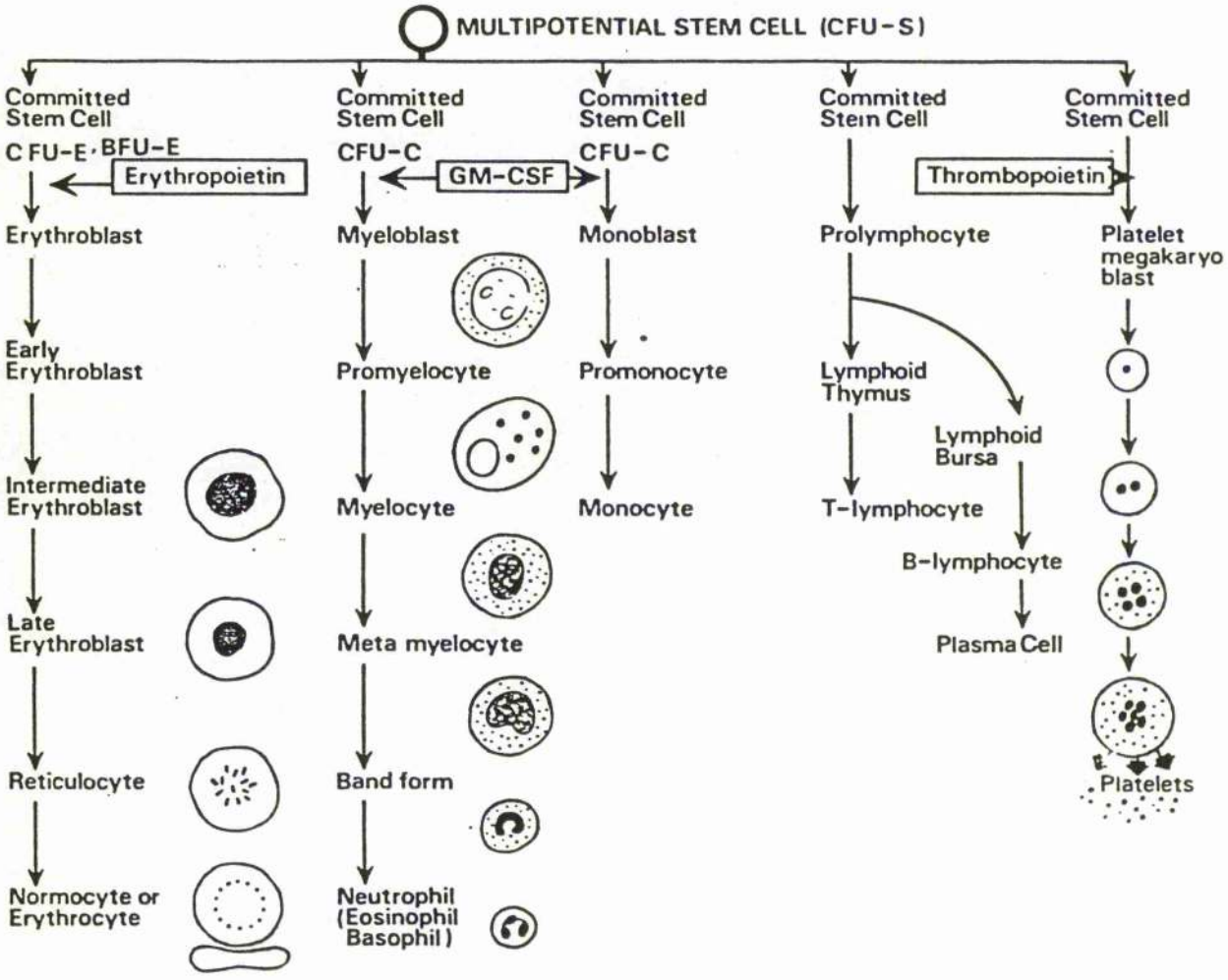

MULTIPOTENTIAL STEM CELL (CFU-S)
Committed Stem Cell
C FU-E · BFU-E
Erythropoietin
Erythroblast
Early Erythroblast
Intermediate Erythroblast
Late Erythroblast
Reticulocyte
Normocyte or Erythrocyte
Committed Stem Cell
CFU-C
GM-CSF
Myeloblast
Promyelocyte
Myelocyte
Meta myelocyte
Band form
Neutrophil (Eosinophil Basophil)
Committed Stem Cell
CFU-C
Monoblast
Promonocyte
Monocyte
Committed Stem Cell
Prolymphocyte
Lymphoid Thymus
T-lymphocyte
Lymphoid Bursa
B-lymphocyte
Plasma Cell
Committed Stem Cell
Thrombopoietin
Platelet megakaryo blast
Platelets

Figure 1

Figure 2: The inter-relationship of early progenitor cells in haematopoiesis.

CFU-S = colony-stimulating unit-spleen.

CFU-GEMM = -granulocyte, erythrocyte, monocyte, myeolcytic.

CFU-C = colony forming unit-culture.

BFU-E = burst forming unit-erythroid.

CFU-E = colony forming unit-erythroid.

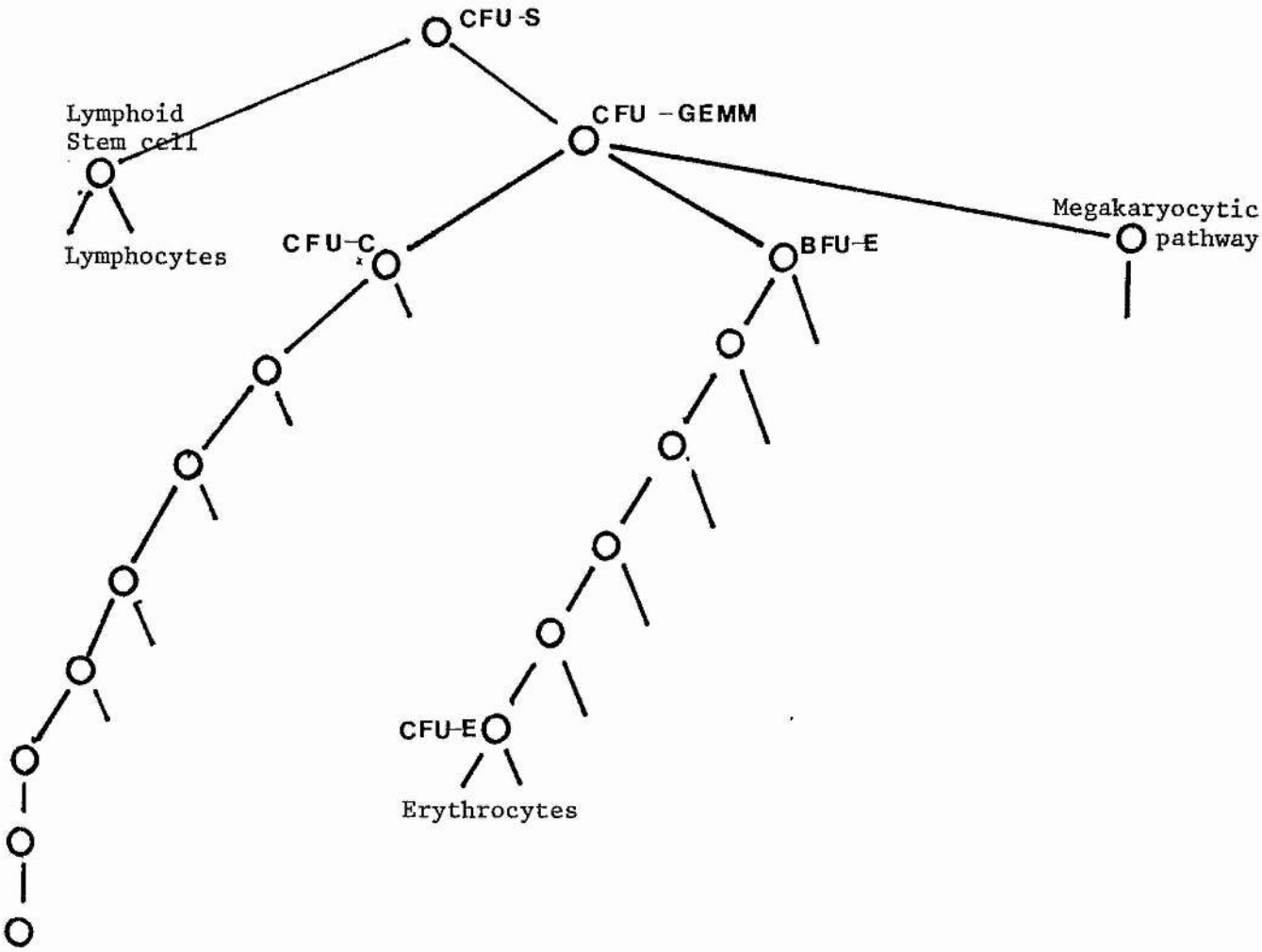

CFU-S
Lymphoid
Stem cell
CFU - GEMM
Lymphocytes
CFU-C
BFU-E
Megakaryocytic
pathway
CFU-E
Erythrocytes

Figure 2

Table 1: Growth Factors in Haematopoiesis.

Growth Factors in Haematopoiesis

Factor	Cell Type	Author
Erythropoietin	Red Blood Cells	Carnot and Deflandre (1906)
Thrombopoietin	Platelets	Abildaard and Simmone (1967)
Thymopoietin	T-lymphocytes	Goldstein (1974)
Interleukin - 2	T-lymphocytes	Gillis and Smith (1977)
Interleukin - 3	Stem cells	Fung et al. (1984)
GM-CSF	Granulocytes and monocytes	Pluznik and Sachs (1966) Bradley and Metcalf (1966)
G-CSF	Granulocytes	Nicola et al. (1983)
M-CSF	Monocytes	Stanley and Heard (1977)
B-cell growth factor	B-lymphocytes	Thoman and Weigle (1983)

Table 1

A BIOCHEMICAL STUDY OF POLYPEPTIDE GROWTH FACTORS RESPONSIBLE FOR THE GROWTH OF HAEMATOPOIETIC CELLS IN CULTURE

INTRODUCTION

INTRODUCTION

Colony-stimulating factor (CSF) is thought to occur as several molecular species which differ in their target cell specificities and in their physicochemical properities. Two distinct murine C.S.F.'s have been extensively purified. One of these C.S.F.'s, the C.S.F. from L - cell conditioned medium, induces monocyte or macrophage colony formation in the murine semi-solid agar culture technique, has an apparent molecular weight of 70,000 and is thought to consist of two similarly charged polypeptide chains, of molecular weight 35,000, held together by disulphide bonds. (Stanley and Heard, 1977). The second murine CSF, a CSF purified from mouse lung - conditioned medium , induces granulocyte, monocyte, macrophage and mixed granulocyte/macrophage colonies in the culture of murine bone marrow cells in the semi-solid agar technique. This CSF is reported as having an apparent molecular weight of 29,000 (Burgess et al , 1977a).

Recently, the gene encoding a murine G.M.-C.S.F. from lung tissue has been cloned, the D.N.A. sequenced and the amino acid sequence deduced (Gough et al.,1984). The deduced amino acid sequence is given in Figure 3.

One of the few human C.S.F's. to be extensively purified is that from human urine. This C.S.F. has a molecular weight of between 40,000 and 85,000. Human urinary C.S.F. induces the formation of murine haematopoietic cell colonies in the semi-solid agar culture

technique (Stanley et al., 1975). Evidence for the induction of human haematopoietic colonies by human urinary C.S.F. is unclear. One group of workers (Motoyoshi et al, 1978) has reported that human urinary C.S.F. induces colony formation in human cultures whereas a second laboratory (Das et al., 1981) has reported only cluster formation in the culture of human bone marrow cells. The colony-stimulating activity from human urine may act indirectly on haematopoietic stem cells, by stimulating the release of colony-stimulating factors from bone marrow monocytes in the semisolid agar culture (Motoyoshi et al., 1982).

Recently Das et al (1981) reported the radioimmunoassay of a C.S.F. which had been extensively purified from human urine. This C.S.F. designated C.S.F.-1 (Stanley, 1979; Das et al, 1980) stimulated the proliferation of murine macrophage cells in vitro. However the role of human urinary C.S.F. in vivo and in the clonal culture of human bone marrow cells remains unclear.

A convenient source of human active C.S.F. is human placental conditioned medium (H.P.C.M.). (Burgess et al., 1977b). This conditioned medium contains at least three different C.S.F.'s which are separable by hydrophobic interaction chromatography. One of these factors, GM-CSF-α, stimulates the formation of granulocyte and macrophage colonies by day 14 of culture; a second factor, designated

GM-CSFβ stimulates the formation of granulocyte and macrophage colonies by day of culture; and a third, designated EO-CSF, stimulates the formation of eosinophil colonies *in vitro*. GM-CSFα and EO-CSF elute in the breakthrough volume from phenyl - sepharose columns which have been equilibrated with 0.03M Tris-HCl buffer pH 7.4, containing 1M NaCl and 0.01 % (w/v) sodium azide. Separation of GM-CSFα and EO-CSF is not possible using this technique. GM-CSFβ is eluted from the columns by including 50% (v/v) ethylene glycol in the equilibrating buffer (Nicola *et al* 1979).

Growth Factors are present in biological fluids in low concentrations. These molecules, therefore, require sensitive techniques for their quantitation. Competitive protein binding assays are highly sensitive and may overcome many of the problems inherent in bioassays. The first competitive protein binding assay was developed by Yalow and Berson in the late 1950's. They noticed that the binding of 131-I-labelled insulin to antibodies which occured in diabetic patients who had received injections of insulin was inhibited by unlabelled insulin. Moreover, recognition that the amount of labelled insulin bound to the antibodies was a quantitative function of the amount of unlabelled insulin added to the reaction mixture when the concentration of antibody was held constant, led to the introduction of a competitive protein binding assay for insulin. (Berson and Yalow, 1958).

In competitive protein binding assays a fixed amount of labelled ligand is incubated in the presence of a known amount of binding protein and an unknown amount of unlabelled ligand. The unlabelled ligand competes with the labelled ligand for sites on the binding protein. The bound material is then separated from the free material and the amount of bound labelled ligand measured. The amount of labelled ligand bound to the protein is inversely proportional to the amount of unlabelled ligand added to the reaction mixture.

The most widely used form of competitive protein binding assay is radioimmunoassay. Radioimmunoassay utilises a radioactively labelled ligand and antibodies as the binding protein. A related technique, the immunoradiometric assay, was developed by Miles and Hales (1968). In this technique the substance to be measured is directly assayed by combination with labelled antibody.

Although antibodies are the most widely used binding proteins, any protein which can bind the molecule to be assayed may be employed. Steriod transport proteins may be used to assay steriod hormones and intrinsic factor may be used to assay Vitamin B12 (Wide and Killander, 1971). Plasma membrane receptors have been used as binding proteins in the assay of a number of polypeptide hormones and this technique is known as radioreceptor assay (Lefkowitz *et al*, 1970). Radioreceptor assays recognise the biologically active site rather than the immunoreactive

site, have high association constants and are more easily obtained than antibodies.

Alternatives to radionuclides have been sought in recent years. Radiolabelled ligands have a relatively short shelf life, require specialised counting facilities and are potentially hazardous to both laboratory workers and the environment. Many candidates have been proposed as alternatives to radionuclides. These include; viruses (Haimovitch et al., 1970) ; metals, (Cais et al., 1977) ; chemiluminescense precursors (Pratt et al., 1978) ; stable free radicals (Leute et al., 1972), and fluorochromes, (Soini and Hemmila, 1979). At present the most promising labels are enzymes, or enzyme inhibitors, cofactors or substrates (Voller et al., 1978).

The aims of the present study are ; to compare colony-stimulating factors present in different conditioned media ; to investigate the feasiblity of a radioimmunoassay for colony-stimulating factor as an alternative to the time-consuming bioassay and to investigate the mechanism of action of colony stimulating factor by studying the binding of labelled colony-stimulating factor to plasma membranes from bone marrow cells.

GM-CSFβ from human placental conditioned medium was purified by calcium phosphate gel absorption, ultrafiltration, phenyl-sepharose chromatography and gel filtration on Sephacryl S-200 superfine to a specific

activity of 7240 colonies/mg. This material gave a single precipitin arc on immunoelectrophoresis. In order to investigate the feasability of estabilishing a radioimmunoassay for this C.S.F. the purified material was radioiodinated and the binding of this radioiodinated GM-CSFβ to an antiserum raised against a less pure preparation has been studied. A radioimmunoassay is a more convenient and more specific means of measuring GM-CSF levels, in biological fluids, than the conventional bioassay.

Abboud et al . (1981) reported that giant cell tumour conditioned medium contains colony stimulating factors similar to those found in human placental conditioned medium. In order to compare the colony-stimulating factors present in giant cell conditioned medium with those present in human placental conditioned medium , C.S.F. was purified from giant cell tumour conditioned medium by ultrafiltration, phenyl-sepharose chromatography, gel filtration and thiolpropyl-sepharose chromatography to give a preparation with a specific activity of at least 4000 colonies/mg. A minor peak which appeared to have high specific activity was resolved on gel filtration. However the insensitivity of the protein assays used in the present study made determination of specific activity impossible.

In order to study the binding of C.S.F. to target cell membranes, C.S.F. from pokeweed mitogen stimulated spleen

conditioned medium was labelled with horseradish peroxidase. The labelled growth factor was observed to bind isolated haematopoietic cell membranes and this binding exhibited a linear relationship with the membrane protein content.

Table 2: Some biological sources of colony-stimulating activity.

Colony - Stimulating Factors

CSF	Source	Colony Cell Type	Species Activity	Reference
CSF-1	Human urine	Macrophage	Murine	Stanley et al. (1978)
CSF-1	Mouse L-cells	Macrophage	Murine	Stanley & Heard (1977)
GM-CSF	Mouse lung	Granulocyte Macrophage	Murine	Burgess et al. (1977a)
G-CSF	Mouse WEHI-3 cells	Granulocyte	Murine	Nicola et al. (1983)
	Human placenta CM	Granulocyte	Human	Nicola et al. (1985)
GM-CSFα	Human placenta CM	Granulocyte Macrophage	Human, Murine	Burgess et al. (1977b)
EO-CSFα	Human placenta CM	Eosinophil	Human	Burgess et al. (1977b)
GM-CSFβ	Human placenta CM	Granulocyte	Human, Murine	Nicola et al. (1985)
GM-CSF	Mo-cell CM	Granulocyte Macrophage	Human	Golde et al. (1978)
GM-CSF	GCT-CM	Granulocyte Macrophage Eosinophil	Human	Abboud et al. (1981)

Table 2

Figure 3: The amino acid sequence of colony-stimulating factor from mouse lung. This sequence was deduced from the nucleotide sequence of a cloned gene. (Gough et al., 1984).

Ile - Ile - Val - Thr - Arg - Pro - Trp - Lys - His - Val -

Glu - Ala - Ile - Lys - Glu - Ala - Leu - Asn - Leu - Leu -

Asp - Asp - Met - Pro - Val - Thr - Leu - Asn - Glu - Glu -

Val - Glu - Val - Val - Ser - Asn - Glu - Phe - Ser - Phe -

Lys - Lys - Leu - Thr - Cys - Val - Gln - Thr - Arg - Leu -

Lys - Ile - Phe - Glu - Gln - Gly - Leu - Arg - Gly - Asn -

Phe - Thr - Lys - Leu - Lys - Gly - Ala - Leu - Asn - Met -

Thr - Ala - Ser - Tyr - Tyr - Gln - Thr - Tyr - Cys - Pro -

Pro - Thr - Pro - Glu - Thr - Asp - Cys - Glu - Thr - Gln -

Val - Thr - Thr - Tyr - Ala - Asp - Phe - Ile - Asp - Ser -

Leu - Lys - Thr - Phe - Leu - Thr - Asp - Ile - Pro - Phe -

Glu - Cys - Lys - Lys - Pro - Ser - Gln - Lys

Figure 3

MATERIALS AND METHODS

MATERIALS AND METHODS

All materials used in the present study were of ANALAR grade and were obtained from B.D.H. Ltd., Poole, England, unless otherwise stated.

PREPARATION OF HUMAN PLACENTAL CONDITIONED MEDIUM

Human placentas were obtained from Caesarean births. The placentas were placed in a sterile plastic bag which was then knotted, stored at 4°C and used within 12 hr of delivery.

The outer membranes were removed from the placentas. The placental tissue was cut into $1cm^3$ pieces. The pieces of placenta were washed (x4) in Hank's balanced salt solution. Six pieces of placenta were placed in a flat bottomed tissue culture flask containing 20ml R.P.M.I.-1640 containing 5% (v/v) foetal calf serum. The flasks were incubated at 37°C for 7 days in a fully humidified atmosphere of 10% CO_2 in air. The conditioned medium was filtered through a double layer of cotton gauze and centrifuged in a M.S.E. Hi-speed centrifuge (M.S.E.,Crawley,England) at 10,000 g for 20 minutes. The supernatant was stored at -70°C until used.

CALCIUM PHOSPHATE GEL ABSORPTION OF HUMAN PLACENTAL CONDITIONED MEDIUM

Human placental conditioned medium (10l) was dialysed against 0.01M sodium phosphate buffer, pH 6.8 (50l).Calcium phosphate gel (4l) was added to the dialysed human placental conditioned medium and the mixture stirred. The calcium phosphate gel was allowed to sediment and the supernatant retained. Sodium phosphate buffer (0.05M), pH 6.8, was added to the calcium phosphate gel, the mixture stirred, allowed to sediment and the supernatant pooled with the supernatant from the first elution. The supernatant was concentrated 200 fold by ultrafiltration in a Amicon DC10 apparatus with an H-10 hollow fibre cartridge. The concentrated eluate was stored at -70°C until used.

PHENYL-SEPHAROSE HYDROPHOBIC INTERACTION CHROMATOGRAPHY OF HUMAN PLACENTAL CONDITIONED MEDIUM

Phenyl-Sepharose CL-4B was packed into a 2.6cm x 13cm glass chromatography column (L.K.B., Ltd., Bromma, Sweden) at a flow rate of 18 ml/hr and equilibrated with 500 ml 0.03M Tris-HCl buffer, pH 7.4, containing 1M sodium chloride.

A sample of human placental conditioned medium in 0.03M Tris-HCl buffer, pH 7.4, containing 1M sodium cholride was applied to the column and eluted with the equilibrating buffer. The elution was continued at a flow

rate of 18 ml/hr and the absorbance constantly monitored using an ISCO UA-4 absorbance monitor (Instrument Specialities Corporation, Lincoln, Nebraska, U.S.A.) at 280 nm. When the absorbance had decreased to baseline level the elution buffer was changed to 50% (v/v) ethylene glycol in 0.03M Tris-HCl buffer, pH 7.4, containing 1M sodium chloride.

PROTEIN DETERMINATION

Proteins were determined by a dye binding technique (Bradford, 1976). Briefly 1 ml of a solution of 0.1% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol and 8.5% (w/v) phosphoric acid was added to a semi -micro cuvette containing 0.1 ml of the solution to be assayed. The contents of the cuvette were mixed by inversion and the absorbance read at 595 nm. A calibration curve was constructed using bovine serum albumin at concentrations of 10-100 µg/ml.

BIOASSAY FOR COLONY-STIMULATING FACTOR

Colony-stimulating factor was assayed by a modification of the method of Stanley *et al* (1972). The material to be assayed was made 0.1% (w/v) with respect to bovine serum albumin (Miles Laboratories,Nashville,TN., U.S.A) and dialysed against 0.02M potassium phosphate buffer, pH 7.3, containing 0.15M sodium chloride. This material was Millipore filtered (0.45µ pore size) and

serially diluted 1:4. An aliquot (0.1ml) of each dilution (including the undiluted material) was added, in triplicate, to 35mm petri dishes (Nunc,Denmark). 7.5×10^4 bone marrow cells from the femours of three-month old C57BL/6 mice (Bainton and Kingham, Hull, England) in 1ml Iscove's modification of Dulbecco's modification of Eagle's minimum essential medium (Flow Laboratories, Irvine, Scotland) containing 15% (v/v) foetal calf serum (Flow Laboratories, Irvine, Scotland) L-asparagine and D.E.A.E. - Dextran (Pharmacia, Uppsala, Sweden) were added to each petri dish. The petri dishes were placed in a sealed container containing 10% CO_2 in humidified air and incubated at 37°C for 7 days. The number of colonies consisting of 40 or more cells were counted using an Olympus dissecting microscope at x 40 magnification. The biological activity of each assay was expressed as the mean of the triplicate determinations. Figure 4 outlines the steps used in the bioassay of colony stimulating activity.

ULTRAFILTRATION AND HYDROPHOBIC-INTERACTION CHROMATOGRAPHY OF GIANT CELL TUMOUR (G.C.T.) CONDITIONED MEDIUM

The procedures used to prepare G.C.T.- conditioned medium were essentially those of Abboud *et al*., (1981). G.C.T. conditioned medium was otained from GIBCO, Paisley, Scotland, and concentrated 5-fold by ultrafiltration (Amicon) using a PM-10 membrane. The concentrated G.C.T. conditioned medium was dialysed against 0.03 M Tris-HCL

buffer, pH 7.4 containing 0.02% (w/v) sodium azide and 0.01% (w/v) polyethlene glycol 6000. This solution was designated G.C.T. - stage I. An aliquot (15ml) of this material was applied to a 2.6cm x 16.0 cm glass chromatography column (L.K.B., Bromma, Sweden) of phenyl-sepharose 4B (Pharmacia, Uppsala, Sweden) at a flow rate of 12ml/hr. Elution was continued using 0.03 M Tris-HCL buffer, pH 7.4 containing 0.02% (w/v) sodium azide and 0.01% (w/v) polyethylene glycol 6000. Fractions (3ml) were collected and the elution monitored by measuring the absorbance at 280nm (Pye-Unicam, Cambridge, England) and by bioassay. Active fractions were pooled, concentrated 10 fold using polyethylene glycol 6000, the biological activity measured and the active fractions stored at -70°C until used. The concentrated material was designated G.C.T.-stage II.

Colony-stimulating activity which bound to the phenyl-sepharose 4B was eluted with 50% (v/v) ethylene glycol in 0.03 M Tris-HCL, pH 7.4 containing 0.02% (w/v) sodium azide and 0.01% (w/v) polyethylene glycol 6000. Fractions containing colony-stimulating activity were pooled and stored at -70°C until used.

GEL PERMEATION CHROMATOGRAPHY OF G.C.T.-STAGE II

A 2.6cm x 66.0cm glass chromatography column was packed with Sephacryl S-200 (Superfine grade; Pharmacia Ltd., Uppsala, Sweden) and equilibrated with 0.03 M

Tris-HCL buffer, pH 7.4, containing 0.5 M NaCl, 0.02% (w/v) sodium azide and 0.01% (w/v) polyethylene glycol 6000 at a flow rate of 18ml/hr. A 0.1% (w/v) solution of Blue Dextran 2000, molecular weight 2 x 10 , was applied to the column to determine the void volume (Vo). Calibration proteins; ribonuclease A (molecular weight 25,000), ovalbumin (molecular weight 43,000 and bovine serum albummin (molecular weight 67,000), were applied to the column at a flow rate of 18ml/hr. The absorbance of each fraction was measured at 280nm using a U.V.-visible spectrophotometer (Pye-Unicam, Cambridge, England) and the elution volume (Ve) of each protein recorded. The data were plotted on a graph of Kav against log molecular weight where;

$$Kav = \frac{V - Vo}{Vt - VO}$$

and Ve = elution volume of protein

Vo = void volume of column

Vt = total bed volume of column.

An aliquot (2ml) of either G.C.T.-stage II or G.M.-C.S.F from human placental conditioned medium was applied to the column. Fractions (3ml) were collected and their absorbance measured at 280nm (Pye-Unicam, Cambridge, England). The colony-stimulating activity of each fraction was assayed and active fractions pooled and stored at -70°C until used. The G.C.T. material fractionated by this technique was designated G.C.T.-stage III.

THIOLPROPYL-SEPHAROSE 6B CHROMATOGRAPHY OF G.C.T.-STAGE III

A 0.9cm x 15cm glass chromatography column (Pharmacia, Uppsala, Sweden) was packed with thiolpropyl-sepharose 6B (Pharmacia, Uppsala, Sweden) and equilibrated with 0.03 M Tris-HCl buffer pH 7.4, containing 0.5 M NaCl, 0.02% (w/v) sodium azide and 0.01% (w/v) polyethylene glycol 6000. An aliquot (4ml) of G.C.T.-stage III was dialysed against 0.03 M Tris-HCL buffer pH 7.4, containing 0.5 M NaCL, 0.02% (w/v) sodium azide and 0.01% (w/v) polyethylene glycol 6000 and applied to the column. The column was eluted with the equilibrating buffer at a flow rate of 8ml/hr and 3ml fractions collected. The fractions were monitored by measuring the protein content (Bradford, 1976) and by assaying for colony-stimulating activity. Those fractions containing colony-stimulating activity were pooled and designated G.C.T. stage IVA. When the breakthrough peak had eluted, the proteins bound to the gel were eluted with the equilibrating buffer containing 25mM cysteine. This

material was designated G.C.T.-stage IVB. An aliquot (2ml) of G.C.T. stage IV A was applied to the gel permeation chromatrography column to verify the molecular weight of the protein.

RAISING OF ANTIBODIES TO COLONY-STIMULATING FACTOR FROM HUMAN PLACENTAL CONDITIONED MEDIUM

Colony-stimulating factor (0.7mg H.P.C.M. IIb) in 1ml phosphate buffered saline (Oxoid Ltd., England) and 1ml of Freund's complete adjuvant (Miles Laboratories Ltd., Nashville, TN., U.S.A.) were mixed by ultrasonication (M.S.E. Crawley, England) to form an emulsin. The emulsin was injected subcutaneously into either a New Zealand white rabbit or a Sandymop rabbit at multiple sites. Four weeks later, 0.7mg of colony-stimulating factor in 1ml of phosphate buffered saline and 1ml incomplete Freund's adjuvant (Miles Laboratories Ltd., Nashville, TN., U.S.A.) were mixed by ultrasonication and injected subcutaneously into the same rabbit. After a further two weeks a sample of blood was collected in a glass centrifuge tube and allowed to clot overnight at 4°C. The blood was centrifuged at 500 x g for 10 minutes and the serum aspirated and stored at -70°C until required. Booster injections of colony-stimulating factor in incomplete Fruend's adjuvant were given at appropriate intervals.

In some experiments the immunoglobulins in the rabbit

serum were precipitated using 50% (w/v) ammonium sulphate (B.D.H. Chemicals Ltd., Poole, England) which had been adjusted to pH7.0 using 6M ammonium hydroxide. The precipitated proteins were collected by centrifugation at 10,000 x g for 10 minutes in a M.S.E. Hi-speed 18 centrifuge (M.S.E. Crawley, England) using a 8 x 50ml rotor. The proteins were reconstituted in either 10ml of 0.12M Tris-HCL buffer pH 7.5, containing 1M sodium chloride, 0.01% (w/v) sodium azide and 0.01% (w/v) polyethylene glycol or in phosphate buffered saline containing 0.02% (w/v) sodium azide. The proteim concentration of each antibody preparation was measured by the dye binding technique of Bradford (1976).

The ability of the antiserum to inhibit colony formation was investigated by adding serial dilutions of the antiserum to the bioassay of C.S.F.

IMMUNODIFFUSION

Two dimensional immunodiffusion was performed by the method described by Ouchterlony and Nilsson (1978). Agarose (1.5gm HGT grade, Miles Laboratories Ltd., Nashville, TN., U.S.A.) was added to 100 ml phosphate buffered saline and dissolved by heating in a boiling water bath. The molten agarose was poured onto a plastic immunodiffusion plate (Miles Laboratories Ltd., Nashville, TN., U.S.A.) and allowed to solidify at room temperature. Holes (5mm diameter) were punched in the agarose using a template and

vacuum pump operated gel punch (Miles Laboratories Ltd., Nashville, TN., U.S.A.) Appropriate dilutions of the various C.S.F. preparations were placed in the central wells and appropriate dilutions of anti-CSF placed in the outer wells. In some experiments antibody was placed in the central well and antigen in the outer wells. The immunodiffusion plates were incubated at room temperature overnight and examined for precipitin lines. Unprecipitated proteins were eluted from the gel by incubating in 2% (w/v) NaCl and the precipitin lines stained by the Coomassie Blue technique described.

IMMUNOELECTROPHORESIS

Immunoelectrophoresis was performed using the technique described by Grabar and Williams (1953). Briefly, 1.5g agarose (H.G.T. grade, Miles Laboratories Ltd., Nashville, TN., U.S.A.) were added to 100 ml 0.003M 5',5' Diethylbarbituric acid/0.017M sodium 5',5' diethylbarbiturate, pH 8.6 containing 0.051% (w/v) calcium lactate dissolved by heating in a boiling water bath. 20ml of the molten agarose were poured onto a 8.0cm x20.0cm glass plate held on a levelling table. The gel was allowed to solidify at room temperature and holes punched using a Grabar and Williams immuno-template. The holes were filled with various C.S.F. preparations and electrophoresis performed at 40 mA for 90 minutes in 0.0075M 5',5' Diethylbarbituric acid/0.042M sodium 5',5' diethylbarbiturate containing 0.083% (w/v) calcium lactate.

The power was switched off, the gel removed and placed on the Grabar and Williams template. A central trough was cut using the template and 50µl anti-CSF placed in each trough. Precipitin arcs were allowed to develop by incubating the gels overnight at room temperature in a humid chamber. Unprecipitated proteins were eluted by incubating the gels in 2% (w/v) NaCl for 2 hours and the precipitin lines stained by the Coomassie Blue technique described. All apparatus was from Shandon Southern Ltd., Runcorn, England.

COOMASSIE BLUE R-250 STAINING

Proteins in immunodiffusion and immunoelectrophoresis gels were stained using Coomassie Blue R-250. The gels to be stained were incubated for 2-24 hours at room temperature in a staining solution comprising 0.2% (w/v) Coomassie Blue R-250 (Sigma Chemical Co., St. Louis, MO., U.S.A.) in 95% (v/v) ethanol to which an equal volume of 20% (w/v) glacial acetic acid had been added immediately before use. The gels were destained in a solution of 400 ml ethanol and 600 ml 5% (v/v) acetic acid overnight.

RADIOIODINATION OF COLONY-STIMULATING FACTOR

GM-CSFβ was radioiodinated by a modification of the chloramine-T method of Greenwood *et al* (1963). A 10µCi aliquot of Na-125-I solution (Amersham International P.L.C., Amersham, England) was dispensed into a polystyrene

tube (B.C.L., Lewes, England). 10µl of 0.25M sodium phosphate buffer, pH 7.5 containing 1.875M dimethyl sulphoxide and 0.01% (w/v) polyethylene glycol 6000 were added and the following added in rapid succession whilst the reaction mixture was continuously stirred; 10µl (0.1 mg/ml) of GM-CSF ; 10µl (50ug) of chloramine-T in 0.05M sodium phosphate buffer, pH 7.5; 100µl (120ug) of sodium metabisulphite in 0.05 M sodium phosphate buffer, pH 7.5. All solutions contained 1.875M dimethyl sulphoxide and 0.01% (w/v) polyethylene glycol 6000. The volume of the solution was adjusted to 1.0ml using 0.2% (w/v) sodium iodide in 0.05 M sodium phosphate buffer, pH 7.5, containing 2% (v/v) horse serum (Flow Laboratories, Irvine, Scotland).

Sephadex G-25 (medium grade, Pharmacia, Uppsala, Sweden) in 0.25 M sodium phosphate buffer, pH 7.5 containing 2% (v/v) horse serum and 0.01% (w/v) sodium azide was packed in a 0.9 cm x 12 cm chromatography column (Pharmacia, Uppsala, Sweden) and equilibrated with the same buffer. The iodination mixture was applied to the column at a flow rate of 18ml/hr and eluted with 0.25M sodium phosphate buffer, pH 7.5, containing 2% (v/v) horse serum and 0.01% (w/v) sodium azide. Fractions (2ml) were collected in polystyrene tubes and the radioactivity of each fraction counted using a Wilj 2101 gammacounter. (Wilj International Ltd., Ashford, England).

BINDING OF RADIOIODINATED GM-CSF TO ANTI-C.S.F.

Aliquots (0.1ml) of serial dilutions (10, 10^2, 10^3, 10^4, 10^5) of anti-CSF and GM-CSFβ were added to each of a series of test tubes. The mixtures were incubated for one hour at 37°C and 0.1ml of 125-I-GM-CSFβ were added to the incubaton mixtures which were further incubated at 37°C for two hours. The incubation mixtures were then transferred to 4°C and incubated for 18-144 hr. On completion of this imcubation 0.1ml of 0.1% (w/v) E.D.T.A., disodium salt and 0.1ml of an appropriate dilution of goat anti-rabbit IgG (Miles Laboratories, Nashville, TN. U.S.A.) were added according to the instructions supplied by the manufacturer of the second antibody. The samples were mixed and incubated at 4°C for 18 hr. An aliquot (1.0ml) of 0.1 M Tris-HCl buffer, pH 7.5 containing 1 M sodium chloride, 0.01% (w/v) sodium azide and 0.01% (w/v) polyethylene glycol 6000 was added and the mixtures were centrifuged at 500 x g for 15 minutes. The supernatant was carefully aspirated and each precipitate was counted using a Wilj 2101 gammacounter (Wilj International Ltd., Ashford, England).

PREPARATION OF PROTEIN-ENZYME CONJUGATES

Glucagon and C.S.F. were labelled with horseradish peroxidase (donor: hydrogen-peroxide oxidoreductase; E.C. 1.11.1.7, type X, Sigma Chemical Co., St. Louis, MO., U.S.A.) Briefly, 1mg of the protein to be labelled and 5mg

enzyme were dissolved in 2ml 0.04M sodium phosphate buffer pH 7.4, containing 2.5% (v/v) glutaraldehyde and shaken at room temperature for 3 hours. This mixture was applied to a 1.6 cm x 40 cm glass chromatography colum (Pharmacia, Uppsala, Sweden) of Sephadex G-150 (Pharmacia, Uppsala, Sweden) equilibrated with 0.04 M sodium phosphate buffer, pH 7.4 containing 0.5M NaCl. The proteins were eluted using the same buffer and 2ml fractions collected using a FRAC-100 fraction collector (Pharmacia, Uppsala, Sweden). The flow rate was maintained at 6ml/hr using a P-1 peristaltic pump (Pharmacia, Uppsala, Sweden). The elution of the proteins was followed by measuring the optical density of each fraction at 280nm on a SP-6 spectrophotometer (Pye Unicam, Cambridge, England).

MEASURMENT OF PEROXIDASE ACTIVITY

The substrate solution was prepared by dissolving 40 mg 5 - aminosalicylic acid in 100 ml 0.02M sodium phosphate buffer, pH6.0 containing 5µl H_2O_2. Substrate solution (1 ml) was added to each sample to be assayed. The absorbance at 450nm was read, after 1 hour at room temperature, using a SP-6 spectrophotometer (Pye-Unicam, Cambridge, England).

BINDING OF ENZYME - LABELLED GLUCAGON TO MEMBRANE RECEPTORS

In an experiment to ascertain the feasability of using enzyme - labelled proteins to study membrane receptors,

glucagon was labelled with horseradish peroxidase and the binding of this conjugate to rat liver plasma membranes investigated.

Plasma membranes were prepared by the method of Lesko et al (1973). A polymer solution was prepared by mixing 200g 20% (w/v) aqueous Dextran T500 (Pharmacia, Uppsala, Sweden), 30% (w/v) aqueous polyethylene glycol 6000, 333ml 0.22 M sodium phosphate buffer pH 6.5 and 179ml glass distilled water in a separating funnel and incubated at 4°C for 96 hours. The two liquid phases were collected and stored at 4°C.

The liver from a rat was removed and washed in 0.001 M sodium bicarbonate buffer pH 7.5, containing 0.0005 M CaCl . The liver was them chopped finely and homogenised in 0.001 M sodium bicarbonate buffer pH 7.5, containing 0.0005 M CaCl to 100x the wet weight of the liver, allowed to stand for 5 minutes at 4°C and the fibrous tissue removed by filtration through surgical gauze.

The filtrate was centrifuged at 1000 x g for 30 minutes in a M.S.E. Hi-speed 18 centrifuge (M.S.E., Crawley, England) using a 8 x 50ml rotor and the supernatant discarded. The membrane rich pellet was resuspended in 0.001 M sodium bicarbonate buffer containing 0.0005 M $CaCl_2$ by gentle homogenisation (4-6 strokes) in a Potter homogeniser and diluted to half the original volume with the same buffer. The homogenate was then centrifuged at 1100 x g for 15 minutes in a M.S.E. Hi-speed 18 centrifuge (M.S.E., Crawley, England) using a 8 x 50 ml

rotor.

The supernatant was discarded and the pellet was suspended in 30ml upper phase polymer by gentle homogenisation.An aliquot (10ml) of lower phase polymer was added to 10ml aliquots of the homogenate in upper phase polymer, mixed and centrifuged at 1100 x g for 15 minutes. Plasma membranes were removed from the polymer interface and resuspended in 30ml upper phase polymer.An aliquot (10ml) of lower phase polymer was added to 10ml aliquots of the plasma membranes in upper phase polymer, mixed and centrifuged at 1100 x g for 15 minutes, the plasma membranes were removed from the polymer interface and added to 30ml of upper phase buffer.An aliquot (10ml) of lower phase buffer was added to 10ml aliquots of the plasma membrane preparation, mixed and centrifuged at 1100 x g for 15 minutes. The plasma membranes were removed from the polymer interface, suspended in 30ml 0.05 M Tris-HCl buffer pH 7.5 and centrifuged at 2400g for 10 minutes in a MSE Hi-speed 18 centrifuge using a 8 x 50ml rotor. The supernatant was discarded and the membrane preparation washed three times in 0.05 M Tris-HCl buffer pH 7.5. The final membrane pellet was resuspended in 10ml 0.05 M Tris-HCl buffer, pH 7.5 and stored in 500ul aliquots at -20° C.

Horseradish peroxidase was coupled to glucagon (Sigma Chemical Co., St. Louis, MO., U.S.A.) as described previously.

In the binding experiment total binding was measured by mixing 500µl membrane suspension, 400ul 2% (w/v) bovine serum albumin (Miles Scientific Ltd., Nashville, TN., U.S.A.) in 0.05M Tris-HCl buffer, pH 7.5 and 100µl horseradish peroxidase-glucagon conjugate. The mixture was incubated at 25°C for 30 minutes, centrifuged at 2400 x g for 10 minutes at 4°C in a M.S.E. Hi-speed 18 centrifuged using a 8 x 50ml rotor (M.S.E., Crawley, England). The pellet was then washed three times in 0.05 M Tris-HCl buffer pH 7.5 and the enzyme activity of the pellets measured. Non-specific binding was measured by mixing 500µl membrane suspension, 300µl 2%(v/v) bovine serum albumin in 0.05 M Tris-HCl, pH 7.5, 100µl horseradish peroxidase-glucagon conjugate and 100µl 1uM glucagon in a test tube, incubating at 25°C for 30 minutes, centrifuging at 2400 x g for 10minutes at 4°C and washing the pellet three times in ice-cold 0.05M Tris-HCl pH 7.5. The peroxidase activity of the final pellet was measured as described previously.

The total peroxidase activity of the horseradish peroxidase-glucagon conjugate added to the reaction mixtures was measured as previously described. Total binding and non-specific binding assays were performed in triplicate.Specific binding was calculated by subtracting the non-specific binding from the total binding and expressing as a percentage of the peroxidase activity added to the reaction mixture.

BINDING OF ENZYME LABELLED COLONY-STIMULATING FACTOR TO

HAEMATOPIETIC CELL MEMBRANE RECEPTORS

Bone marrow cells were extracted from the femours of 20 C57BL/6 mice (Baintin and Kingham, Hull, England) and collected in 0.001 M sodium bicarbonate buffer pH 7.5, containing 0.0005 M CaCl . The bone marrow cells were homogenised using 20-30 strokes of a Potter homogeniser and plasma membranes prepared by the same method used to prepare the rat liver membranes.

Colony-stimulating factor was prepared from pokeweed mitogen stimulated spleen cell conditioned medium as described by Burgess *et al* (1980). This C.S.F. preparation was labelled with horseradish peroxidase and the binding of the enzyme labelled growth factor to bone marrow cell membranes was studied using an identical protocol to that used to study the binding of enzyme - labelled glucagon to plasma membrane receptors.

Figure 4: A diagram illustrating the bioassay of colony-stimulating activity.

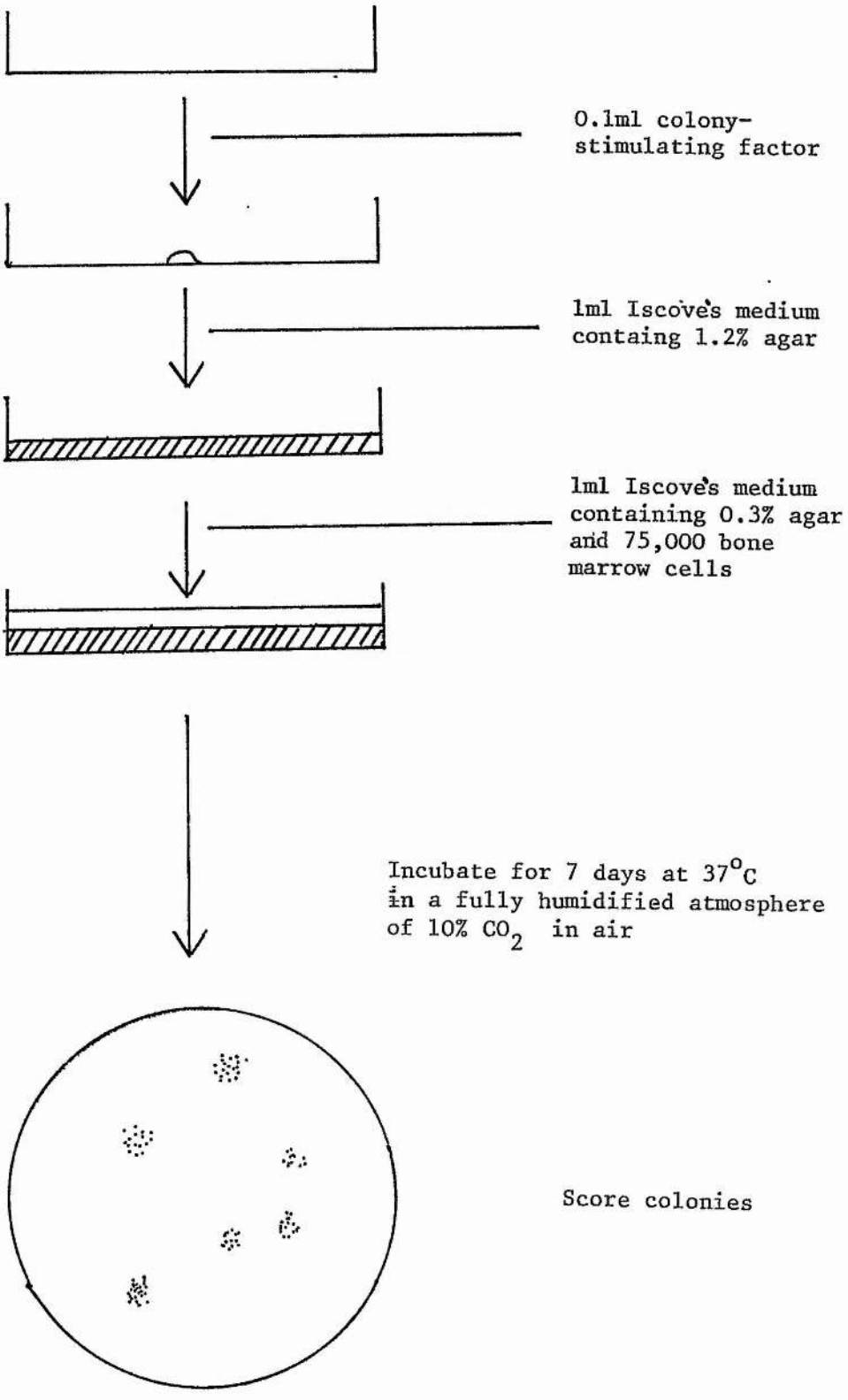

0.1ml colony-stimulating factor
1ml Iscove's medium containg 1.2% agar
1ml Iscove's medium containing 0.3% agar and 75,000 bone marrow cells
Incubate for 7 days at 37°C in a fully humidified atmosphere of 10% CO_2 in air
Score colonies

Figure 4

RESULTS

RESULTS

PREPARATION OF HUMAN PLACENTAL CONDITIONED MEDIUM

The human placental conditioned medium contained maximal colony-stimulating activity by day 4 to day 7 of culture. The conditioned medium was harvested at day 7 and pooled with other human placental conditioned media containing active material. The pooled material, designated H.P.C.M.-I, was stored at -70°C until further processed.

CALCIUM PHOSPHATE GEL ABSORPTION

The H.P.C.M.-I was dialysed against four changes of 0.01 M sodium phosphate buffer pH 6.8. Calcium phosphate gel was then added to the H.P.C.M.-I in the 0.01M sodium phosphate buffer pH 6.8. Colony-stimulating activity remained in the supernatant whilst most of the protein bound to the gel. The supernatant (designated H.P.C.M.-II) was removed and concentrated by ultrafiltration (Amicon) using a PM-10 membrane. The concentrated material was designated H.P.C.M.-IIb and stored in 15ml aliquots at -70° C until required for future experiments. For practical reasons this was regarded as the starting material for further purification procedures.

PHENYL-SEPHAROSE HYDROPHOBIC INTERACTION CHROMATOGRAPHY OF H.P.C.M.-IIb

Figure 5 shows a typical elution profile obtained when H.P.C.M.-IIb was applied to a phenyl-sepharose 6B column. Two peaks of colony-stimulating activity were identified on hydrophobic interaction chromatography of H.P.C.M.-IIb. The breakthrough peak was pooled and the biological activity of the pooled material determined. The bound material was eluted with 50% (w/v) ethylene glycol and the protein was monitored by absorbance at 280nm. Fractions were pooled and the biological activity measured. The colony-stimulating activity which eluted in the breakthrough peak was shown to contain eosinophil and granulocyte-macrophage colony-stimulating factors and designated G.M.-C.S.F by Nicola et al ., (1979). The material eluted by ethylene glycol from the phenyl-sepharose column was shown to contain mainly granulocyte and/or macrophage colony-stimulating factors and designated G.M.-C.S.F.β by Nicola et al ., (1979). Active fractions were pooled as indicated in Figure 5 and stored at -70^{o}C until used. This purification was preformed 8 times during the present study and all experiments gave similar elution profiles to that shown in Figure 5.

PROTEIN DETERMINATION

Figure 6 shows a typical calibration curve obtained using bovine serum albumin as standards in the dye-binding technique for protein determination (Bradford, 1976) Each point represents the mean of duplicate determinations.

BIOASSAY OF COLONY-STIMULATING ACTIVITY

The bioassays were performed as described in the Materials and Methods section. Each assay was performed in triplicate and each of the biological activities reported in the present study is the mean of these three observations. The number of colonies consisting of greater than 40 cells were counted and activity expressed as the number of colonies formed per ml of material. Specific activity was expressed as the number of colonies formed per mg protein in the C.S.F. preparation.

ULTRAFILTRATION AND PHENYL-SEPHAROSE CHROMATOGRAPHY OF G.C.T.-CONDITIONED MEDIUM

Chromatography of G.C.T.-conditioned medium was performed by the technique described by Abboud *et al.*, (1981). The fractions consisting the breakthrough peak were pooled and the colony-stimulating activity of this material found to be 582 colonies per mg protein.

GEL PERMEATION CHROMATOGRAPHY OF H.P.C.M.-IIB AND G.C.T.-STAGE II

Figure 7 shows a typical calibration curve (experiment preformed 8 times) for determination of molecular weight using gel permeation chromatography on Sephacryl S-200 superfine gels. A linear relationship was estabilished

between the Kav and the log molecular weight of the calibration proteins. The molecular weights of proteins could be determined from this graph.

Gel filtration chromatography of H.P.C.M.-IIb gave a single peak of colony-stimulating activity (Figure 8). This chromatgraphy was performed 6 times and similar elution profiles obtained in each experiment. The molecular weight of the active material was confirmed as 30,000.

Gel filtration chromatography of G.C.T. stage-II gave two peaks of biological activity (Figure 9). The major peak was found to have a specific activity of 4000 colonies/mg and a molecular weight of 63,000 whereas the minor peak has a biological activity of 280 colonies/ml and appeared to have a molecular weight of 30,200. The experiment was repeated 4 times with similar results.

THIOLPROPYL - SEPHAROSE 6B AFFINITY CHROMATOGRAPHY OF G.C.T.-STAGE III

The majority of the colony stimulating activity from G.C.T.-stage III eluted in the breakthrough peak (Figure 10). The specific activity of this material was found to be 2,700 colonies/mg. A minor peak of biological activity bound to the column and was eluted with the equilibrating buffer containing 25mM cysteine. This minor peak had a specific activity of 846 colonies/mg Figure 10 shows a typical elution profile obtained for 2 separate experiments

which gave similar results.

Tables summarising the purification scheme for both H.P.C.M. (Table 3) and G.C.T. (Table 4) are given.

RAISING OF ANTIBODIES TO C.S.F.

The harvested antisera were examined by a number of immunochemical techniques which are described in the following sections. The antiserum inhibited the formation of colonies in the semi-solid agar culture technique. The marked precipitin lines, however, may be due to an antibody other than an anti-C.S.F.

IMMUNODIFFUSION

Figure 11 shows the precipitin line pattern obtained when anti-C.S.F., raised in rabbits against H.P.C.M. IIb was placed in the central wells of an Ouchterlony-type immunodiffusion plate and various C.S.F. preparations from human placental conditioned medium and giant cell tumour conditioned medium were placed in the outside wells. It can be seen that precipitin lines were obtained when the anti-C.S.F. was reacted with human placental conditioned medium IIb, giant cell tumour conditioned medium, G.M.-C.S.F. α from human placental conditioned medium and G.M.-C.S.F.β from human placental conditioned medium. Lines of identity were demonstrated between; (i) human placental conditioned medium IIb and giant cell tumour conditioned

medium, (ii) human placental conditioned medium and G.M.-C.S.F.α, and (iii) between human placental conditioned medium IIb and G.M.-C.S.F.β.

IMMUNOELECTROPHORESIS

Figure 12 shows the results of the immunoelectrophoresis.Multiple precipitin arcs were visible when the H.P.C.M. IIb was reacted with the antiserum. A single precipitin arc was demonstrated when G.M.-C.S.F.α was reacted with the antiserum and a single precipitin line appeared to be present when the antiserum was reacted with G.M.-C.S.F.β.

COOMASSIE BLUE R-250 STAINING

Precipitin lines and protein stained with Coomassie Blue-R-250. The precipitin lines were easier to visulise than unstained preparations.

RADIOIODINATION OF C.S.F.

Colony-stimulating factor was radioiodinated by a modification of the chloramine-T method. The radioiodinated protein was separated from unreacted 125-I by Sephadex G-25 gel permeation chromatography (Figure 13). The addition of polyethylene glycol 6000 and dimethylsulphoxide to the reaction mixture prevented loss of biological activity during the iodination procedure.

BINDING OF C.S.F. TO ANTI-C.S.F.

Figure 14 shows a typical binding curve obtained when a fixed amount of 125-I-C.S.F. was reacted with anti-C.S.F. diluted 1:10,000 in reaction buffer and a known amount of C.S.F. preparation.

PREPARATION OF PROTEIN-ENZYME CONJUGATES

Glucagon was coupled to horseradish peroxidase as described in the materials and methods section of this report. The results of the separation of the products of the crosslinking reaction by gel permeation chromatography are illustrated in Figure 15. Fractions 62-66 were pooled and used in susequent binding experiments.

The chromatography, on Sephadex G-150, of the products of the crosslinking reaction between C.S.F. and horseradish peroxidase is illustrated in Figure 16. Fractions 23-46 were pooled and used in subsequent experiments.

ASSAY OF PEROXIDASE ACTIVITY

The results of the enzyme assays are incorporated into the results of the binding experiments.

BINDING OF ENZYME LABELLED GLUCAGON TO MEMBRANE RECEPTORS

The specific binding of horseradish peroxidase labelled glucagon to rat hepatocyte membranes (Figure 17) exhibited a linear relationship to the membrane protein concentration. A rat hepatocyte membrane protein concentration of 70µg/ml was sufficient to bind 48% of the enzyme-labelled glucagon using a 1:1 dilution of enzyme - labelled glucagon.

BINDING OF ENZYME LABELLED C.S.F.TO BONE MARROW MEMBRANE RECEPTORS

The specific binding of horseradish peroxidase labelled colony-stimulating factor to murine bone marrow cell membranes exhibited a linear relationship to the membrane protein concentration (Figure 18). A membrane protein concentration of 54 µg/ml was sufficient to bind 50% of the peroxidase labelled C.S.F. when the dilution of conjugate was 1:1.

Figure 5 : Phenyl-sepharose 4B chromatography of H.P.C.M. IIb. The figure shows results from a typical experiment. Protein (-●-) was monitored at 280 nm and biological activity measured as described in the text. In the experiment described in the figure, the shaded areas (▒) were pooled and used in subsequent purification procedures. The fractions from the breakthrough material had a biological activity of 400 +30 colonies/ml. The material from the peak eluted by ethylene glycol was split into two pools. Pool 1 had a biological activity of 302+17 colonies/ml and Pool 2 had an activity of 197+15 colonies/ml. The experiment was performed 8 times. In all experiments the biological activity and protein eluted at similar volumes to those indicated in this Figure.

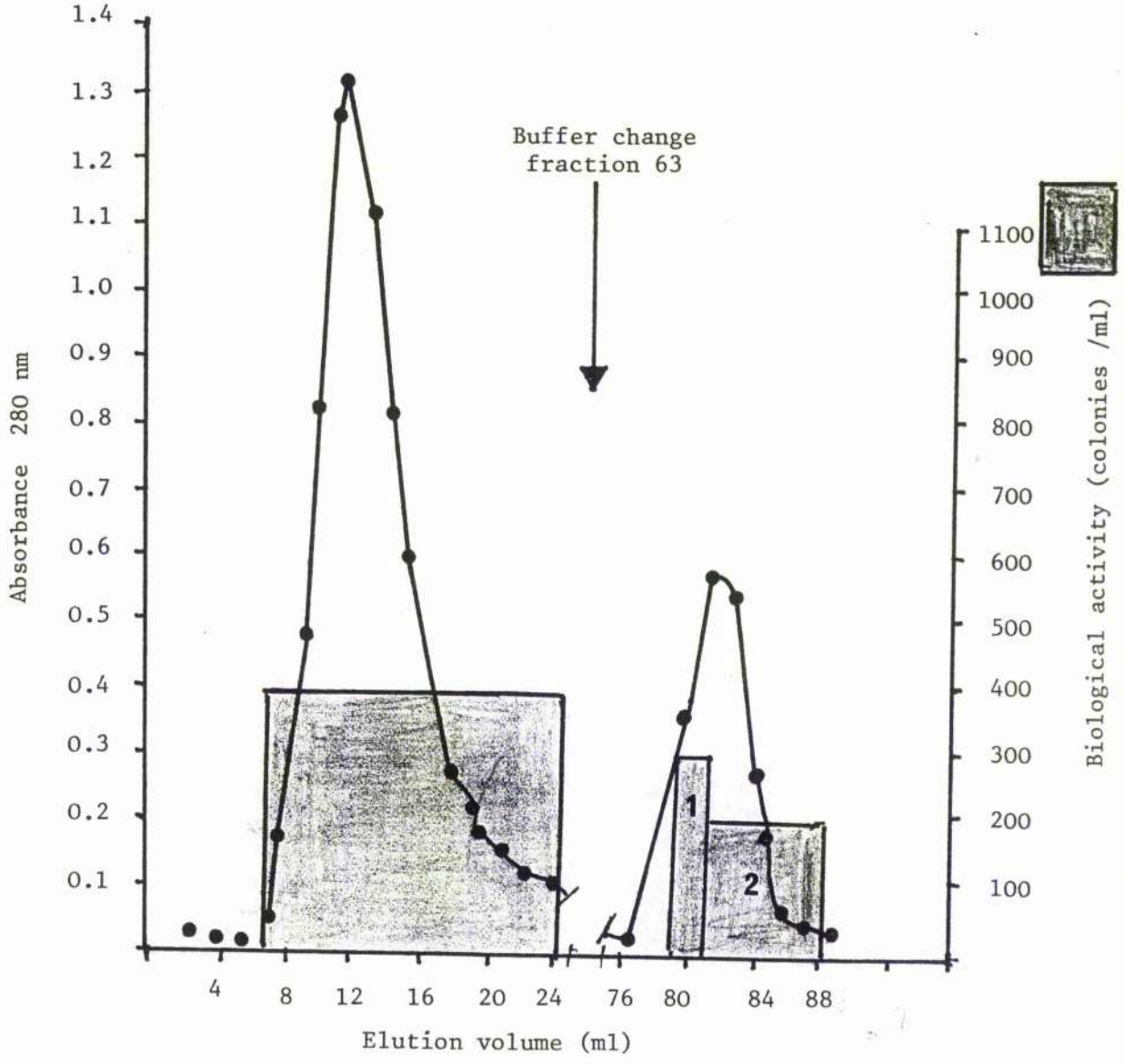

Absorbance 280 nm
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Buffer change
fraction 63
1
2
1100
1000
900
800
700
600
500
400
300
200
100
Biological activity (colonies /ml)
4
8
12
16
20
24
76
80
84
88
Elution volume (ml)

Figure 5

Figure 6 : Calibration curve for the determination of protein using the dye - binding method of Bradford (1976). Bovine serum albumin used as standards. The figure shows a typical experiment and each point on the graph is the mean of two observations. Protein determinations were preformed a number (>50) of times during the course of this study and a calibration curve was constructed on each occassion.

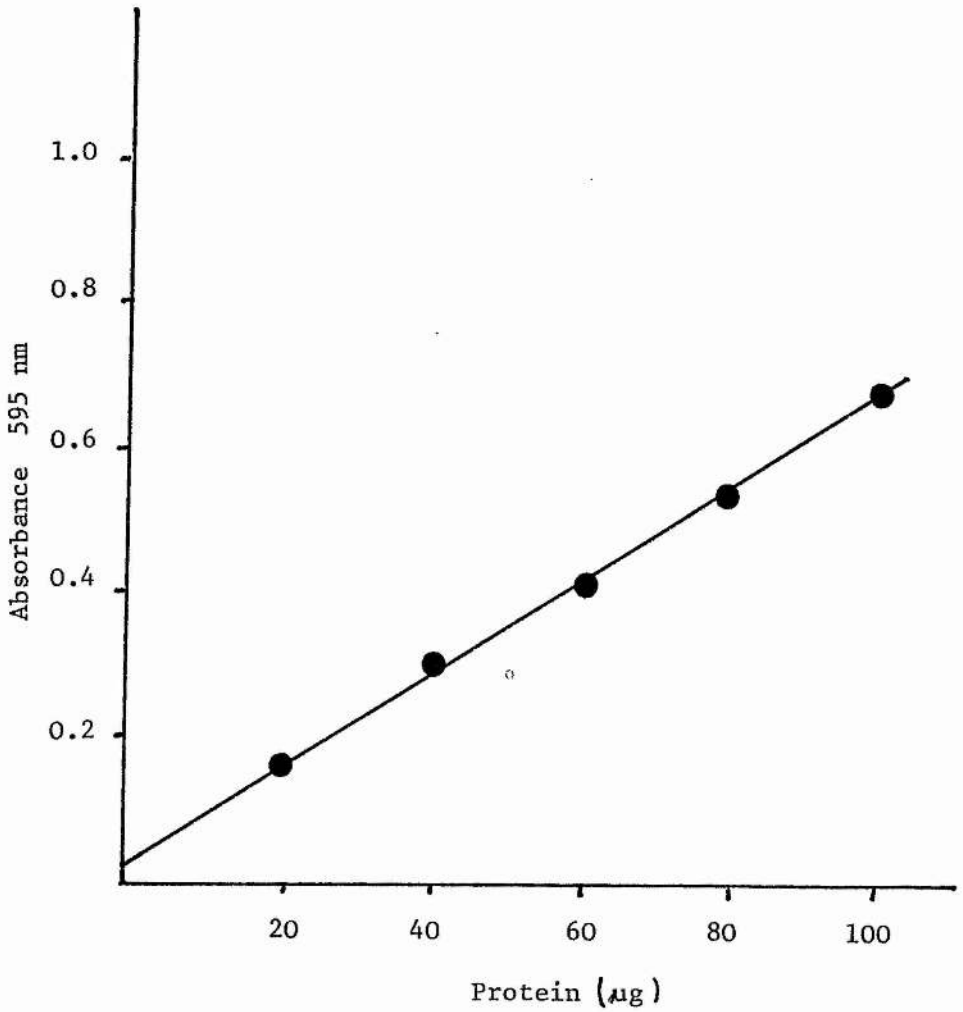


Figure 6

Figure 7 : Typical calibration curve for the determination of molecular weights of proteins using gel permeation chromatography on Sephacryl S-200 superfine columns. This experiment was performed 8 times.

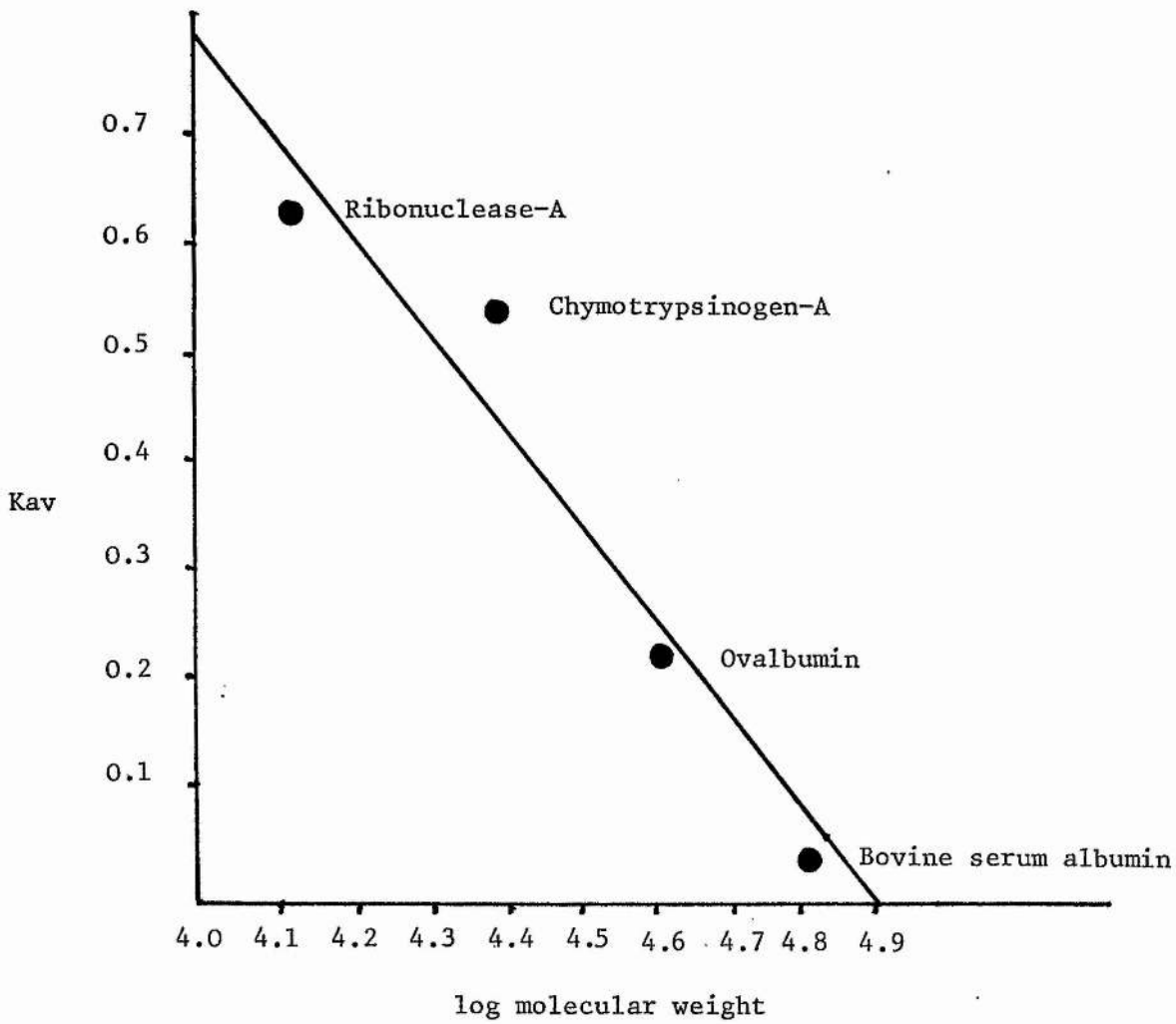

0.7
0.6
0.5
0.4
Kav
0.3
0.2
0.1
Ribonuclease-A
Chymotrypsinogen-A
Ovalbumin
Bovine serum albumin
4.0 4.1 4.2 4.3 4.4 4.5 4.6 4.7 4.8 4.9
log molecular weight

Figure 7

Figure 8 : Gel permeation chromatography of H.P.C.M. IIb. This diagram shows a typical elution profile obtained when H.P.C.M. IIb was chromatographed on Sephacryl S-200 superfine. The biological activity eluted with an apparent molecular weight of 30,000 (± 78). The experiment was performed 6 times and similar elution profiles obtained in all experiments.

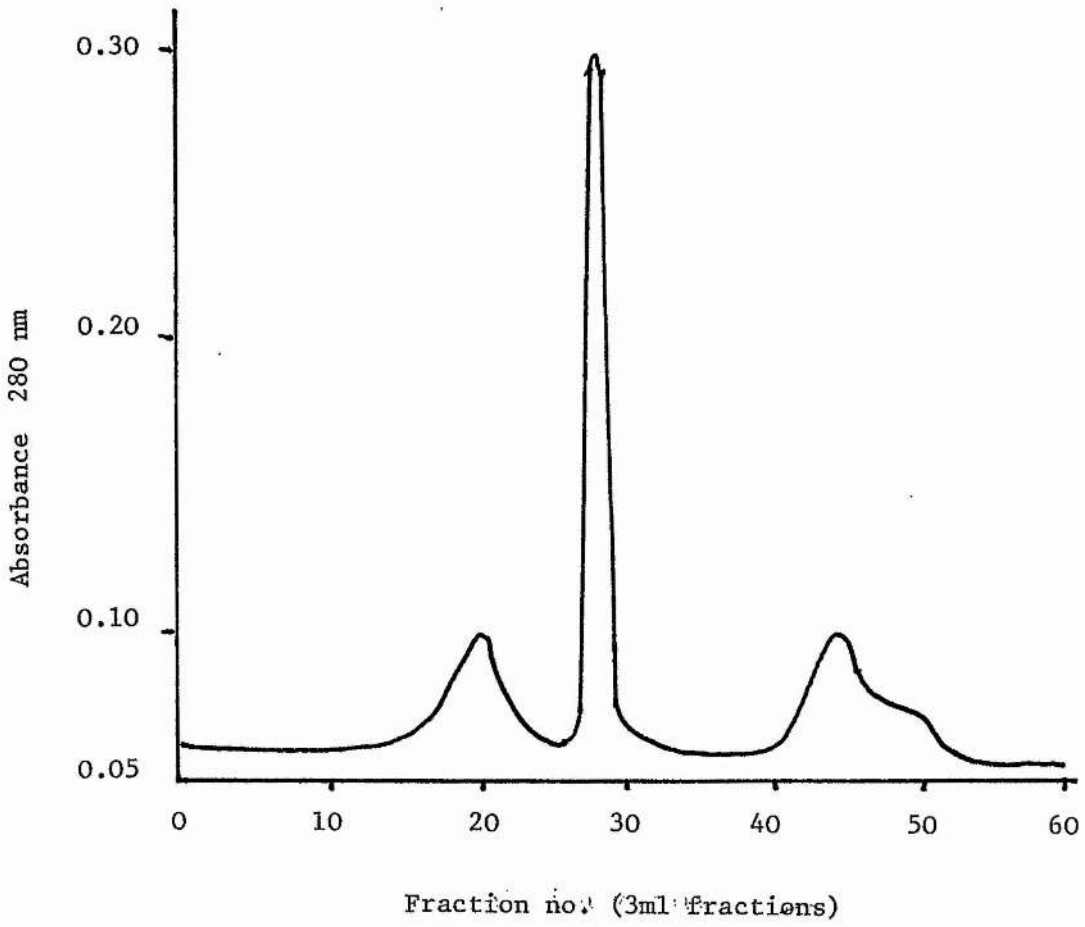

0.30
0.20
0.10
0.05
Absorbance 280 nm
0
10
20
30
40
50
60
Fraction no. (3ml fractions)

Figure 8

Figure 9 : Gel permeation chromatography of G.C.T. stage II. The elution was followed by monitoring protein at 280 nm (-●-) and by measuring the biological activity (-■-) of each fraction. Bars indicate standard errors. The experiment was performed 4 times and each C.S.F. assay was expressed as the mean of triplicate observations.

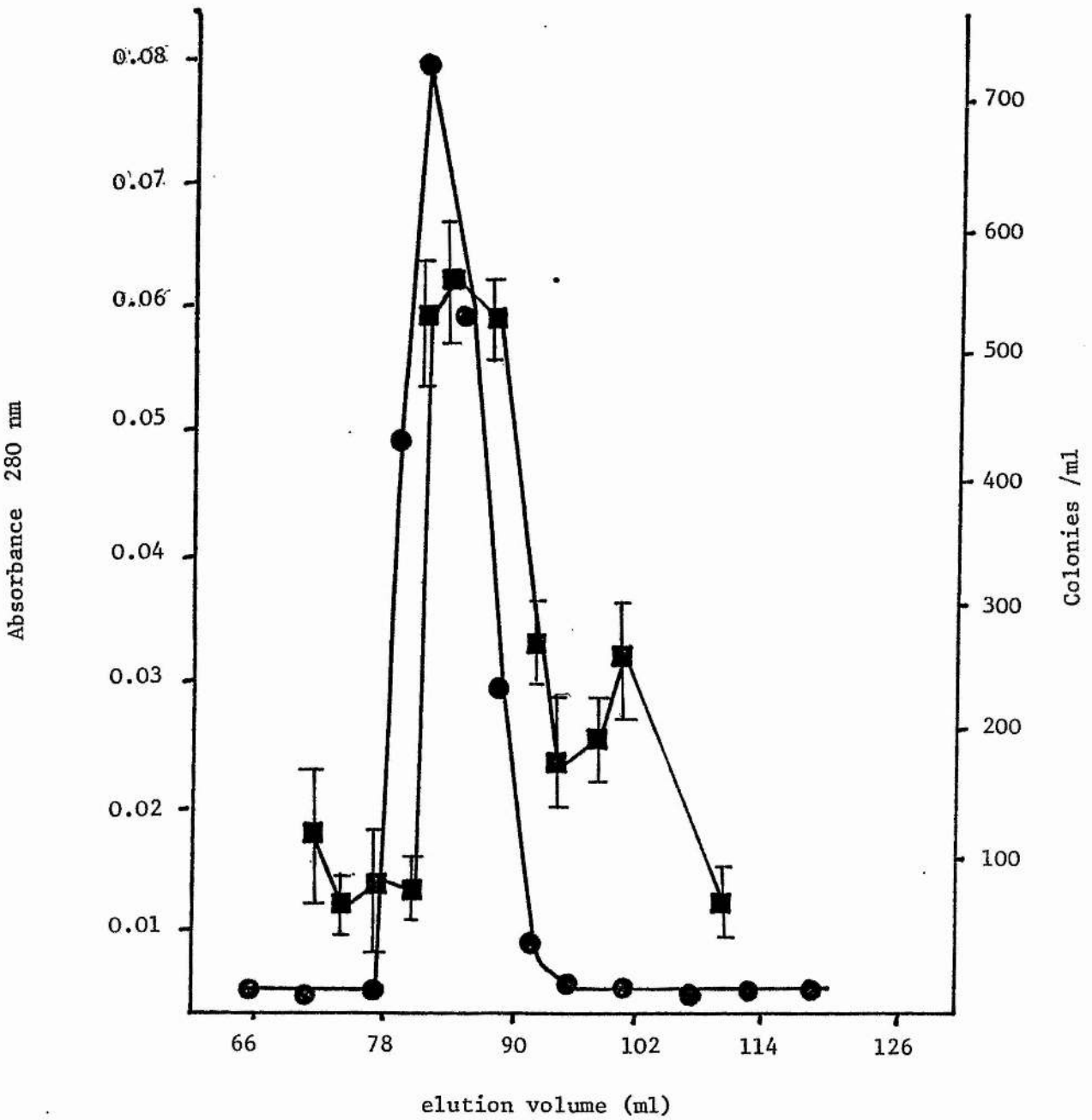

0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
Absorbance 280 nm
700
600
500
400
300
200
100
Colonies /ml
66
78
90
102
114
126
elution volume (ml)

Figure 9

Figure 10 : Thiopropyl-sepharose 6B chromatography of G.C.T. stage III. The protein content (—●—) was measured by the dye binding technique of Bradford (1976). The biological activity (■) was measured as described in the text and each point on the graph is the mean of three observations. Bars indicate standard errors. This experiment was performed 4 times and similar elution profiles obtained in each experiment.

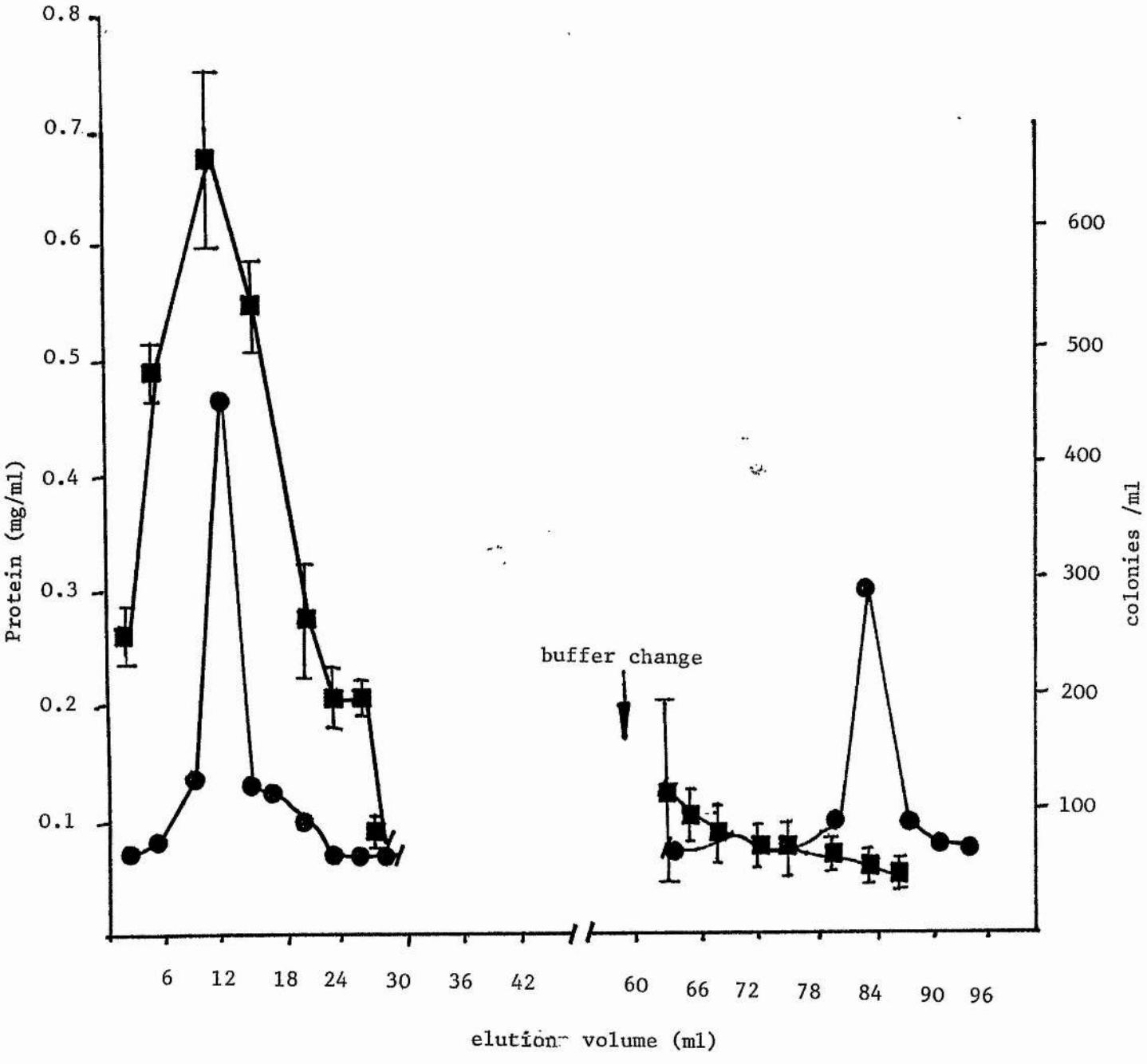

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Protein (mg/ml)
600
500
400
300
200
100
colonies /ml
buffer change
6
12
18
24
30
36
42
60
66
72
78
84
90
96
elution volume (ml)

Figure 10

Table 3: Summary of the purification of colony-stimulating factors from human placental conditioned medium.

Step		Protein mg/ml	Colonies /ml	Specific Activity /mg	Yield %
HPCM IIb		4.7	650	138	100
Phenyl GM-CSFα Sepharose		0.7	400	571	233
Phenyl Sepharose GM-CSFβ	1	0.31	300	967	20
	2	0.28	200	714	35
gel filtration		0.1	724	7240	73

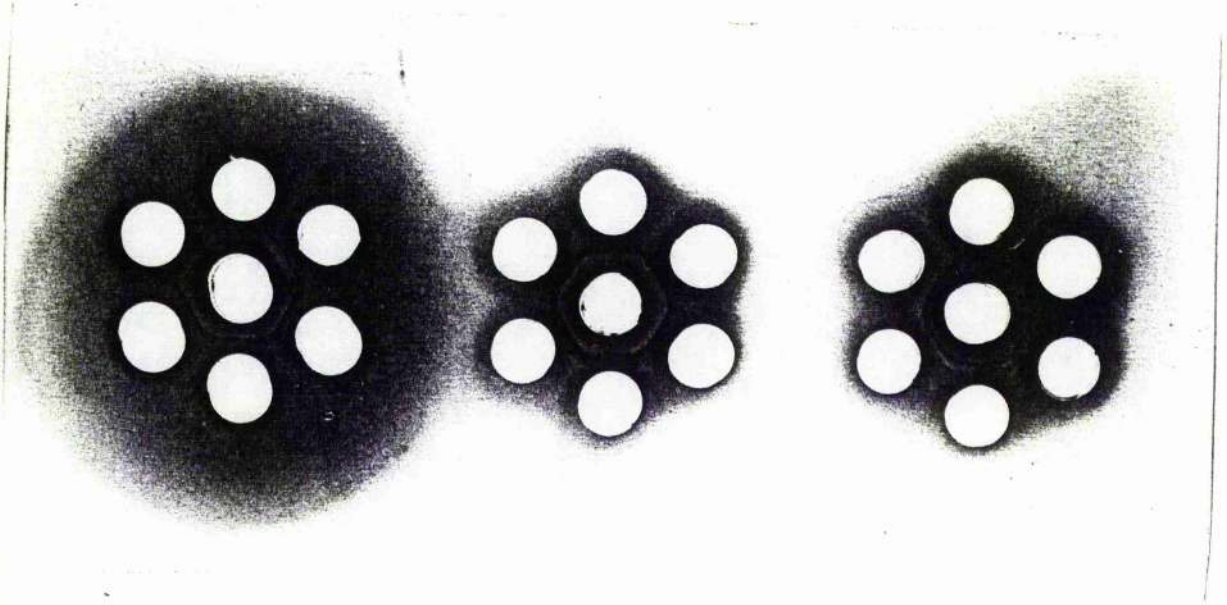
Table 3

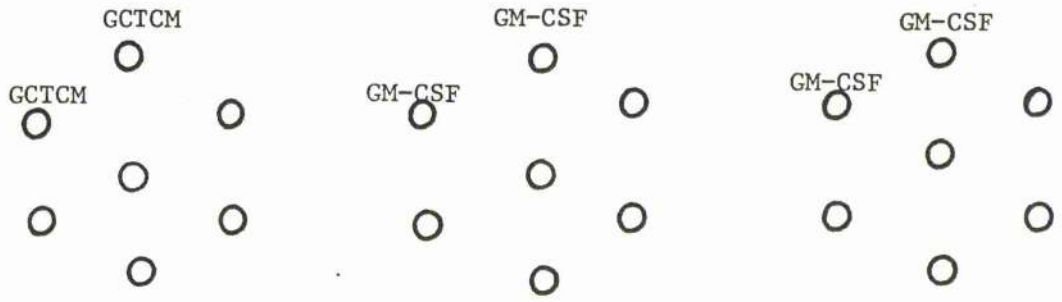
Table 4: Summary of the purification of colony-stimulating factors from G.C.T. conditioned medium.

Purification Stage	Protein mg/ml	Colonies /ml	Specific Activity /mg	Yield %
Crude GCT	1.30	1110	854	100
Ultrafiltrate GCT-Stage-I	1.60	1530	956	137
Phenyl-Sepharose GCT-Stage-II	0.91	530	582	48
Gel Filtration GCT-Stage-III	major peak 0.15	600	4000	54
	minor peak 0.005	250	not known	not known
Affinity Chrom.				
(1) GCT Stage IVA	0.20	540	2700	49
(2) GCT Stage IVB	0.13	110	846	10

Table 4

Figure 11 : Immunodiffusion in 1.5% (w/v) agarose gels. Antiserum was placed in the central wells and various C.S.F. preparations placed in the outside wells according to the key given. The plate shown here was stained with Coomassie Blue as described in the text.

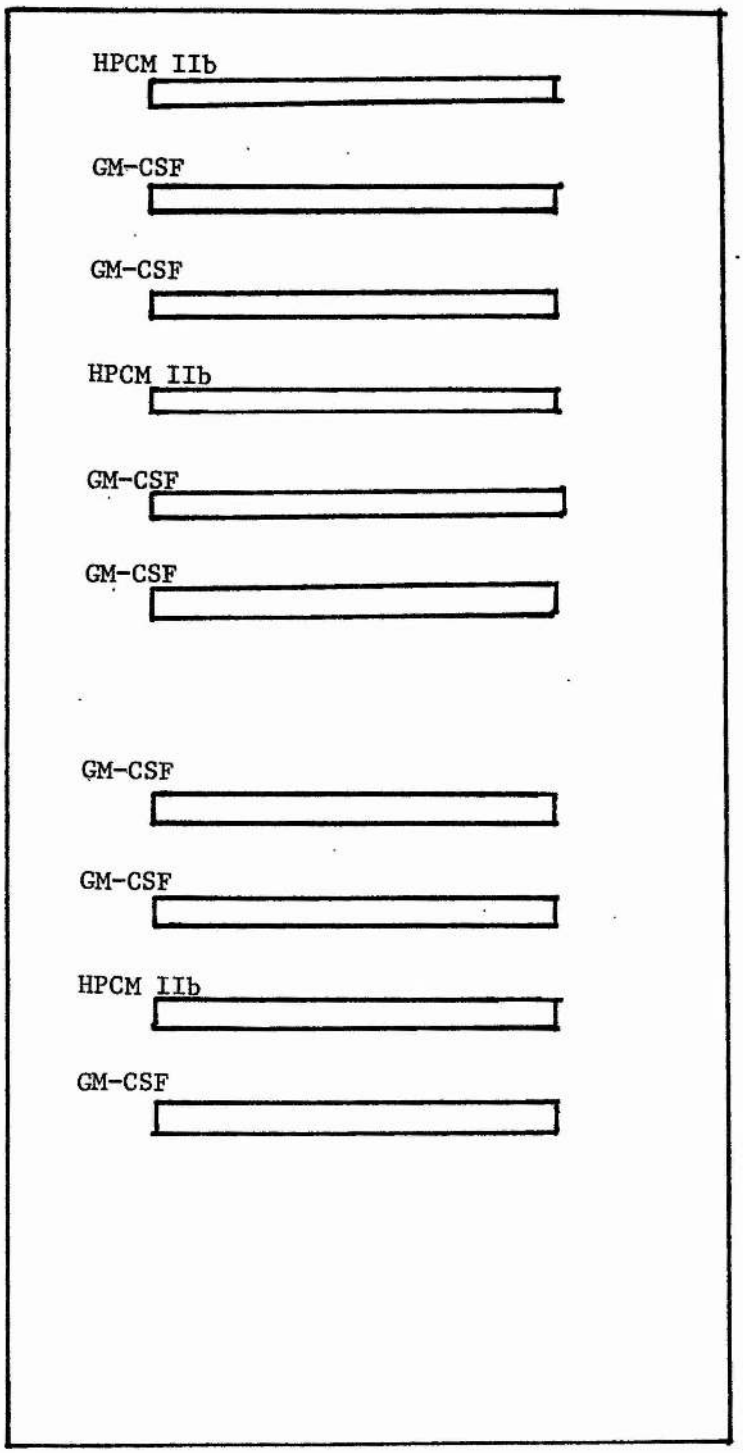




HPCM IIb in all other outside wells

Figure 11

Figure 12 Immunoelectrophoresis in 1.5% (w/v) agarose gels. Antiserum was placed in the central trough and various C.S.F. preparations placed in the small wells according to the key given. The plate shown here was stained with Coomassie Blue as described in the text.



Key to Figure 12

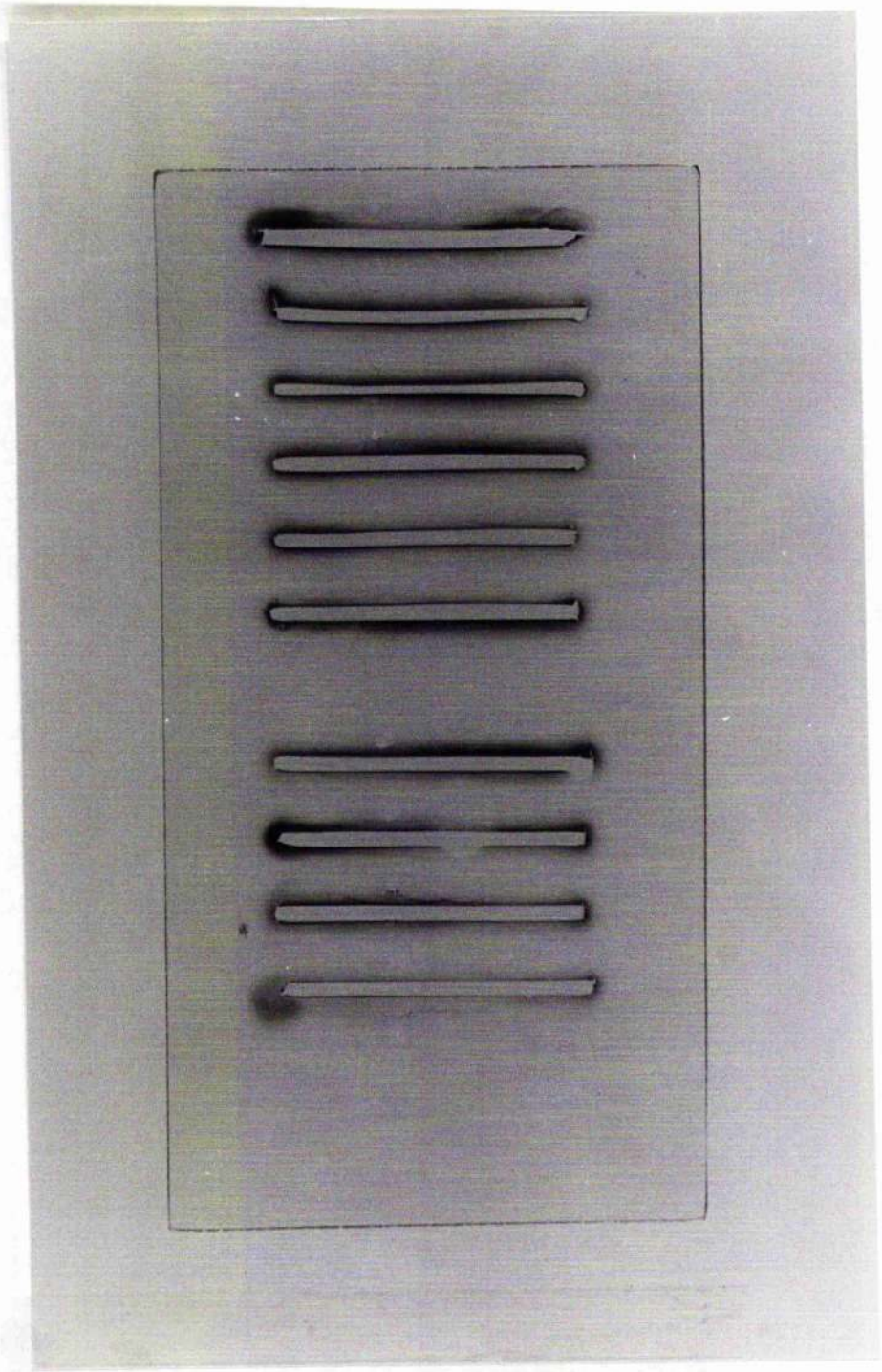


Figure 12

Figure 13 : The separation of 125-I labelled colony-stimulating factor from unreacted Na 125-I on a Sephadex G-25, medium grade, column. Fractions from the early peak were pooled, assayed for biological activity and used for subsequent binding experiments.The figure shows results from a typical experiment. Radioiodination of C.S.F. was performed on 22 occassions during the present study.

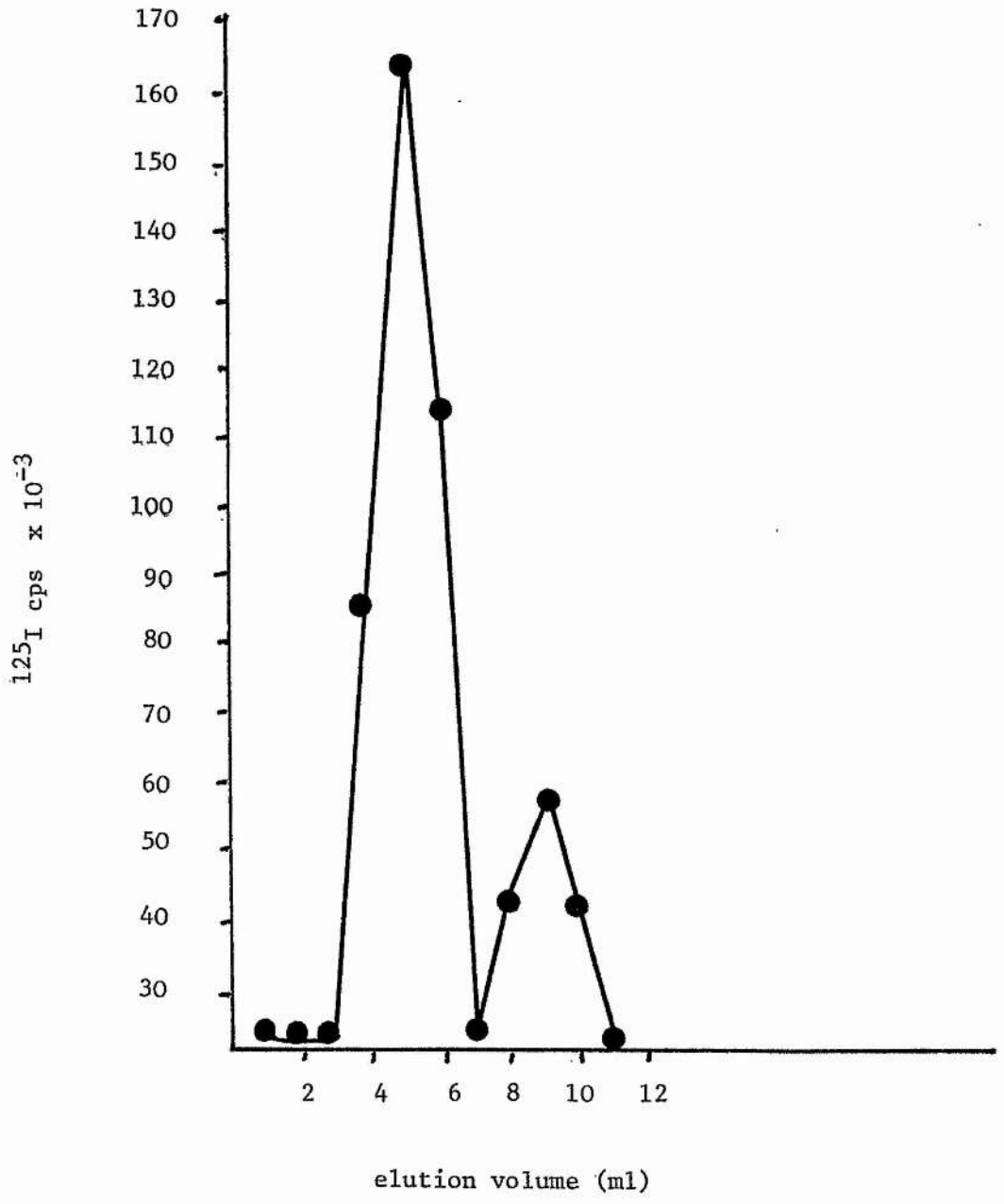

170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
^{125}I cps x 10^{-3}
2
4
6
8
10
12
elution volume (ml)

Figure 13

Figure 14 : Typical binding curve obtained when 0.1ml C.S.F. were added to 0.1ml antiserum (diluted 1:10,000 in phosphate buffered saline) and the mixture incubated for 1 hr at 37° C. An aliquot (0.1ml) of 125-I-C.S.F. was added to the incubation mixture and incubated for a further 2 hr at 37°C. Goat anti rabbit IgG (0.1ml) was used to precipitate the 125-I-C.S.F. bound to the antiserum. After centrifugation the bound material was counted using a gamma counter. Bars indicate standard errors.

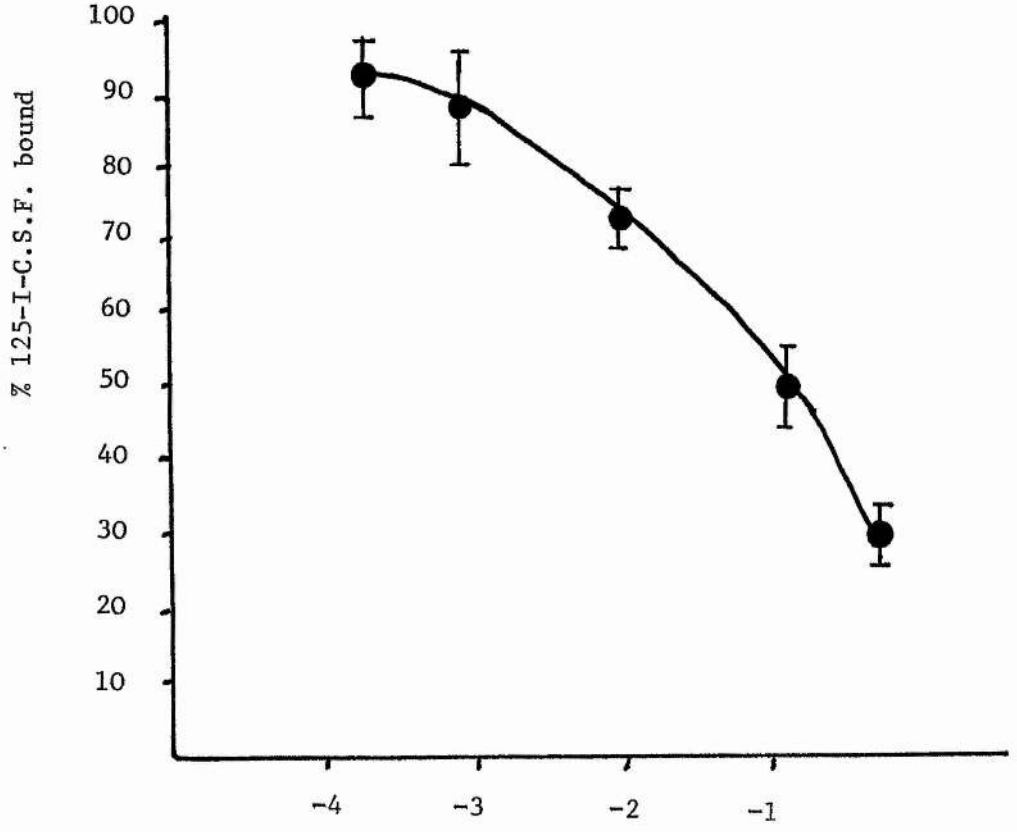

% 125-I-C.S.F. bound
100
90
80
70
60
50
40
30
20
10
-4
-3
-2
-1
log GM-CSF concentration (mg protein)

Figure 14

Figure 15 : Chromatography on Sephadex G-150 of the products of the crosslinking reaction used to couple horseradish peroxidase to glucagon. The material indicated was pooled and used in the binding experiments. This experiment was performed on 3 occassions and the figure shows a typical elution profile.

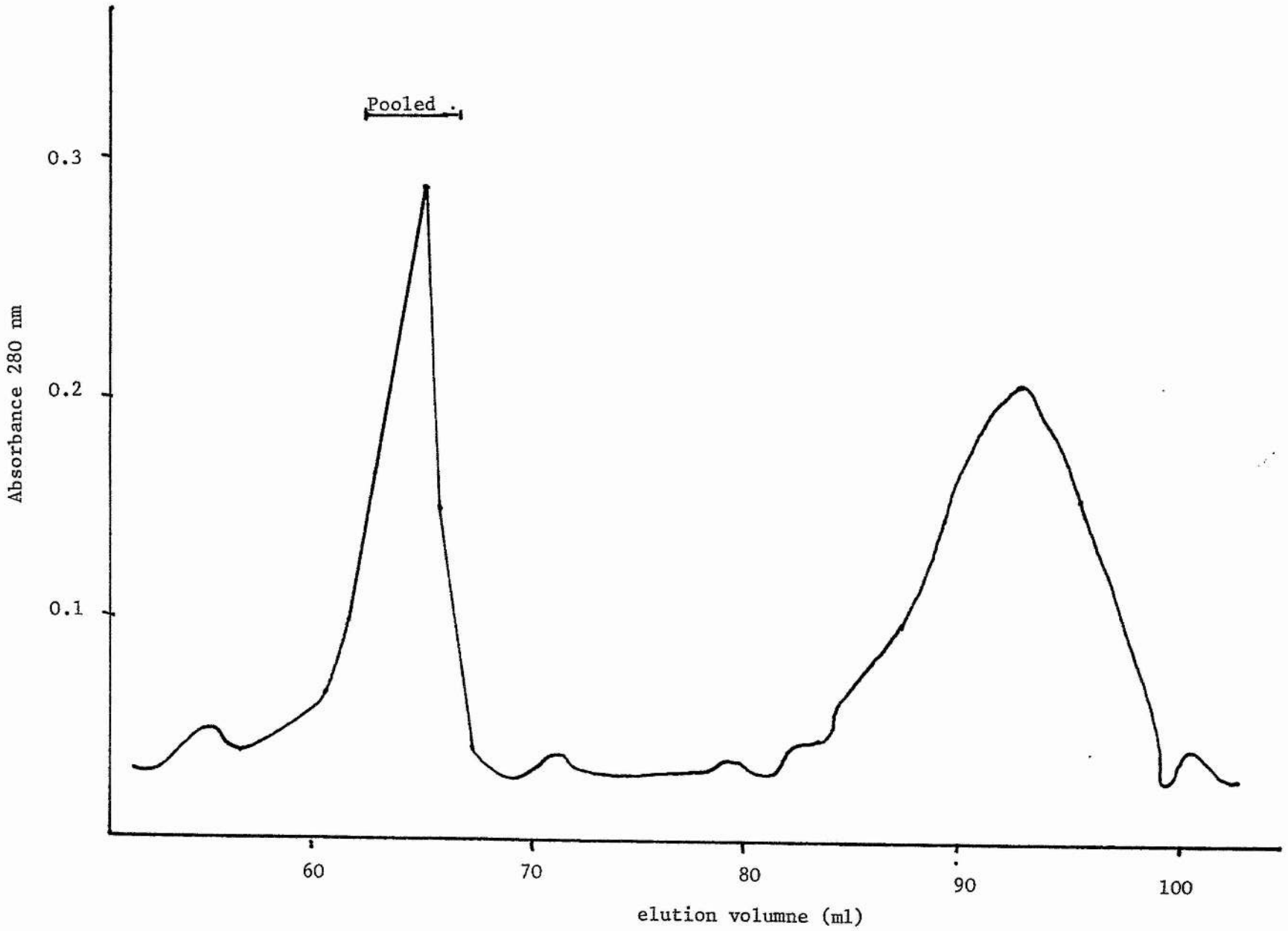

Pooled
0.3
0.2
0.1
Absorbance 280 nm
60
70
80
90
100
elution volumne (ml)

Figure 15

Figure 16 : Sephadex G-150 gel filtration chromatography of the products of the crosslinking reaction used to link horseradish peroxidase to C.S.F. from pokeweed mitogen-stimulated spleen conditioned medium. The fractions indicated were pooled and used in further experiments. This experiment was performed on 6 occassions and the figure shows a typical elution profile.

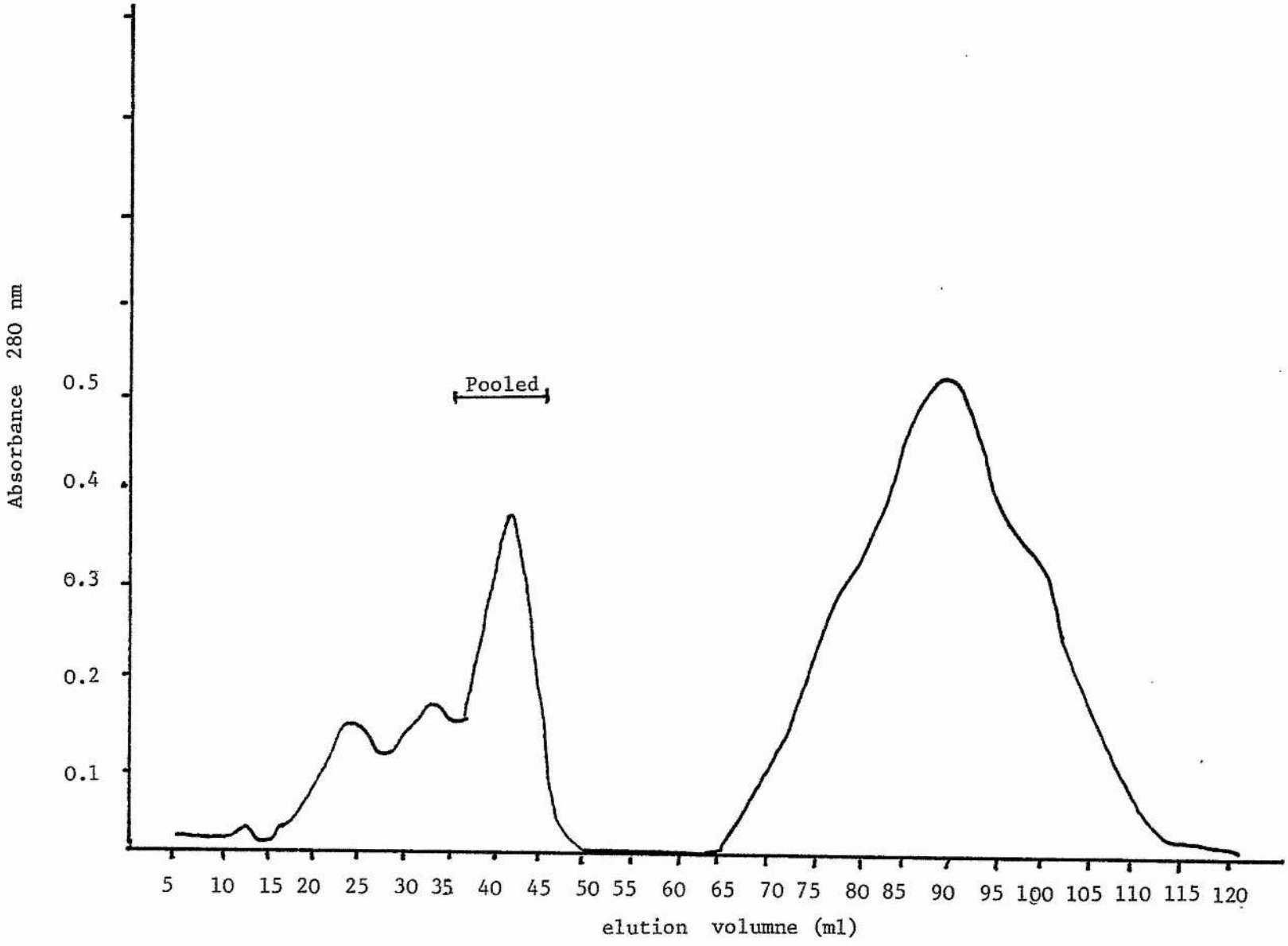

Absorbance 280 nm
0.5
0.4
0.3
0.2
0.1
Pooled
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120
elution volumne (ml)

Figure 16

Figure 17 : The binding of horseradish peroxidase-glucagon conjugates to a rat liver membrane preparation (a) at a conjugate dilution of 1:1, (b) at a conjugate dilution of 1:10 and (c) at a conjugate dilution of 1:20. The membrane protein concentrations are plotted against the specific binding of the labelled glucagon to the membrane preparation. Specific binding was expressed as the percentage of the peroxidase activity present in each assay. Bars indicate standard errors. Each point is the mean of 3 observations.

Specific binding = Total binding - Non-specific binding

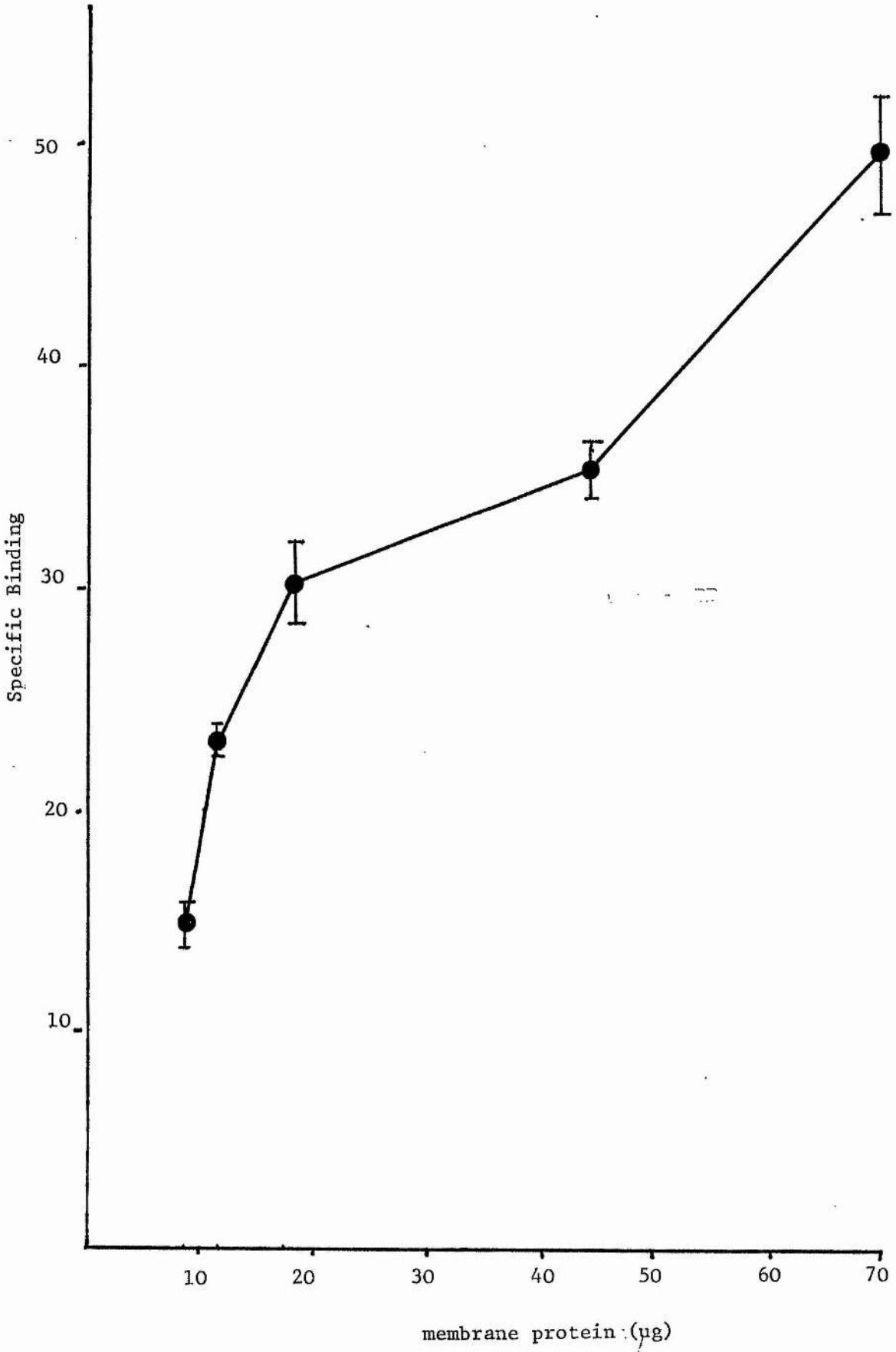

Specific Binding
50
40
30
20
10
10
20
30
40
50
60
70
membrane protein (µg)

Figure 17(a)

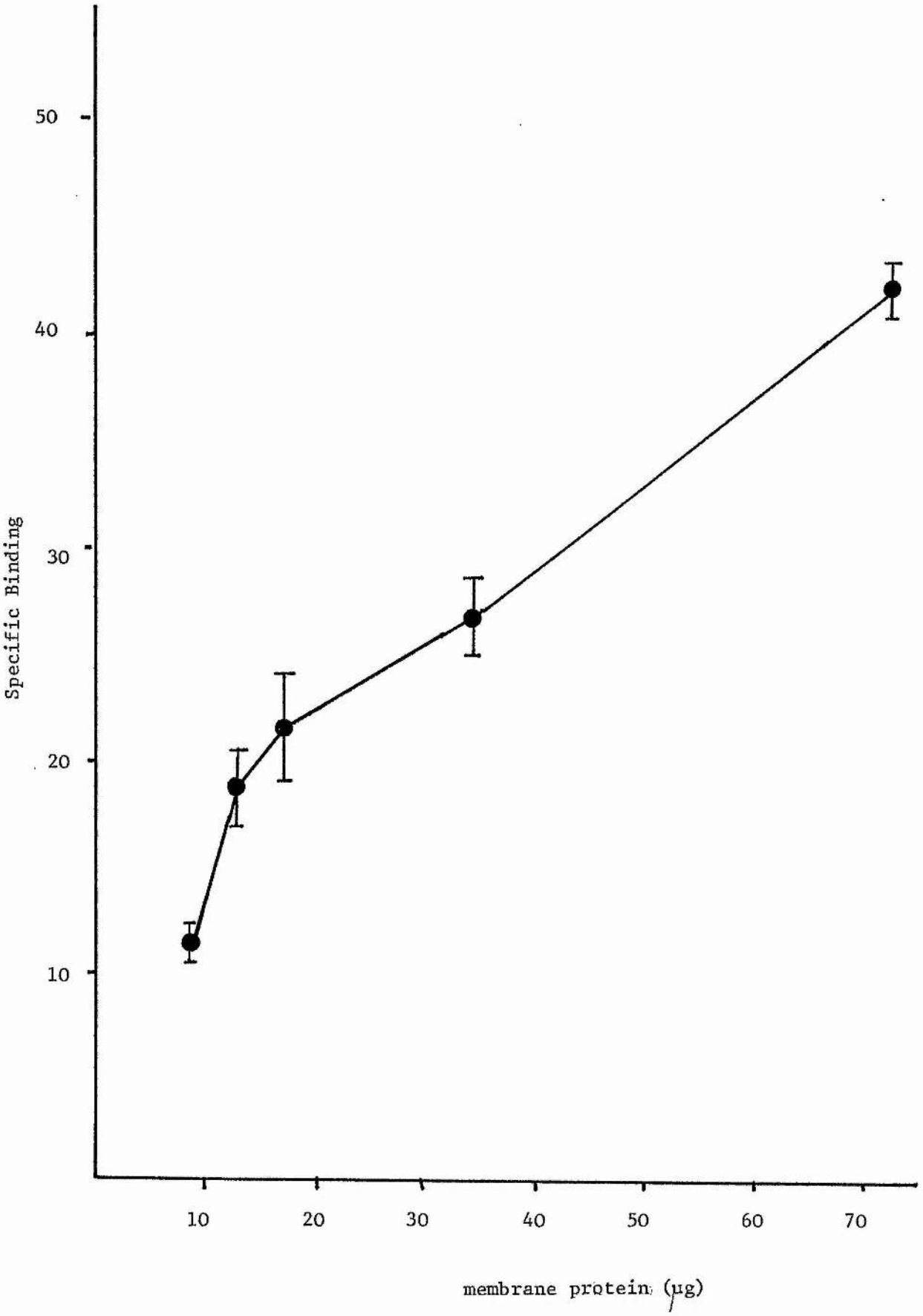

50
40
30
20
10
Specific Binding
10
20
30
40
50
60
70
membrane protein (µg)

Figure 17(b)

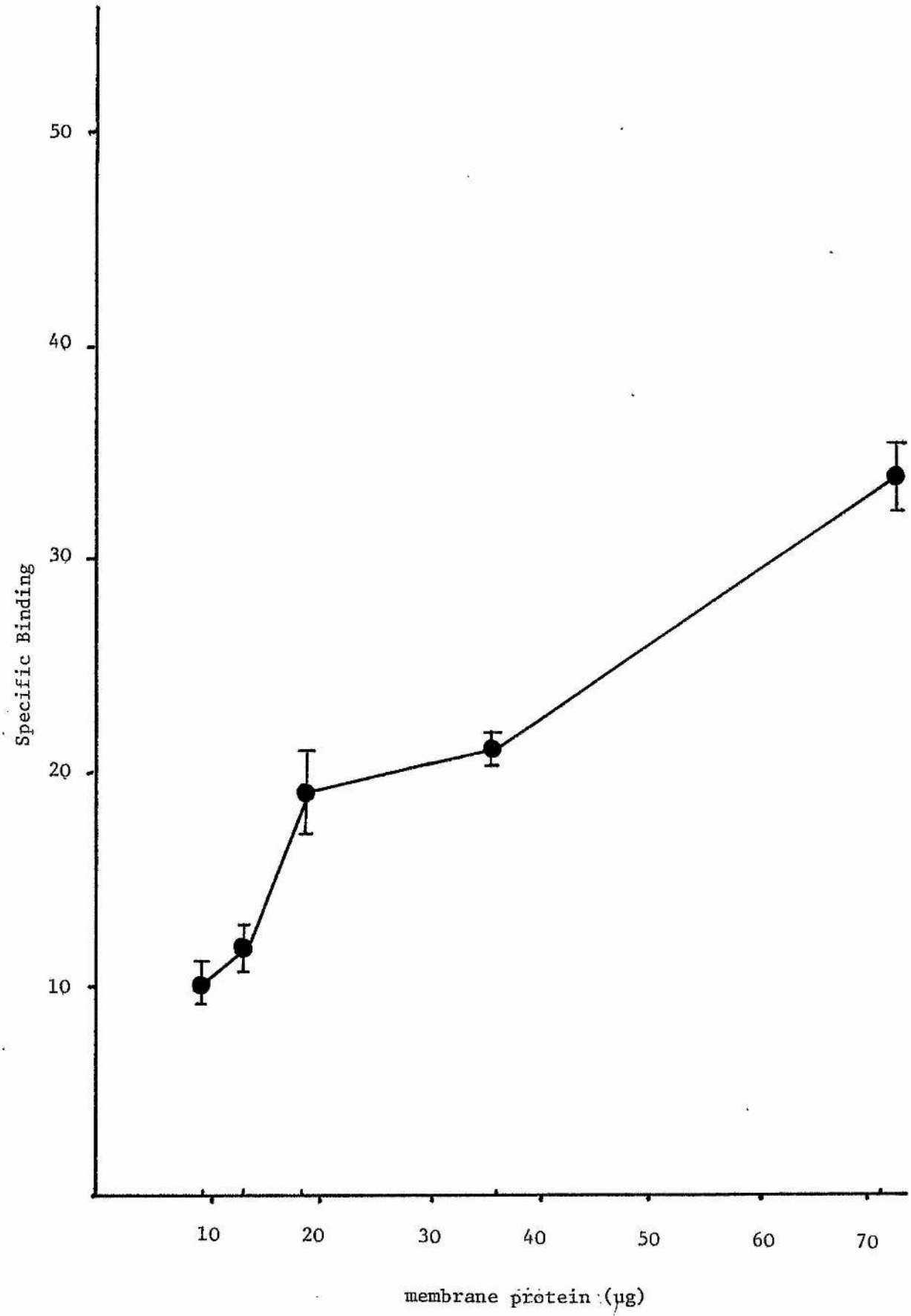

50
40
30
20
10
Specific Binding
10
20
30
40
50
60
70
membrane protein (µg)

Figure 17 (c)

Figure 18 : The binding of horseradish peroxidase - C.S.F. conjugates to a membrane preparation from bone marrow cells, (a) at a conjugate dilution of 1:1, (b) at a conjugate dilution of 1:10, (c) at a conjugate dilution of 1:20, and (d) at a conjugate dilution of 1:40. The membrane protein concentrations were plotted against specific binding of the labelled-C.S.F. to the membranes. Bars indicate standard errors. Specific binding was expressed as a percentage of the total peroxidase activity present in each assay. Each point on the graphs is the mean of 3 observations.

Specific binding = Total binding - Non-specific binding

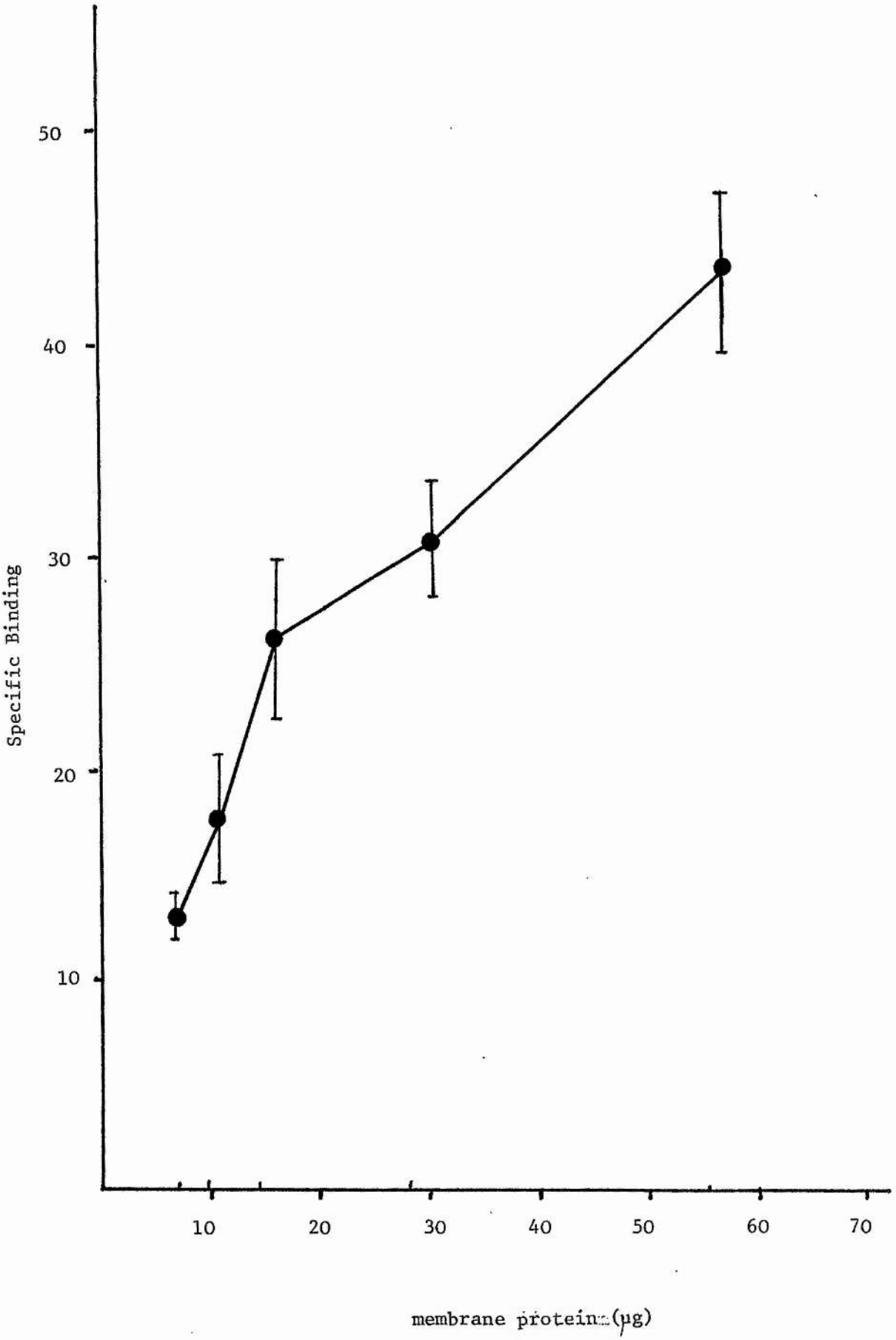

50
40
30
20
10
Specific Binding
10
20
30
40
50
60
70
membrane protein (µg)

Figure 18 (a)

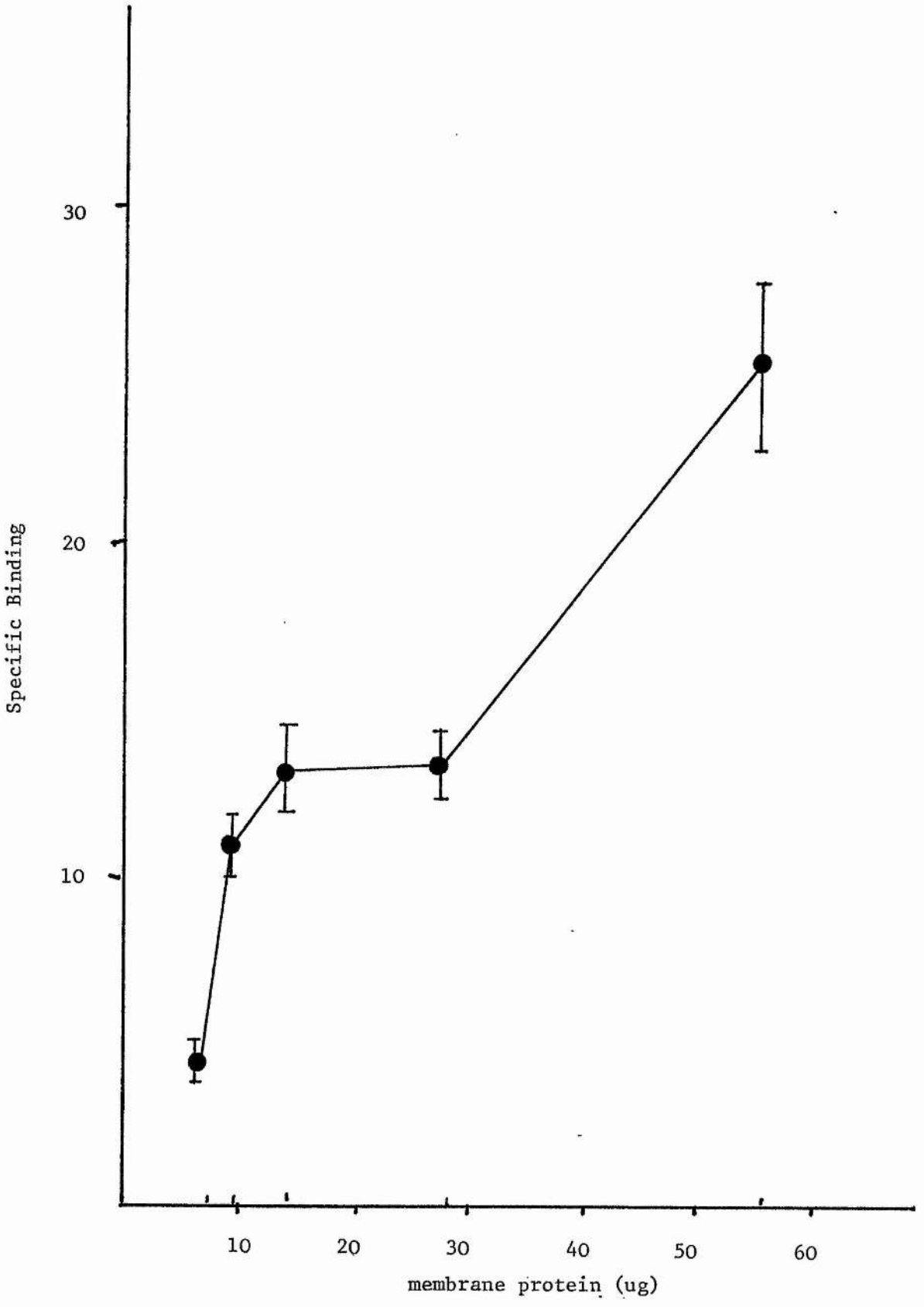

Specific Binding
30
20
10
10
20
30
40
50
60
membrane protein (ug)

Figure 18 (b)

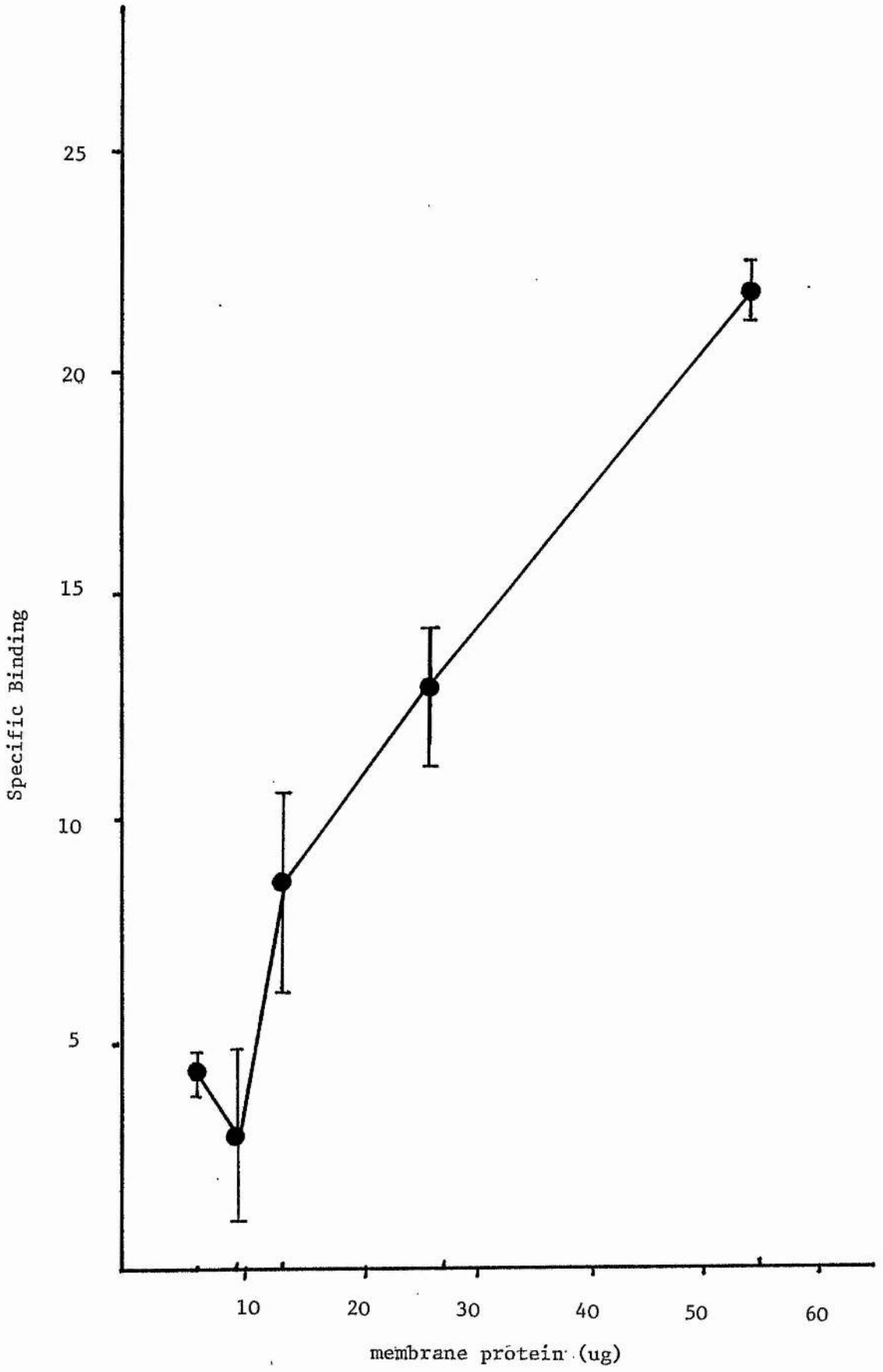

25
20
15
10
5
Specific Binding
10
20
30
40
50
60
membrane protein (ug)

Figure 18 (c)

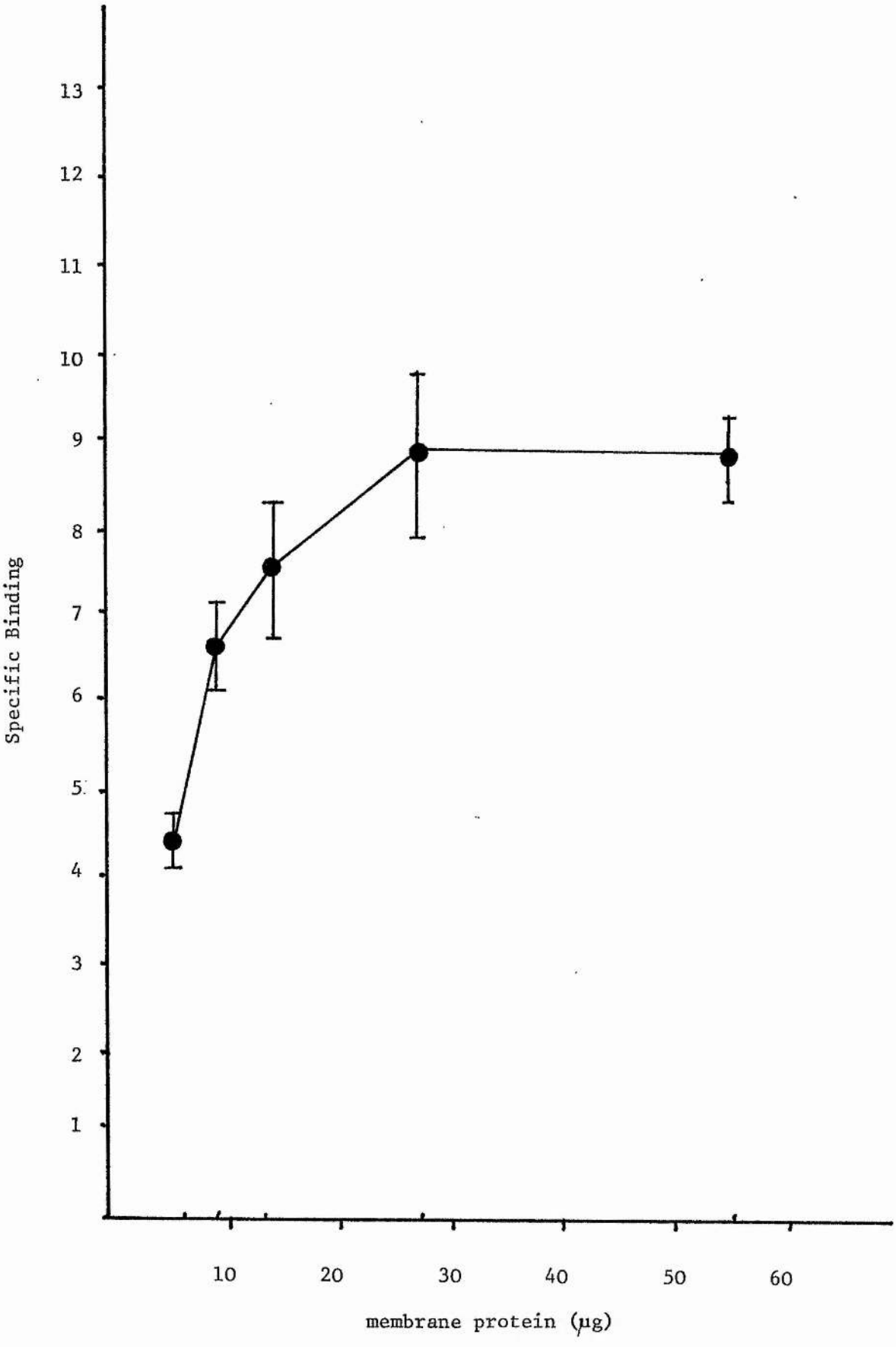

Specific Binding
13
12
11
10
9
8
7
6
5
4
3
2
1
10
20
30
40
50
60
membrane protein (µg)

Figure 18 (d)

DISCUSSION

DISCUSSION

In order to investigate the mechanism of action of a protein, to determine it's amino acid sequence, or to establish an immunoassay for the protein sufficient quantities of pure material must be available.

Colony-stimulating factors are polypetide growth factors present in low concentrations in urine, serum, and cell culture media conditioned by a number of cell types. A comparison of the relative specific activities of different sources of colony-stimulating factor is difficult because of lack of standardisation of the bioassay.

The choice of starting material is important in any protein purification scheme but is even more important in the purification of colony-stimulating factors since they are present in such low concentrations in biological fluids. Urine contains about 0.6ng CSF-1 per ml, serum about 4ng CSF-1 per ml and serum-free L-cell conditioned medium about 23ng CSF-1 per ml (Stanley and Guilbert, 1981). The richest known sources of colony - stimulating factors are conditioned media. In particular, the G.C.T. cell conditioned medium (Abboud *et al*, 1981) and Mo cell conditioned media (Golde *et al*.,1978) are rich sources of human colony-stimulating factors. However large volumes of starting material are always necessary when attempting to purify colony-stimulating factor.

The techniques which may be employed to concentrate these large volumes include pressure dialysis, ultrafiltration and lyophilisation. Ethanol or ammonium sulphate precipitation is impractical during the early stages of purification of colony-stimulating factor (Stanley and Metcalf, 1969; Stanley *et al*. 1971; Burgess *et al*. 1977b). The batchwise concentration of starting material by treatment with DEAE - cellulose or calcium phosphate gel may also purify the colony-stimulating factors (Burgess *et al*. 1976).

Vacuum rotary evaporation at 40 C may also be employed to concentrate colony-stimulating factors. In this technique some proteins are denatured at 40°C resulting in some purification of colony-stimlulating factors (Stanley and Heard, 1977). In these early purification steps it is important to remove any material which might be inhibitory to the bioassay. This inhibitory material may consist of small toxic molecules which are removed by dialysis or larger molecules which are removed by denaturation or by the chromatographic media. There has been much speculation on the role of this inhibitory material and of the role of inhibitors of granulopoiesis *in vivo*. For example, Broxmeyer *et al*. (1982) have proposed isoferritins as "feedback regulators" of granulopoiesis and monocytopoiesis. It may be that these acidic isoferritins may simply remove an essential molecule,(perhaps iron) from the medium in which the cells are grown (Jacobs, 1984).

Chromatographic procedures used in the later stages of colony-stimulating factor purification include; ion-exchange chromatography on DEAE - cellulose, calcium phosphate gel chromatography, gel filtration and hydrophobic interaction chromatography. Group specific bioaffinity chromatography on lectin-sepharose columns may be employed for some glycosylated colony-stimulating factors (Stanley *et al*, 1975) and although other types of colony-stimulating factor do not bind lectin columns this chromatographic step may remove any contaminating glycoproteins from the starting material. The use of analytical high performance liquid chromatography (H.P.L.C.) columns would be useful in the purification of small amounts of material especially in the latter stages of purification since the amount of protein is small. A major problem of working with small amounts of protein is the loss of biological activity due to denaturation or the absorption on to the surfaces of vessels and tubing used in the experiments.

In the present study the inclusion of polyethylene glycol 6000 in all buffers prevented the loss of biological activity in dilute solutions (Stanley *et al*. 1975; Stanley and Heard, 1977 and Burgess *et al*. 1977a). In addition, all glassware was washed in detergent, rinsed in large volumes of double glass distilled water, heated at 180^{o}C for 2 hours, allowed to cool and, finally, rinsed in 0.1%((w/v) bovine serum albumin (Sigma Chemical Co., St.

Louis, MO.,U.S.A.) before being allowed to dry at room temperature. All chromatographic tubing etc. was treated with 0.1% (w/v) bovine serum albumin before use.

A pure preparation of any colony-stimulating factor will help to define the specificities of the polypeptide growth factors acting during haematopoiesis. Different colony-stimulating factors act on different target cells. Some, for example Interleukin - 3, act on early stem cells such as the pluripotent stem cells and are mainly responsible for the self renewal of these cells although interleukin - 3 may induce some differentiation (Fung et al . 1984). Others, like the granulocyte-macrophage colony-stimulating factors act on cells which are programmed to differentiate along either the granulocyte or macrophage pathways (Burgess et al . 1977a) whilst others, for exammple macrophage colony-stimulating factor (M-CSF or CSF-1) (Stanley and Heard, 1977) and granulocyte colony-stimulating factor (Nicola et al . 1983 ; Nicola et al . 1985) act on cells programmed to differentiate along a single pathway. These growth factors have a somewhat more limited action and their main responisibility may be as inducers of cellular differentiation (Sachs and Lotem, 1984).See Figures 1 and 2.

Conditioned media may contain different haematopoietic growth factors showing different target cell specificities (Burgess et al . 1980) and physico-chemical properties.The G.C.T. conditioned medium used in the

present study gave two peaks of activity on gel filtration. The apparent molecular weights of these were 63,000 and 30,200 respectivly. These may represent two distinct molecular species or, the larger molecular weight moiety may represent a dimer of the smaller molecule. The active material from human placental conditioned medium, on the other hand, gave a single peak of biological activity on gel filtration. The apparent molecular weight of this material was 30,000. In addition to containing colony - stimulating factors that appeared in the breakthrough volume of hydrophobic gels, both G.C.T. conditioned medium and human placental conditioned medium contained colony - stimulating factors which bound to these gels.

The purification procedures used in the present study yielded GM-CSF from human placental conditioned medium with a specific activity of 7240 colonies / mg and this exhibited a single precipitin arc against an antiserum raised against a crude CSF preparation. The specific activity of the C.S.F. from G.C.T. conditioned medium was 4,000 colonies / mg. Although these specific activities are somewhat lower than those reported by other workers (Nicola et al.,1979 ; Wu and Fischer, 1980) it may be that a comparison between CSF preparations from different laboratories is not possible because of the variation in bioassays. Both sources of C.S.F. used in the present study were found to be reliable and gave similar results on purification using hydrophobic chromatography and gel filtration. However, G.C.T. conditioned medium may prove to

be a better source of C.S.F. if continuous cultures of the cells can be grown in serum - free medium. Gel filtration proved to be the most effective purification procedure for both sources of C.S.F.

The possible use of haematopoietic growth factors as therapeutic agents in the treatment of acute myeloid leukaemia by causing the differentiation of the malignant cells was proposed by Sachs in 1978. In 1983, Nicola et al . (1983) showed murine granulocyte-colony-stimulating factor (G-CSF) could induce the differentiation of the myeloid leukaemia cell line WEH1-3B. Recently these workers (Nicola et al . 1985) have identified a human analogue of this molecule and have shown it to be colony-stimulating factor (G.M.-C.S.F.β). It is therefore important large quantities of this factor are obtained to enable clinical trails to be carried out.A purified C.S.F. may also be useful in the treatment of immune disorders such as acquired immune deficiency syndrome, (A.I.D.S.). In 1982, Motoyoshi and coworkers reported the first clinical trials of C.S.F. In this study they administered human urinary C.S.F. to patients with leukopenia due to cytotoxic drug administration or irradation and obtained some success in restoring leukocyte numbers in these patients.

The bioassay for colony-stimulating factor is complex and takes between four and fourteen days to complete. This presents difficulties during the purification and limits

the assay for routine uses. In addition, the assay is difficult to standardise and is susceptible to various conditions such as inhibitory material in the sample to be assayed and lack of specificity. Radioimmunoassays are less time consuming, faster, easier to standardise, not susceptible to inhibitors and are specific. A pure preparation of colony-stimulating factor is nesscessary to establish a radioimmunoassay for this protein.

In the present study a colony-stimulating factor (G.M.-C.S.F.β) from human placental conditioned medium (γ (Nicola et al., 1979) was radioiodinated and the feasability of a radioimmunoassay investigated. The antiserum was raised in New Zealand white rabbits against H.P.C.M.-IIb and examined by immunodiffusion (Figure 11) and immunoelectrophoresis (Figure 12). The immunoelectrophoresis gave several precipitin arcs with the anti-C.S.F. against the H.P.C.M.-IIb and one precipitin arc with anti-C.S.F. against G.M.-C.S.F.α and one precipitin arc with the antiserum against G.M.-C.S.Fβ. Although this anti-C.S.F. inhibited colony formation in the C.S.F. bioassay the precipitin lines observed may be due to an antibody raised against a contaminating protein. Other workers have shown anti-C.S.F. preparations to be inhibitory in bioassays (Shadduck et al 1979) but it can not be said with certainty that the inhibition was due to the anti-C.S.F. antibodies alone. To rule out the possibilty of components of the rabbit serum being inhibitory in the bioassay it would be necessary to include

a sample of rabbit serum in a bioassay prior to the commencement of the immunisation program.

In the preliminary binding studies the purest preparation of G.M.-C.S.F. was radiolabelled with 125 - I and reacted with various dilutions of anti-C.S.F. The radioiodination procedure used was that of Greenwood et al., (1963). This method utilised chloramine-T and included dimethyl sulphoxide and polyethylene glycol 6000 to preserve biological activity. In the binding experiments the concentration of antiserum used was a 1:10 dilution of the neat preparation. This is the concentration of antiserum which bound 50% of a trace amount of radiolabelled G.M.-C.S.F. . Figure 14 shows a typical curve obtained when log G.M.-C.S.F. concentration was plotted against percent labelled polypeptide bound. From these preliminary studies it can be seen that a more highly purified C.S.F. preparation and/or a more specific antibody are required before a sensitive radioimmunoassay for C.S.F. can be developed.

To date there has been only one report of a radioimmunoassay for a human C.S.F. (Das et al .,1981) and there are many difficulties in adopting this approach. A pure preparation of C.S.F. must be available in sufficient quantities to raise an antiserum and to provide suitable standards. The advent of hybridoma technology may permit a monoclonal antibody to be raised against an impure C.S.F. preparation and genetic engineering techniques may

provide sufficient quantities of pure C.S.F. for use in immunoassays.

Radioimmunoassay has several disadvantages over other conventional methods of assaying biological material. The use of radioisotopes presents a small but significant hazard to workers and the environment. During the present study all work was carried out in specialised laboratories designated for radioisotope work. Radioiodine was used in fume hoods and the thyroid and femur monitored at weekly intervals. Disposal of radioactive material was carried out using procedures stipulated by the appropriate authorities. Other disadvantages of radioimmunoassay include the use of expensive counting equipment and the limited shelf-life of reagents due to the relatively short half lives of some of the radioisotopes used.

Alternative labels have been sought in recent years. The most widely used of these labels are enzymes (Voller et al. 1978; Wisdom, 1976). An enzyme-linked immunoassay has been established for a colony-stimulating factor C.S.F.-1 (Chen et al.,1983). In the present study colony-stimulating factor from pokeweed mitogen stimulated spleen conditioned medium was labelled with horseradish peroxidase and the binding of this labelled growth factor to murine bone marrow cell membrane preparations studied.

Another problem associated with radioimmunoassay is that the immunologically reactive material may not be

directly comparable to the biological activity. In 1970 Lefkowitz *et al*. showed that radiolabelled adrenocorticotropic hormone binds to receptors extracted from adrenals. The finding led to the establishment of a variant of the competitive protein binding assay, the radioreceptor assay. Radioreceptor assays have the principal advantage over radioimmunoassays in that they may reflect, more accurately, the biological activity of the material. Moreover, competitive protein binding techniques which employ membrane receptors may be useful in investigating conditions in which an abnormal receptor can lead to disease. In the present study the binding of horseradish peroxidase labelled C.S.F. to bone marrow cell membranes was investigated.

Cell-surface receptors, located in or on the plasma membrane have been shown to exist for a number of biologically active molecules including hormones, growth factors and neurotransmitters (Kahn, 1976). The binding of these molecules to the receptor may trigger the desired physiological response via a number of intracellular events. On binding to the receptor one or more of the following internal events may take place: (1), The internalisation of the molecule and it's subsequent transport to the cell nucleus where, presumeably, a genetic template is uncovered and gene expression altered (Carpenter and Cohen, 1979). (2), Phosphorylation of intracellular proteins (Cohen, 1982; Carpenter *et al*., 1979). (3), The synthesis of second messengers such as

cyclic nucleotides (Rodbell, 1980 ; Ross and Gilman, 1980) and inositol lipids (Berridge and Irvine, 1984).

Receptor molecules, therefore, play a central role in biological processes. In the present study the binding of a haematopoietic growth factor, colony-stimulating factor, to it's putative target cells, bone marrow cells, was investigated. Colony-stimulating factor from pokeweed mitogen-stimulated mouse spleen conditioned medium was labelled with horseradish peroxidase and the binding of this labelled material to mouse bone marrow membranes was studied. Although a radioreceptor assay for C.S.F. has been reported by Das et al . (1980), there is no report of a receptor-bound assay for C.S.F. using enzyme-labelled colony-stimulating factor.

In order to establish that enzyme - labelled polypeptides could be used in place of radionuclide labelled molecules for membrane receptor binding studies the following experiment was performed : Glucagon was labelled with horseradish peroxidase and the binding of this material to a rat hepatocyte membrane preparation was investigated. It was demonstrated that horseradish peroxidase labelled glucagon binds to rat hepatocyte membrane preparations and that the specific binding of enzyme labelled glucagon exhibits a linear relationship to the concentration of membrane protein. There was no significant non-specific binding observed in these studies.

Horseradish peroxidase labelled C.S.F. binds to bone marrow cell membranes and this specific binding exhibits linear relationship with membrane protein concentrations. This finding confirms the existance of membrane receptors for C.S.F. (Stanley, and Guilbert, 1981 ; Das *et al*. 1980). Isolated membranes were used in this study because they offer the advantage of stability on storage at -20 C and the receptors are more concentrated than in intact cells (Gardner, 1979).

Our understanding of the physiological events in haematopoiesis will be greatly enhanced by the study of membrane receptors for C.S.F. Different stem cell populations may respond to different colony-stimulating factors by possessing a membrane bound receptor for the particular C.S.F. involved. Other stem cells may respond to more than one subclass of haematopoietic growth factor.

In addition to leading to the development of simple assays for C.S.F. and it's receptor, the molecular mechanisms which underlie human disease can be investigated using receptor-bound studies.Downward *et al*. (1984) have proposed that an abnormal epidermal growth factor receptor may lead to cancer. Antibodies directed against a receptor for a growth factor such as C.S.F. may lead to disease (Belcher, 1979). It is interesting, therefore, to spectulate on the role of receptors for haematopoietic growth factors in diseases like leukaemia.

For example, the Friend leukaemia cell line is a mouse erythroleukaemia which does not respond to erythropoietin (Marks et al., 1974) and, therefore, this leukaemia may be due to an abnormal receptor for erythropoietin. The use of immunoassay to measure the amounts of C.S.F. in serum from leukaemia patients and the use of receptor assays to study membrane receptors for C.S.F. on leukaemic cells may greatly increase our understanding of leukaemia and related disorders. Indeed, it may be possible to use enzyme labelled C.S.F.'s to visulise receptors on cells using cytochemical techniques.

Leukaemia is a group of neoplastic blood disorders resulting in the uncontrolled proliferation and differentiation of blood cell precursors. The disease is classified by the clinical course of the disease (acute or chronic) and by the type of cell involved (Table 5). Although the cause of leukaemia is unkown it now seems likely that this disease may arise due to the expression of an abnormal gene. The abnormal gene may result from radiation damage to DNA, chemical damage to DNA, somatic mutation or the integration of foreign DNA into the genome by oncogenic viruses

In 1911 Rous discovered a RNA virus which gave rise to tumours in chickens. This retrovirus became known as the Rous sarcoma virus (R.S.V.) and since its discovery a number of viruses which cause cancer in a variety of vertebrates have been recognised (Bishop, 1983). Two

viruses which cause cancer in man have been recognised. One of these is a DNA virus, the Epstein-Barr virus, which causes glandular fever in normal hosts but which can cause Burkitt's lymphoma in patients with poor T-cell immunoregulation due to Plasmodium falciparum malaria (Whittle *et al*. 1984). The second human active oncogenic virus is the Human T-cell leukaemia virus-type I which causes adult T-cell leukaemia in restricted areas of Japan and in some other individuals (Poiesz *et al*., 1980, Poiesz *et al*., 1981, and Haynes *et al*., 1983).

Oncogenic viruses may possess a DNA genome or a RNA genome. The DNA viruses include the SV40 virus which causes tumours in monkeys and the Epstein-Barr virus. The RNA tumour viruses include the Rous sarcoma virus and the human T-cell leukaemia virus - type I. The mechanisms whereby these tumour causing viruses act are gradually becoming unveiled. In 1969 Huebner and Todaro proposed their oncogene hypothesis. They postulated the existance of oncogenes in normal cells and their activation by viruses, radiation or carcinogens to produce cancer. They further postulated that these cellular oncogenes could be transduced by viruses and so become part of the viral genome.

The discovery of reverse transcriptase led Temin (1971) to postulate the protovirus theory.Temin proposed that normal cells contain potential oncogenes which could

become viral or cellular oncogenes by somatic mutation and the action of reverse transcriptase.

The first viral oncogene to be biochemically defined was the src gene of the Rous sacroma virus (Duseberg and Vogt, 1970; Martin and Duesberg, 1972; Lai et al. 1973). The src gene was shown to be necessary for the viruses ability to cause tumours but was not essential for the survival of the virus. Since the recognition of the src gene about 16 other oncogenes have been characterised (Bishop, 1983). In addition to these oncogenes being present in viruses (v - onc) they have been shown to exsist in normal cells of all species studied, including tumours, as part of the normal genome (Scolnick et al 1973; Scolnick and Parks, 1974; Tsuchida et al., 1974; Scolnick, 1981; Stehelin et al., 1976; Frankel and Fischinger, 1976). The positions of some of these cellular oncogenes (c-onc) on human chromosomes are shown in Figure 19.

Some of the proteins encoded by these oncogenes have recently been identified. Waterfield et al., (1983) have shown that platelet-derived growth factor is encoded by c-sis. The oncogenes c-fos and c-myc have been shown to be nuclear proteins. Downward et al., (1984) have shown the erb-B product to be a portion or sub-unit of the epidermal growth factor receptor and McGrath et al., (1984) have provided evidence that the ras product acts in a similar fashion to the G protein which regulates adenyl

cyclase.

Phosphorylation and dephosphorylation reactions are present in the switching on and off of many cellular and physiological processes including cell proliferation and differentiation. In particular, the phosphorylation of tyrosine residues seem to be prevalent in mitogenic responses. Many tumour causing viruses have been shown to phosphorylate tyrosine residues and epidermal growth factor has been shown to activate a protein kinase which phosphorylates tyrosine residues of cellular proteins (Ushiro and Cohen, 1980).

It is interesting to speculate on the role of colony-stimulating factors and their receptors in haematopoiesis in the light of this new information on oncogenes and growth factors. However, the currently available sources are inadequate for the purification of sufficient quantities of these growth factors to enable any worthwhile investigations to be undertaken. Currently, the most promising possibility is the production of C.S.F. by genetic engineering techniques.

Table 5: The classification of the human leukaemias.

Classification of the Leukaemias

Acute Leukaemias

M_1	Acute myeloblastic (poorly differentiated)
M_2	Acute myeloblastic (well differentiated)
M_3	Acute promyelocytic
M_4	Acute myelomonocytic
M_5	Acute monocytic
M_6	Erythroleukaemia
	Acute megakaryoblastic
	Acute lymphoblastic : common (non-T, non-B, cALL positive)
	null - ALL (non-T, non-B)
	Thy - ALL
	B -ALL

Chronic Leukaemias

Chronic myelocytic
Chronic lymphocytic
Hairy cell leukaemia
Prolymphocytic
Dysmyelopoietic syndromes

Table 5

Figure 19: The position on human chromosomes of the cellular oncogenes; c-myc (Neel et al., 1982), c-abl (de Klien et al., 1982), c-ets (de Taisne et al., 1984), c-myb (Harper et al., 1983) and c-sis (Dallas-Favera, et al., 1981).

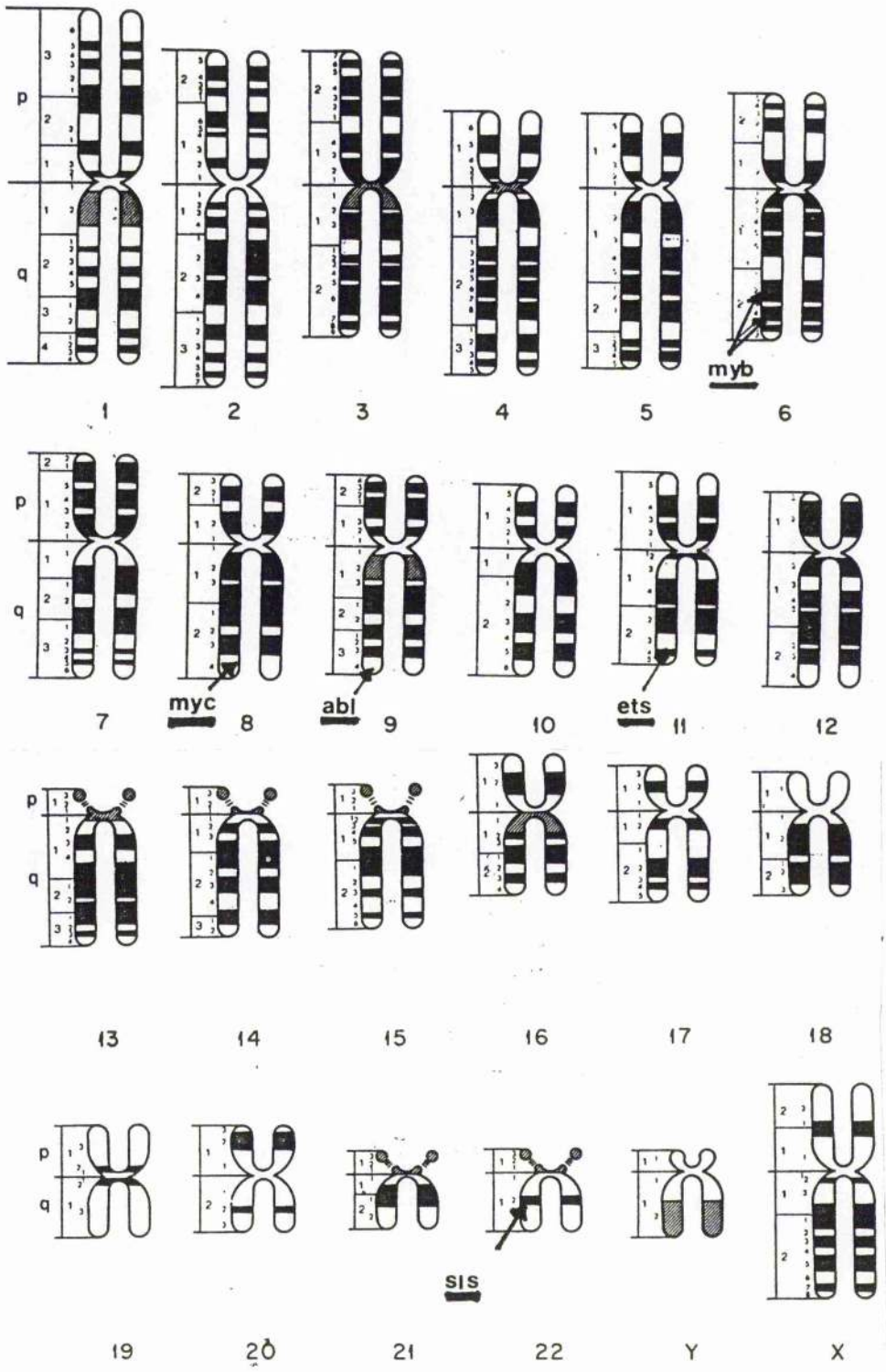
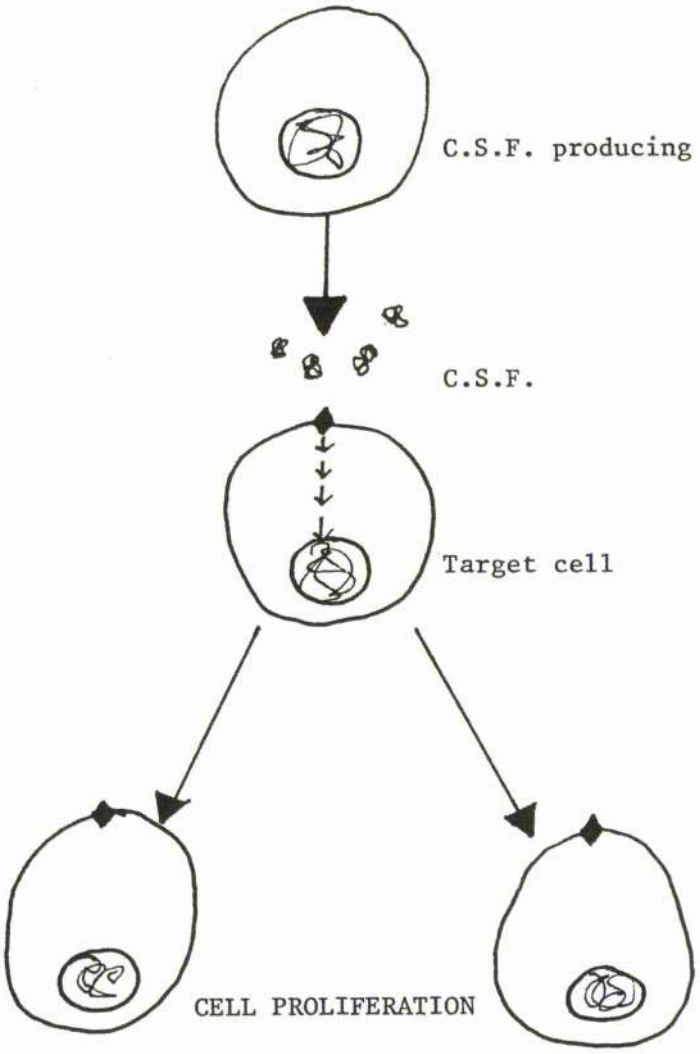

p
q
myb
1
2
3
4
5
6
myc
abl
ets
7
8
9
10
11
12
13
14
15
16
17
18
sis
19
20
21
22
Y
X

Figure 19

Figure 20: Some possible roles of C.S.F's in leukaemia.



Possible defects in leukaemia

abnormal C.S.F.
abnormal receptor
abnormal internal events
abnormal inhibitor
abnormal gene
excess C.S.F.
low inhibitors

Figure 20

GROWTH OF CHICK MYOCARDIAL CELLS IN SERUM - FREE CULTURE

INTRODUCTION

INTRODUCTION

The study of mechanisms which control cellular division and differention has been facilitated by the introduction of cell culture techniques (Paul , 1972) and cell culture has become an increasingly more important laboratory tool used in a wide variety of disciplines including embryology , laboratory medicine and biotechnology.

The growth of muscle in cell culture has provided biologists with an interesting model for cellular differentiation. There are three principal types of muscle. These are, smooth muscle, cardiac muscle and skeletal muscle. Most information regarding the growth and differentiation of muscle is available for skeletal muscle. Here, the myoblasts fuse to give rise to myotubes which, in turn give rise to the myofibrils.

Cardiac muscle has been, successfully, grown in cell culture (DeHaan, 1967). These cultured cells are capable of spontaneous activity and can be observed to beat (DeHaan, 1967).Cardiac cells in culture have applications in pharmacology to monitor the effects of drugs on the heart (Acosta *et al*., 1974) and in cardiology to monitor the effects of electrical countershock treatment (Jones *et al*., 1978).

The use of cultured chick embryonic heart cells to study the effects of electrical field stimulation led Jones

et al. (1976) to develop adult-type cardiac cell culture. These workers found it easier to insert microelectrodes into the adult type cells than into the embryonic heart muscle cells (Jones et al., 1978). They harvested myocardial cells from 8 day - old chick embryos and cultured the cells, first, in Leibowitz L-15 tissue culture medium containing 10% (v/v) foetal calf serum for two days and then in Leibowitz L-15 (without histidine) containing methionine and 0.1% (v/v) foetal calf serum. In this medium the cells differentiate into cells exhibiting adult-type morphology.

The culture of cardiac muscle cells may provide a useful tool for the study of cellular proliferation and differentiation. In studying cells in culture it is desireable to define the culture conditions. Most mammalian and avian cell cultures require serum, such as foetal calf serum, to support their growth. Serum is a complex mixture of components and probably provides, hormones, growth factors, cell attachment factors, transport proteins plus a mixture of different nutrients. The development of a serum-free growth medium would, therefore, help to define the culture conditions.

In the present study, a serum-free cell culture medium was developed for chick myocardial cells. The defined medium contained Leibowitz L-15, antimycotic solution, L-glutamine, sodium pyruvate, sodium selenite, phosphatidyl choline, glucagon, somatotropin and insulin.

In this medium the embryonic cells were observed to differentiate into adult - type myocardial cells.

MATERIALS AND METHODS

MATERIALS AND METHODS

BASIC PROCEDURE FOR THE CULTURE OF CHICK MYOCARDIAL CELLS

All culture work was carried out under a perspex hood swabbed down with 70 % (v/v) ethanol. Pipettes and dissection equipment were flamed before use. The work surface and dissection instruments were sterilised with 70% (v/v) ethanol.

The serum-containing medium was prepared by adding 1ml of L-glutamine 1ml of antibiotic / antimycotic solution, and 11ml of foetal calf serum to 100 ml Leibowitz L-15 medium. Additional L-glutamine was added after approximately four days. All these solutions were obtained, already prepared, from GIBCO, Paisley, Scotland.

An eight day old fertilised chicken egg (Wright Chicks Ltd., Newtownards, Northern Ireland) was placed in a 50 ml beaker and soaked with 70% (v/v) ethanol. Scissors were soaked in 70% (v/v) ethanol and flamed. The egg was flamed, an incission made, and the top of the egg removed. The embryo was removed, by forceps, and placed on a 70 mm plastic petri dish (Nunc, Denmark) containing 4 ml culture medium.The head of the embryo was removed and the beating heart was then carefully dissected without excess tissue. The heart was transferred to a second 70 mm plastic culture dish. The embryo was then discarded, and a further five hearts obtained in this manner.The hearts were added to a

vial tube to which sufficient 0.05% (w/v) trypsin solution had been added to cover the contents of the tube. The vial tube was incubated at 37°C for 10 minutes with constant stirring on a magnetic stirrer (M.S.E., Crawley, England) at 5 cycles per second. The supernatant became cloudy. This supernatant was discarded, and 1 ml 0.05% (w/v) trypsin solution were added and the trypsinisation procedure repeated. This supernatant was aspirated and added to a centrifuge tube containing 2ml of culture medium. This procedure was repeated until the supernatant became clear. The contents of the centrifuge tube were centrifuged for ten minutes at 2500 x g at 4°C. The supernatant was aspirated and the myocardial cells resuspended in approximately 0.5 ml fresh culture medium. Two drops of cell suspension were added to each of twelve 70mm plastic petri dishes (two dishes per heart). The culture medium was changed daily. To prevent fibroblast growth the concentration of foetal calf serum was reduced from 10% to 0.1%. The cultures were examined using an inverted phase contrast microscope (Wild, Heerburg, Switzerland).

ESTABILISHMENT OF A SERUM FREE CELL CULTURE MEDIUM FOR CHICK MYOCARDIAL CELLS

Serum was gradually replaced with, sodium pyruvate, sodium selenite, bovine serum albumin, transferrin, phosphatidyl choline, glucagon, somatotropin and insulin at various concentrations. All chemicals were of the purest grade available and were obtained from Sigma Chemical Co.

Ltd., St. Louis, MO., U.S.A. Culture and examination of the cells were the same procedures described for serum-containing cultures.

PRETREATMENT OF GLASS COVERSLIPS

In some experiments the cells were grown on glass coverslips to facilitate staining and light microscopic examination.The glass coverslips were placed in L-15 medium contaig foetal calf serum (10%), and incubated for 24 hours at room temperature. These cover slips were then washed in sterile water to remove excess medium and allowed to dry in an incubator at 37°C.

STAINING OF CELLS FOR LIGHT MICROSCOPY

A coverslip containing a monolayer of cells was submerged into Gendre fluid for approximately 15 seconds to fix the cells, and washed twice with distilled water. The cells were then immersed in toluidine blue, pH 9.2, for 30 seconds, and washed (x2) in distilled water another two times. The stained cells were then mounted using D.P.X. mounting medium. The stained preparation was then examined under a Watson Nulux 70 light microscope.

PREPARATION OF CELLS FOR TRANSMISSION ELECTRON MICROSCOPY

Primary fixation of the cells was carried out in 2.5% (w/v) glutaraldehyde, 2% (w/v) paraformaldehyde and 3%

(w/v) sucrose in 0.1m sodium cacodylate buffer, pH 7.2, at room temperature for two hours.The cells were then washed for one hour, at room temperature, in 0.1m sodium cacodylate buffer, pH 7.2 containing 3% (w/v) sucrose. Secondary fixation was performed at room temperature for one hour in 1% (v/v) osmium tetroxide on 0.1m sodium cacodylate buffer, pH 7.2.The cells were dehydrated by being taken from 70% (v/v) alcohol, 90% (v/v)alcohol to absolute alcohol. The cells spent one hour in each of these solutions, and whilst in the 100% alcohol they received three changes over the one hour period. The cells were embedded in resin by incubating in; absolute ethanol, 50% absolute ethanol/50% Spurr resin, 25% absolute ethanol/75% Spurr resin, and in 100% Spurr resin.The cells were polymerised by incubating at $70^{o}C$ for 24 hours. Silver sections (60-90nm) were cut on the L.K.B. Nova Ultramicrotome (L.K.B., Bromma, Sweden) using glass knives.The sections were double stained in uranyl acetate and lead citrate. The stained sections were then examined using a Jeol Transmission electron microscope 1005 (Jeol, Tokyo, Japan).

RESULTS

RESULTS

BASIC TECHNIQUE FOR THE CULTURE OF CHICK MYOCARDIAL CELLS

Chick myocardial cells grew readily in serum-containing cell culture medium. The average seeding density of the cells was 670 per dish. The cells formed a monolayer within 24 hours of seeding and areas of beating cells could be seen.The cells could be seen to differentiate after 24 hours in culture.

ESTABLISHMENT OF A SERUM-FREE CELL CULTURE MEDIUM FOR CHICK MYOCARDIAL CELLS

After replacing serum with various concentrations of chemicals which may be required by cells in culture, it was found that the cells grew in Leibowitz L-15 medium containing, 0.0011% (w/v) sodium pyruvate, 0.00000173% (w/v) sodium selenite, 0.004% (w/v) bovine serum albumin, 0.00001% (w/v) human transferrin, 0.001% (w/v) phosphatidyl choline, 0.000032% (w/v) glucagon, 0.0001% (w/v) somatotropin and 0.005% (w/v) insulin.The cells grew readily in this medium. They formed a monolayer after 24 hours in culture and showed spontaneous activity. The cells were well differentiated within 24 hours and were indistinguishable from cells grown in serum-containing cells.

STAINING OF THE CELLS FOR LIGHT MICROSCOPY

Staining of the cells in culture enabled the cultures to be assesed more readily than by observing cultures under the inverted phase contrast microscope. A few fibroblasts were noted in the serum-containing cultures but their growth was inhibited in serum-free cultures and in cultures with reduced serum content.

ELECTRON MICROSCOPY OF CHICK MYOCARDIAL CELLS IN CULTURE

The cells from serum-free culture were indistinguishable, by transmission electron microscopy, from cells grown in serum-containing medium. The ultrastructural features shown by electron microscopy are given in Figures 21 - 25. Figure 21 shows a coated pit and glycogen granules. Intercellular attachments may be seen in Figure 22. Longitudional sections, and transverse sections of muscle fibres can be seen in Figure 23. Figure 24 shows muscle striations with a Z line and Figure 25 shows the thick and thin filaments of cardiac muscle.

DISCUSSION

DISCUSSION

Serum in cell and tissue culture provides the growing cells with hormones, growth factors, attachment factors, transport proteins, detoxifying agents,and trace nutrients. The development of serum-free culture is desireable if we are to adequately describe the molecular mechanisms which regulate cell growth *in vitro* . See: Barnes and Sato,1980a,b.

Cardiac muscle cells in culture have been used to monitor the probable effects of drugs on heart tissue (Acosta *et al* .,1974) and to study the effects of defibrillators on the heart muscle (Jones *et al* .,1978). The devolopment of defined culture comditions for these cells may make the interpretation of these studies easier.

Early attempts to define the molecules, in serum responsible for the growth of cells in culture involved trying to isolate these factors from serum. These studies proved unsuccessful (Temin, 1972). For a reveiw of serum factors in cell culture see Gospodarowitz and Moran, 1976. However, Sato and other workers used a different approach. These workers replaced serum with various substances, and , in this way were able to develope serum-free media (Barnes and Sato, 1980a,b.

In the present study a serum-free cell culture medium was developed for chick heart cells by adding various

substances to the medium in place of serum. This empirical approach was successful in estabilishing a defined growth medium for these cells. The cells grew only on the defined medium reported in the previous section.The cultured cells were examined by light and transmission microscopy. The ultrastructural features are given in the results section and a selection of micrographs displayed in Figures 21-25.

These cultured cells were seen to beat spontaneously and may provide a useful model for further studies.It may be a useful line of study to investigate the role of polypeptide growth factors which might be responsible for the growth and/or differentiation of heart cells in culture. In the present study, cardiac cells grew and differentiated in serum-free culture. It would be likely that such growth factors (if they exist) are produced by the cardiac cells themselves.

Figure 21: Electrom micrograph, of cultured myocardial cells grown in serum-free medium, showing a coated vesicle (CV) and glycogen granules (GL). Magnification x 100,000.

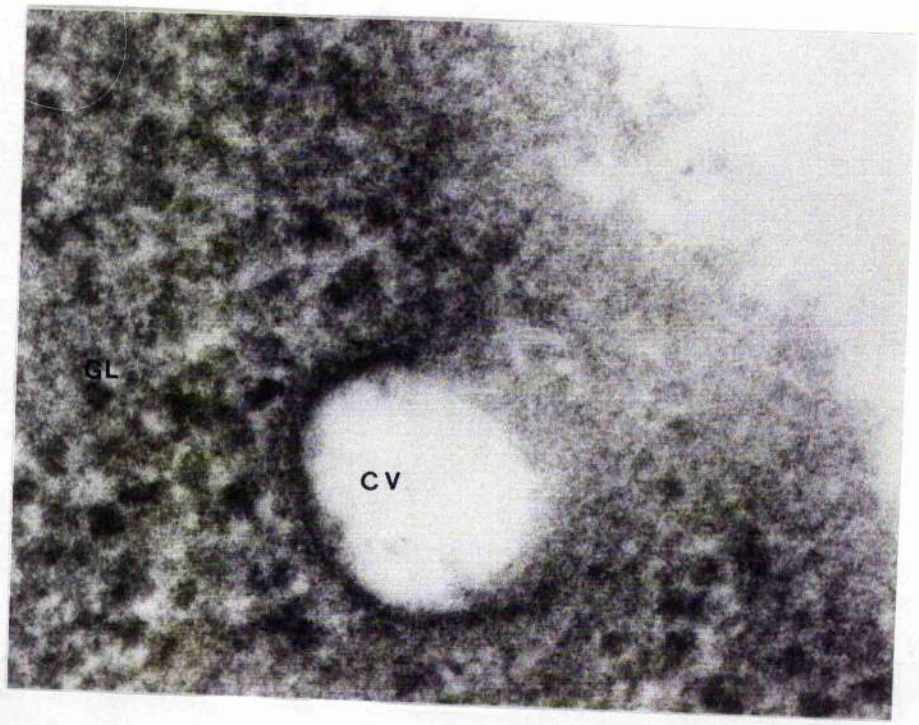


Figure 21

Figure 22: Electron micrograph of chick myocardial cells grown in serum-free medium. Intercellular attachments can be seen. Magnification x 30,000.

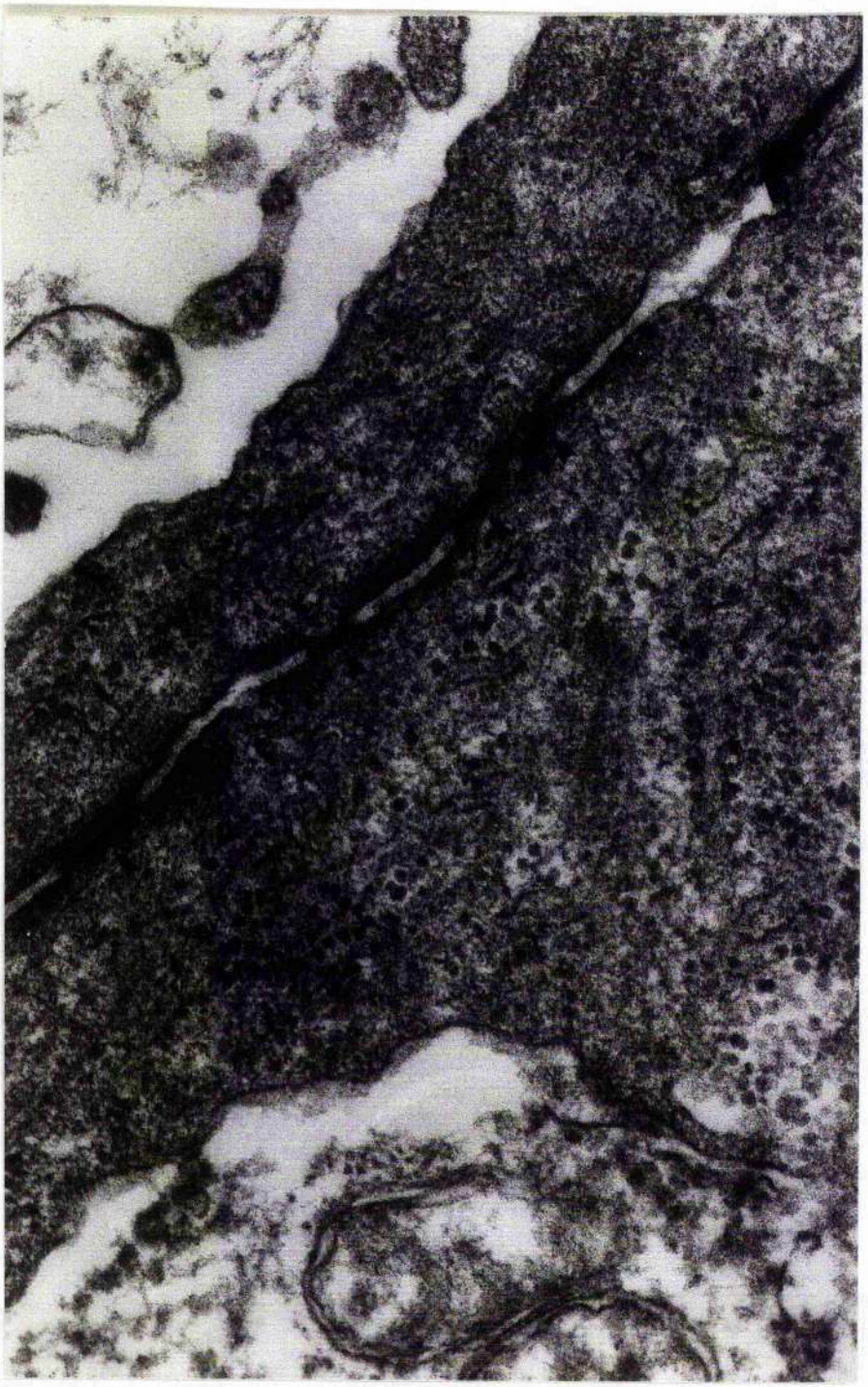


Figure 22

Figure 23: Low magnification electron micrograph showing longitudional striations (LS) and transverse sections (TS) of muscle from chick myocardial cells grown in serum-free culture. Magnification x 5000.

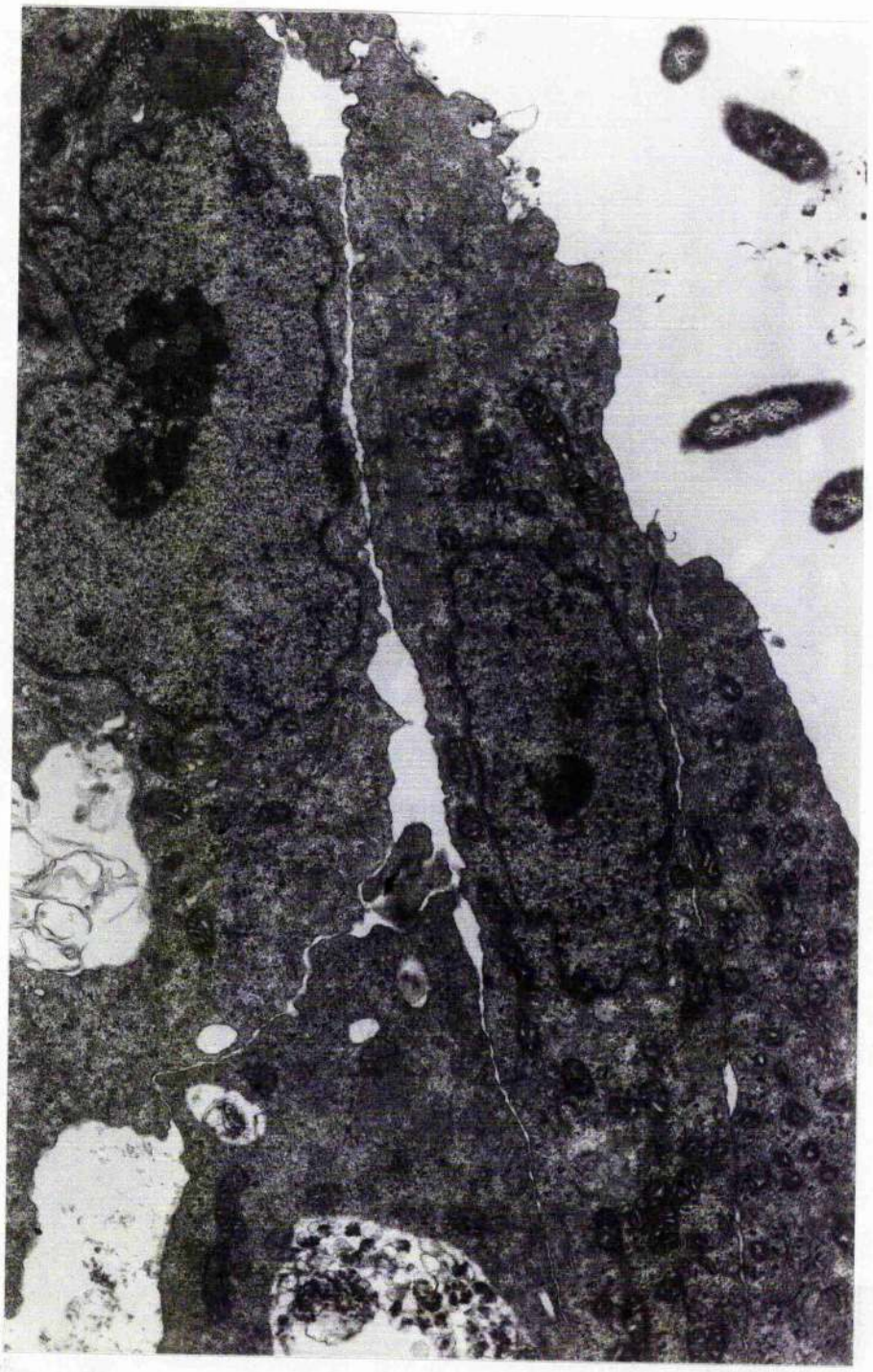


Figure 23

Figure 24: Electrom micrograph of chick myocardial cells grown in serum-free cell culture medium showing muscle striations with Z line (Z). Magnification x 20,000.

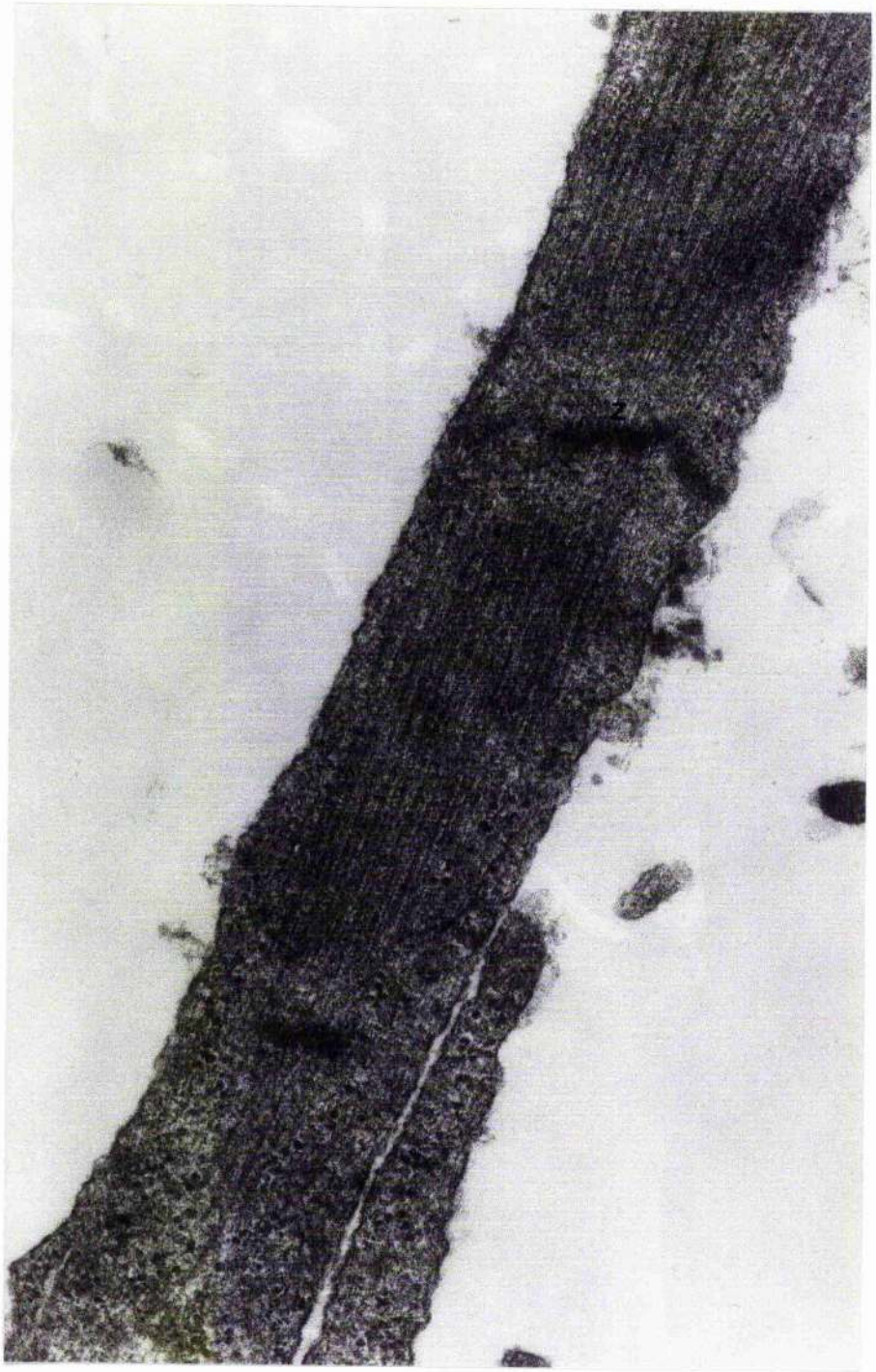


Figure 24

Figure 25: An electron micrograph of chick myocardial cells grown in culture showing the deposition of thick and thin muscle filaments. Magnification x 25,000.

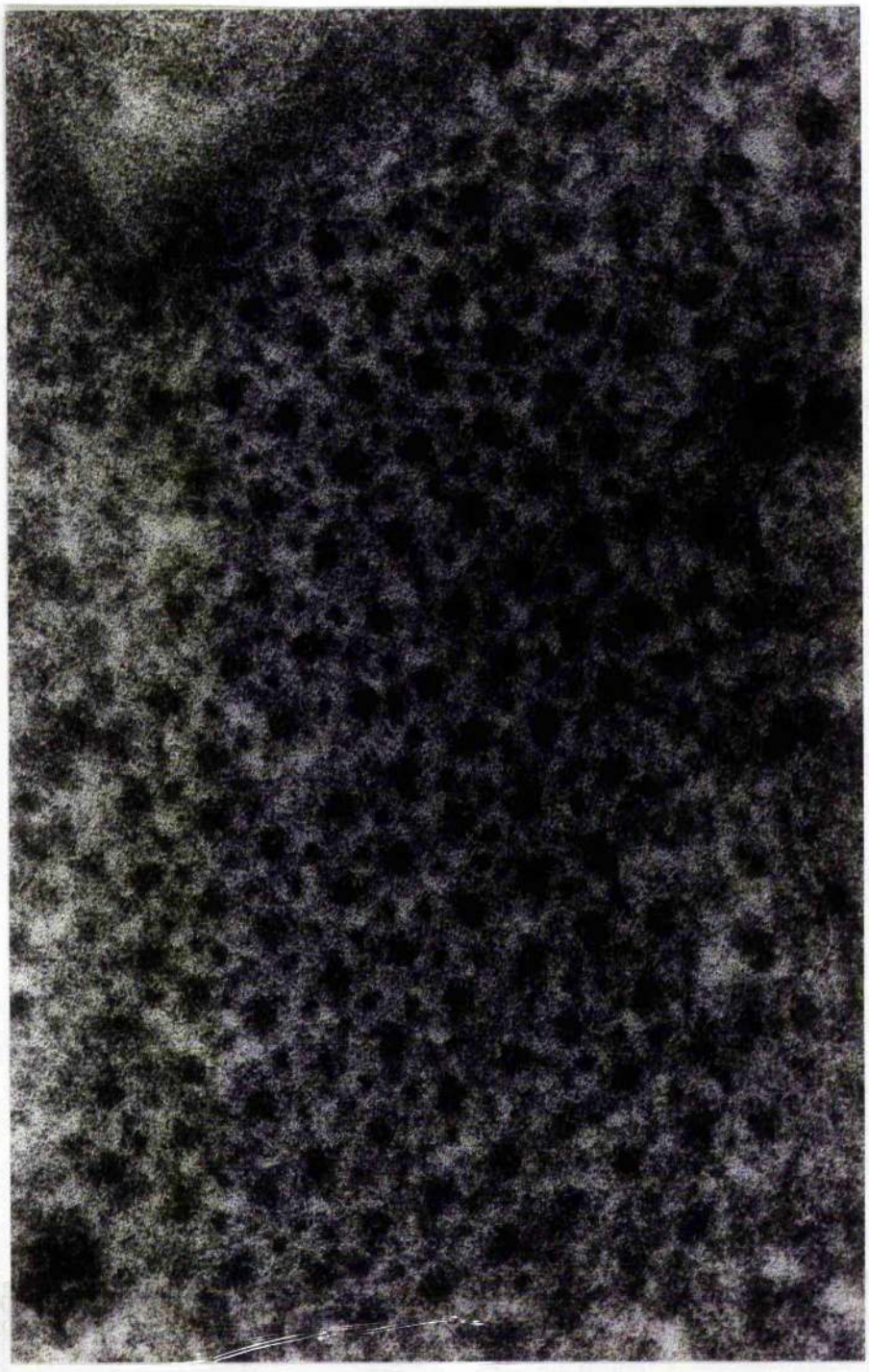


Figure 25

BIBLIOGRAPHY

ABBOUD, C.N., BRENNAN, J.K., BARLOW, G.H. and LICHTMAN, M.A.(1981). Hydrophobic adsorption chromatography of colony-stimulating activities and erythroid-enhancing activity from the human monocyte-like cell line,GCT. Blood 58 , 1148-1154.

ABILGAARD, C.F. and SIMMONE, J.V.(1967). Thrombopoiesis. Seminars in Haematology 4 ,424-452.

ACOSTA.D., WENZEL, D.G. and WHEATLEY, J.W.(1974).Beating duration of cultured rat heart cells as affected by drugs and other factors. Pharmacological Research Communications 6 263-271.

BARNES, D.W.H., FORD, C.E., GRAY, S.M. and LOUTIT, J.F.(1952). Spontaneous and induced changes in cell populations in heavily irradiated mice. Progress in Nuclear Energy (Biology), 2 , 1-10.

BARNES, D. and SATO, G. (1980a). Methods for the growth of cultured cells in serum free medium. Analytical Biochemistry 102 , 255-270.

BARNES, D. and SATO, G. (1980b). Serum-free cell culture: a unifying approach. Cell 22 , 649-655.

BENVENISTE, R. and TODARO, G.J. (1973). Homology between type-C viruses of various species as detected by molecular hybridisation. Proceedings of the National Academy of

Sciences U.S.A. 67 , 207-219.

BERSON, S.A. and YALOW, R.S. (1958). Advances in Biological and Medical Physics. 6 349-430.

BEVERLEY, P.C.L., EINCH, D. and DELIA, D. (1980). Isolation of human haematopoietic progenitor cells using monocolonal antibodies. Nature 287 , 332-333.

BISHOP, J.M. (1983). Cellular oncogenes and retroviruses. Annual Review of Biochemistry 52 , 3011-354.

BELCHER, M. (1979) Cell-surface receptors in health and disease. Clinical Chemistry 25 , 11-19.

BERRIDGE, M.J. and IRVINE, R.F. (1984). Inositol triphosphate, a second messenger in cellular signal transduction. Nature 312 , 315-321.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein - dye binding. Analytical Biochemistry 72 , 248-254.

BRADLEY, T.R. and METCALF, D. (1966). The growth of mouse bone marrow cells in vitro. Australian Journal of Experimental Biology and Medical Science 44 , 287-300.

BRADSHAW, R.A. (1978). Nerve growth factor. Annual Review of Biochemistry 47 , 191-216.

BROXMEYER, H.L., BOGNACKI, J., RALPH, P., DORNER, M.H., LU L.,and CASTRO-MALASPINA, H. (1982). Monocyte-macrophage - derived acidic isoferritins: normal feedback regulators of granulocyte - macrophage progenitor cells in vitro. Blood 60 , 595-607

BURGESS, A.W., CAMAKARIS, J. and METCALF, D. (1977a). Purification and properties of colony-stimulating factor from mouse lung-conditioned medium. The Journal of Biological Chemistry 252 , 1998-2003.

BURGESS, A.W. and METCALF, D. (1980). The nature and action of granulocyte - macrophage colony-stimulating factors. Blood 56 , 947-958.

BURGESS, A.W., METCALF, D., RUSSELL, S.H.M. and NICOLA, N.A. (1980). Granulocyte / macrophage-, negakaryocyte-, eosinophil- and erythroid- colony-stimulating factors produced by mouse spleen cells. Biochemical Journal 85 , 301-314.

BURGESS, A.W., WILSON, E.M.A. and METCALF, D. (1977b). Stimulation by human placental conditioned medium of hemopoietic colony formation by human marrow cells. Blood 49 , 573-583.

CAIS, M., DANI ,S., EDEN, Y., GANDOLFI, O., HORN, M., ISAACS, E.E., JOSEPHY, Y., SAAR, Y., SLOVIN, E. and SNARSKY, L.(1977) Metalloimmunoassay. Nature 270 534-535.

CARNOT,P. and DEFLANDRE O. (1906). Sur l'activite hemopoietique de differents organes au cours de la regeneration du sang. Comptes Rendous d'Academie Science 143 ,432-435

CARPENTER, G. and COHEN, S. (1979). Epidermal growth factor. Annual Review of Biochemistry 48 , 193-216.

CARPENTER, G., KING, L. and COHEN,S. (1978) Epidermal growth factor stimulates phosphorylation in membrane preps. in vitro. Nature 276 , 409-410.

CHEN, D-M.B. HSU, S, and LIN, H-S. (1983). Enzyme-linked immunoadsorbent assay for colony stimulating factor (CSF-1). Journal of Immunological Methods 56 , 347-357.

COHEN, P. (1982). The role of protein phosphorylation in neural and hormonal control of cellular activity. Nature 296 , 613-620.

COHEN, S. (1959). The Journal of Biological Chemistry 234 , 1129-1137

COHEN, S., LEVI-MONTALCINI, R. and HAMBERGER, V. (1954).

Proceedings of the National Academy of Sciences U.S.A. 40 , 1014

DALLA FAVERVA, R.D. GELMAN, E.P., GALLO, R.C. and WONG-STAAL, F.(1981).A human oncogene homogolous to the trans forming gene (v-sis) of simian sarcoma virus. Nature 292 31-35.

DAS, S.K., and STANLEY, E.R. (1982). Structure-function studies of a colony stimulating factor (CSF-1). The Journal of Biological Chemistry 257 , 13679-13684.

DAS, S.K., STANLEY, E.R., GUILBERT, L.J. and FORMAN, L.W. (1980). Discrimination of a colony stimulating factor subclass by a specific receptor on a macrophage cell line. Journal of Cellular Physiology 104 , 359-366.

DAS, S.K., STANLEY, E.R., GUILLBERT, L.J. and FORMAN, L.W. (1981). Human colony stimulating factor (CSF-1) radioimmunoassay; resolution of three subclasses of human colony-stimulating factors. Blood 58 , 630-641.

DE HAAN, R.L. (1967). Regulation of spontaneous activity and growth of embryonic chick heart cells in tissue culture. Developmental Biology 16 , 216-249.

DE KLIEN, A., VAN KESSEL, A.G., GROSVELD, G., BARTVAM, C.R., HAGEMEIJER, A., BOOTSMA, D., SPURR, N.K., HEISTERKAMP, N., GROFFEN, J. and STEPHENSON, J.R. (1982).

A cellular oncogene is translocated to the philadelphia chromosome in chronic myelocytic leukaemia. Nature 300 , 765-767.

DE TAISNE, C., GEGONNE, A., STEHELIN, D., BERNHEIM, A., and BERGER, R. (1984). Chromosomal localization of the human proto-oncogene c-ets. Nature 310 , 581-583.

DEUSBERG, P.H. and VOGT, P.K. (1970). Differences between the ribonucleic acids of transforming and nontransforming avian tumour viruses. Proceedings of the National Academy ofSciences, U.S.A. 67 1673-1680.

DOWNWARD, J, YARDEN, Y., MAYES, E., SCRACE, G.,TOTTY, N., STOCKWELL, P., ULLRICH, A., SCHLESSINGER, J. and WATERFIELD, M.D. (1983). Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. Nature 307 521-527.

FLEMMING, W.(1882). Zellsubstanz, Kern und Zelltheilung, Vogel-Verlag, Leipzig.

FRANKEL, A. and FISCHINGER, P.J.(1976). Nucleotide sequences in mouse D.N.A. and R.N.A. specific for Moloney sarcoma virus. Proceedings of the National Academy of Sciences U.S.A. 73 , 3705-3709.

FRASSONI, F., BACIGALUPO, A., PODESTA, M., VAN LINT, M.T., PIAGGO, G. and MARMONT, A. (1982). Generation of CFU-C

suppressor T-cells in vitro III. Failure of mitogen-primed T-cells from patients with chronic granulocytic leukaemia to inhibit the growth of normal CFU-C. Blood 60 , 1447-1452.

FUNG, M.C., HAPEL, A.J., YMER, S., COHEN, D.R., JOHNSON, R.M., CAMPBELL, H.D., and YOUNG, I.G. (1984). Molecular cloning of cDNA for murine interleukin-3. Nature 307 , 233-237.

FURTH, J. (1953). Conditioned and autoinomous neoplasms. Cancer Research 13 , 477-492.

GARDNER, J.D.(1979). Receptors for gastrointestinal hormones. Gastroenterology 76 ,202-214.

GILL, G.N. and LAZAR, C.S. (1981). Increased phosphotyrosine content and inhibition of proliferation in EGF-treated A431 cells. Nature 293 , 305-307.

GILLIS, S. and SMITH, K. (1977). Long term culture of tumor-specific T-cells. Nature 268 , 154-156.

GOLDE, D.W., QUAN, S.G., and CLINE, M.J.(1978). Human T-lymphocyte cell line producing colony-stimulating activity. Blood 52 ,1068-1072.

GOLDSTEIN, G. (1974). Isolation of Bovine Thymin; a polypeptide hormone of the thymus. Nature 247 , 11-14.

GOSPODAROWICZ, D. and MORAN, J.S. (1976). Growth factors in mammalian cell cultrure. Annual Review of Biochemistry 45 , 531-556.

GOUGH, N.M., GOUGH, J., METCALF, D., KELOS, A., GRAIL, D., NICOLA, N.A., BURGESS, A.W. and DUNN, A.R. (1984). Molecular cloning of cDNA encoding a murine haematopoietic growth regulator, granulocyte-macrophage colony stimulating factor. Nature 399 , 763-767.

GRABAR, P. and WILLIAMS, C.A. Jr.(1952). Methode permettant l'etude conjugee des propertes electrophoretiques et immunochimiques d'un melange de proteines; application au serum sanguin. Biochimica et Biophysica Acta 10 , 193-194.

GREENWOOD, F.C., HUNTER, W.M. and GLOVER J.S.(1963). The preparation of 125-I-labelled human growth hormone of high specific activity. Biochemical Journal 89 , 114-123.

GUILBERT, L.J. and ISCOVE, N.N. (1976). Partial replacement of serum by selenite, transferin, albumin and lecithin in haemopoietic cell cultures. Nature 263 , 594-595.

HAIMOVITCH, J., HORVITZ, E., NOVIK, N. and SELA, M.(1970). Use of protein bacteriophage conjugates for detection and quantitation of proteins. Biochimica et Biophysica Acta 207 115-124.

HARPER, M.E., FRANCHINI, G., LOVE, G., SIMON, M.I., GALLO, R.C. and WONG-STAAL, F. (1983). Chromosomal sublocation of human c-myb and c-fes cellular oncogenes. Nature 304 , 169-171.

HAYASHI, I., SATO, G.H. (1976). Replacement of serum by hormones permits growth of cells in a defined medium. Nature 259 , 132-134.

HAYNES,B.F., MILLER, S.E., PALKER, T.J., MOORE, J.O., DUNN, P.H., BOLOGNESI,D.P. and METZGAR, R.S.(1983) Identification of human T-cell leukemia virus in a Japanese patient with adult T-cell leukemia and cutaneous lymphomatous vasculitis. Proceedings of the National Academy of Sciences, U.S.A. 80 2054-2058.

HUEBNER, R.J. and TODARO, G.J. (1969). Oncogenes of RNA tumour viruses as determinants of cancer. Proceedings of the National Academy of Sciences U.S.A. 64 , 1087-1094.

ICHIKAWA, Y., PLUZNIK, D.H. and SACHS, L. (1966). In vitro control of the development of macrophage and granulocyte colonies. Proceedings of the National Academy of Sciences U.S.A. 56 , 488-495.

ICHIKAWA, Y., PLUZNIK, D.H. and Sachs, L. (1967). Feedback inhibition of the development of macrophage and granulocyte colonies. I. inhibition by macrophages. Proceedings of the

National Academy of Sciences U.S.A. 58 , 1480-1486.

JACOBS, A.(1984) Do acidic isoferritins regulate haemopoiesis ? British Journal of Haematology 55 , 199-202.

JAMES, R. and BRADSHAW, R.A. (1984). Polypeptide growth factors. Annual Review of Biochemistry 53 , 259-292.

JONES, J.L., LEPESCHKIN, E., JONES, R.E. and RUSH, S. (1978). Response of cultured myocardial cells to countershock-type electric field stimulation. The American Journal of Physiology 235 , H214-H222.

JONES, J.L. PROSKAUER, C., JONES, R.E. PAULL, W.K. WILLIAMS, E.H. and LEPESCHKIN, E.(1976). Differentiation of myocardial cells in monolayer culture. Federation Proceedings 35 , 628.

JOYNER, A., KELLER, G., PHILLPS, R.A. and BERNSTEIN, A. (1983). Retrovirus transfer of a bacterial gene into mouse haemopoietic cells. Nature 305 , 556-558.

KAHN, C.B.(1976). Membrane receptors for hormones and neurotransmitters. Journal of Cell Biology 70 , 261-286.

KLEIN, G. (1981) The role of gene dosage and genetic transpositions in carcinogenesis. Nature 294 , 313-318.

LAI, M.M., DUESBERG, P.H., HORST, J. and YOGT, P.K. (1973). Avian tumour virus RNA: A comparison of three sarcoma viruses and their transformation-defective derivatives by oligonucleotide fingerprinting and DNA-RNA hybridisation. Proceedings of the National Academy of Sciences U.S.A. 70 , 2266-2270.

LEFKOWITZ, R.J., ROTH, J. and PASTAN, I. (1970). Radioreceptor assay of adrenocorticotropic hormone: New approach to assay of polypeptide hormones. Science 170 , 633-635.

LEONARD, W.J., DEPPER, J.M., CRABTREE, G.R., PUMPHREY, J., ROBB, R.J., KRONKE, M., SVETLIK, P.B., PEFFER, N.J., WALDMANN, T.A. and GREEN, W.C. (1984). Molecular cloning and expression of cDNA's for the human interleukin - 2 receptor. Nature 311 , 626-631.

LESKO, L., DONLON, M., MARINETTI, G.V. and HARE, J.D.(1973). A rapid method for the isolation of rat liver membranes using an aqueous two-phase polymer system. Biochimica et Biophysica Acta 311 , 173-275.

LEUTE, J.C., ULLMAN, E.F.and GOLDSTEIN, A.(1972). Spin immunoassay of opiate narcotics in urine and saliva. The Journal of the American Medical Association 221 , 1231-1234.

MARKS, P.A., RIFKIND, R.A. and BANK, A. (1974). Mammalian erythroid cell differentiation. In: M.T.P. International Reveiw of the Sciences. Biochemistry of Cell Differention; Paul, J. (ed.). Biochemistry series 1. 9 129-160.

MARTIN, G.S. and DUESBERG, P.H. (1972). The subunit in the RNA of transforming avian tumour viruses: I: Occurence in different strains. II Spontaneous loss in nontransforming variants. Virology 47 , 494-497.

MILES, L.E.M., and HALES, C.N. (1968). Labelled antibodies and immunological assay systems. Nature 219 , 186-189.

MESSNER, H.A., TILL, J.E., MCCULLOCH, E.A. (1973). Interacting cell populations affecting granulopoietic colony formation by normal and leukaemic human marrow cells. Blood 42 , 701-710.

METCALF, D. (1976). Characteristics of B-lymphocyte colony growth in vitro. Experimental Hematology 4 , 243-245.

METCALF, D. (1977). Hemopoietic colonies: in vitro cloning of normal and leukemic cells. Springer-Verlag, Berlin.

METCALF, D., PARKER, J., CHESTER, H.M. and KINCADE, P.W. (1974). Formation of Eosinophilic-like colonies by mouse bone marrow cells in vitro. Journal of Cellular Physiology 84 , 275-290.

METCALF, D., RUSSELL, S. and BURGESS, A.W. (1978). Production of hemopoietic stimulating factors by pokeweed mitogen stimulated spleen cells. Transplantation Proceedings 10 , 91-94.

MOTOYOSHI, K., SUDA, T., KUSUMOTO, K., TAKAKU, F. and MIURA, Y. (1982). Granulocyte-macrophage colony-stimulating and binding activities of purified human urinary colony-stimulating factor to murine and human bone marrow cells. Blood 60 , 1378-1386.

MOTOYOSHI, K., TAKAKU, F., MIZOGUCHI, H. and MIURA, Y. (1978). Purification and some properties of colony stimulating factor from normal human urine. Blood 52 , 1012-1020.

MCGRATH, J.P., CAPON, D.J., GOEDDEL, D.V. and LEVINSON, A.D. (1984). Comparative biochemical properties of normal and activated human ras p 21 protein. Nature 310 , 644-649.

NEEL, B.G., JHARNWAR, S.C., CHAGANTI, R.S.K. and HAYWARD,W.S.(1982). Two human c-onc genes are located on the long arm of chromasome 8. Proceedings of the National Academy of Sciences U.S.A. 79 7842-7846.

NEWBOLD, R. (1984) Mutant ras proteins and cell transformation. Nature 310 , 628-629.

NICOLA, N.A., BEGLEY, C.G., and METCALF, D. (1985). Identification of the human analogue of a regulator that induces differentiation in murine leukaemia cells. Nature 314 , 625-628.

NICOLA, N.A., METCALF, D., JOHNSON, G.R. and BURGESS, A.W. (1978). Preparation of colony stimulating factors from human placental conditioned medium. Leukaemia Research 2 , 313-322.

NICOLA, N.A., METCALF, D., JOHNSON, G.R. and BURGESS, A.W. (1979). Separation of functionally distinct human granulocyte-macrophage colony-stimulating factors. Blood 54 , 614-627.

NICOLA, N.A., NETCALF, D., MATSUMOTO, M. and JOHNSON, G.R. (1982). Purification of a factor inducing differentiation in murine myelomonocyte leukaemia cells- identification as granulocyte colony stimulating factor. The Journal of Biological Chemistry 258 , 9017-9023.

NIKAIDO, T., SHIMIZU, A., ISHIDA, N., SABE, H., TESHIGAWARA, K., MAEDA, M.;, UCHIYAMA, T., YODOI, J. and HONJO, T. (1984). Molecular cloning of cDNA encoding human interleukin - 2 receptor. Nature 311 , 631-638.

OUCHTERLONY, O. and NILSSON, L.- A.(1978). Immunodiffusion and immunoelectrophoresis. In: Handbook of Experimental Immunology, 3rd edn. (Weir, D.M. ed.) pp.

19:1-19:44.Blackwell Scientific Publications, Oxford, London, Edinburg and Melbourne.

PAUL, J. (1972). General introduction. In: Growth, nutrition and metabloism of cells in culture. (Rothblat, G.H. and Cristofalo, V.J., eds.), vol. 1, pp. 1-9, Academic Press, New York, San Francisco and London.

PAWELETZ, N. and LITTLE, M. (1980). Cell division problems and solutions. Interdisciplinary Science Reviews 5 , 138-148.

PELICCI, P.G., LANFRANCOME, L., BRATHWAITE, M.P.., WOLMAN, S.R. and DALLA FAVERA, R. (1984). Amplyficahton of the c-myc oncogene in a case of acute myelogenous leukaemia. Science 224 , 1117-1121.

PIGOLI, G., WAHEED, A. and SHADDUCK, R.K. (1982). Observations on the binding and interaction of radiocodinated colony-stimulating factor with murine bone marrow cells in vitro. Blood 59 , 408-420.

PIKE, B.L., and ROBINSON, W.A. (1970) Human bone marrow colony growth in agar gel. Journal of Cellular Physiology 76 , 77-84.

PLUZNICK, D.H. and SACHS, L. (1965). The cloning of normal mast cells in tissue culture. Journal of Cellular Physiology 66 , 319-324.

PLUZNIK, D.H. and SACHS, L. (1966). The induction of clones of normal mast cells by a substance from conditioned medium. Experimental Cell Research 43 , 553-563.

POIESZ, B.J., RUSSCETTI, F.W., GAZDAR, A.F., BUNN, P.A., MINNA, J.D. and GALLO, R.C. (1980). Detection and isolation of type-c retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. Proceedings of the National Academy of Sciences U.S.A. 77 , 7415-7419.

POIESZ, B.J., RUSSETTI, F.W., REITZ, M.S., KALYANARAMAN, V.S. and GALLO, R.C. (1981). Isolation of a new type-c retrovirus (HTLV) in primary uncultured cells of a patient with Sezary T-cell leukaemia. Nature 294 , 268-271.

PRCHAL,J.T., THROCKMORTON, D.W., CARROLL, A.J., FUSSON, E.W., GAMS, R.A. and PRCHAL, J.F. (1978). A common progentor for human myeloid and lymphoid cells. Nature 274 , 590-591.

PRATT, J.J., WOLDRING, M.G.and VILLERIUS, L.(1978). Chemiluminescence-linked immunoassay. The Journal of Immunological Methods 21 179-184.

ROBINSON, W.A. and PIKE, B.L. (1970) Leukopoietic activity in human urine. The New England Journal of Medicine 282 , 1291-1297.

RODBELL, M.(1980). The role of hormone receptors and G.T.P.-regulatory proteins in membrane transduction. Nature 284 17-22.

ROSS, E.M. and GILMAN, A.G. (1980). Biochemical properties of hormone - sensitive adenylate cyclase. Annual Reveiw of Biochemistry 49 533-564.

ROUS, P. (1911). A sarcoma of the fowl transmissible by an agent separable from tumour cells. Journal of Experimental Medicine 13 , 397-411.

RUDLEY, H.E. (1983). Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature 304 , 602-606.

SACHS, L. (1978). Control of normal cell differectiation and the phenotypic reversion of malignancy in myeloid leukaemia. Nature 274 , 535-539.

SACHS, L. and LOTEM, J.(1974). Haematopoietic growth factors. Nature 312 , 407.

SCATCHARD, G. (1949). The attractions of proteins for small molecules and ions. Annals of the New York Academy of Sciences 51 , 660-672.

SCHNEIDER, A. (1873). Untersuchungen uber Plathelminthem.

Jahrb, Oberhess, Ges. Natur - Heik 14.

SCOLNICK, E.M. and PARKS, W.P. (1974). Harvey sarcoma virus; A second murine type C sarcoma virus with rat genetic information. Journal of Virology 13 , 1211-1219.

SCOLNICK, W.M., RANDS, E., WILLIAMS, D. and PARKS, W.P. (1973). Studies on the nucleic acid sequences of Kirsten sarcoma virus; a model for formation of a mammalian RNA-containing sarcoma virus. Journal of Virology 12 , 458-463.

SHADDUCK, R.K., WAHEED, A., PIGOLI, G., BOEGEL, F. and HIGGINS, L. (1979). Fractionation of antibodies to L-cell CSF by affinity chromatography. Blood 53 , 1182-1190.

SHERWOOD, J.B. and GOLDWASSER, E. (1979). A radioimmunoassay for erythropoietin. Blood 54 , 885-893.

SREDNI, B., KLAECHMAN, Y., MICHLIN, H. et al (1976). Development of colonies in vitro of mitogen stimulated mouse T-lymphocytes. Nature 259 , 130-132.

STANLEY, E.R. (1979). Colony-stimulating factor (CSF) radioimmunoassay-detection of a CSF subclass stimulating macrophage production. Proceeding of the National Academy of Sciences (USA) 76 , 2969-2973.

STANLEY, E.R., BRADLEY, T.R. and SUMNER, M.A. (1971).

Properties of the mouse embryo conditioned medium factor(s) stimulating colony formation by mouse bone marrow cells in vitro. Journal of Cellular Physiology 78 , 301-318.

STANLEY, E.R., CHEN, D.M. and LIN, D.S. (1978). Induction of macrophage production and proliferation by a purified colony stimulating factor. Nature 274 , 168-170.

STANLEY, E.R., and GUILBERT, L.J. (1980). Regulation of macrophage production by a colony-stimulating factor, in Van Furth, R (ed) : Mononuclear Phagocytes. Functional Aspects, part 1. The Hague, Martinus N., Hoff pp.417-433.

STANLEY, E.R. and GUILBERT, L.J. (1981). Methods for the purification, assay, characterization and target cell binding of a colony-stimulating factor (CSF-1). Journal of Immunological Methods 42 , 253-284.

STANLEY, E.R., HANSEN, G., WOODCOCK, J. and METCALF, D. (1975). Colony stimulating factor and the regulation of granulopoiesis and macrophage production. Federation Proceedings 34 , 2272-2278.

STANLEY, E.R. and HEARD, P.M. (1977). Factors regulating macrophage production and growth. Purification and some properties of CSF from medium conditioned by mouse L cells. Journal fo Biological Chemistry 252 , 4305-4312.

STANLEY, E.R. and METCALF, D. (1969) Partial purification

and some properties of the factor in normal and leukaemic human urine stimulating mouse bone marrow colony growth in vitro. Australian Journal of Biological and Medical Science 47 , 467-483.

STANLEY, E.R., METCALF, D., MARITZ, J.S. and YEO, G.F. (1972). Standardised bioassay for bone marrow colony stimulating factor in human urine; levels in normal man. Journal of Laboratory and Clinical Medicine. 79 , 657-668.

STEHELIN, D., VARMUS, H.E., BISHOP, J.M. and VOGT, P.K. (1976). DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. Nature 260 , 170-173.

STRASSBURGER, E. (1875). Zellbilding und Zelltheilung, I. Aufl, Fischer-Verlay, Jena.

TEMIN, H.M (1971). The protovirus hypothesis: speculation on the significance of RNA-directed DNA synthesis for normal development and for carinogenesis. Journal of the National Cancer Institute 46 , 3-7.

TEMIN, H.M, PIERSON JR., R.W., and DULAK, N.C. (1972). The role of serum in the control of multiplication of avian and mammalian cells in culture. In; growth, nutrition and metabolism of cells in culture (Rothblat, G.H. and Cristofalo, V.J., eds.), vol.1, pp 49-81. Academic Press, New York, San Franciso and London.

THOMAN, M.L. and WEIGLE, W.O. (1983). Identification of a B-cell growth-promoting material utilizing the BCL cell line. The Journal of Immunology 130 , 233-236.

TILL, J.E. and MCCULLCOH, E.A. (1961). Spleen colony technique demonstrates the exsistance of pluri-potent stem cell which can give rise to colonies of erythrocytes and grannulocytes. Radiation Research 14 , 213-222.

TSUCHIDA, N., GILDEN, R.V. and HATANAKA, M. (1974). Sarcoma-virus-related RNA sequences in normal rat cells. Proceedings of the National Academy of Sciences USA 71 , 4503-4507.

SOINI, E,. and HEEMMILA, I. (1979). Fluroimmunoassay: Present status and key problems. Clinical Chemistry 25 , 353-361.

USHIRO, H. and COHEN, S. (1980). Identification of phosphotyrosine as a product of epidermal growth factor-activated protein kinase in A-431 cell membranes. The Journal of Biological Chemistry 255 , 8363-836.

VOLLER, A., BARTLET, A. and BIDWELL, D.E. (1978). Enzyme immunoassays with special reference to the ELISA techniques. Journal of Clinical Pathology 31 507-520.

WATERFIELD, M.D., SCRACE, G.T., WHITTLE, N., STROOBANT, P.,

JOHNSSON, A., WASTESON, A., WESTERMARK, B., HELDIN, C-H., HUANG, J.S. and DEUEL, T.F. (1983). Platelet-derived growth factor is structurally related to the putative transforming protein p28 of simian sarcoma virus. Nature 304 , 35-39.

WAYMOUTH, C. (1972). Construction of tissue culture media: In growth, nutrition and metabolism of cells in culture (Rothblat, G.H. and Cristofalo, V.J. eds.), vol. 1. pp 11-47, Academic Press, New York, San Francisco and London.

WHITTLE, H.C., BROWN, J., MARSH, K., GREENWOOD, B.M., SEIDELIN, P., TIGHE, H. and WEDDERBURN, L. (1984). T-cell control of Epstein-Barr virus-infected B cells is lost during P. Falciparum malaria . Nature 312 , 449-450.

WIDE, L. and KILLANDER, A. (1971). A radiosorbent technique for the assay of serum Vit-B12. The Scandinavian Journal of Clinical and Laboratory Investigations 27 , 151-159.

WISDOM,G.B. (1976). Enzyme-immunoassay. Clinical Chemistry 22 , 1243-1255.

WU, M.-C., and FISCHER, R.A. (1980). Granulocyte and macrophage colony-stimulating factor from human placental conditioned medium. Biochemistry 19 , 3846-3850.