THE CHARACTERISATION OF NOVEL GROWTH FACTORS FROM NORMAL AND TUMOR TISSUES

by

© JOHN M. ROWE, B.Sc, M.Sc.

A thesis submitted to the Faculty of Graduate Studies, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Department of Physiology
University of Manitoba

July, 1981

THE CHARACTERISATION OF NOVEL GROWTH FACTORS FROM NORMAL AND TUMOR TISSUES

BY

JOHN M. ROWE

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

ACKNOWLEDGEMENT

Sincere appreciation is extended to my thesis advisor, Dr. Henry G. Friesen for his guidance, constructive criticism and direction which have unfailingly contributed to the completion of this study.

I am also indebted to Drs. Robert Shiu, Sam Balk, and Gavin Cheng for their excellent advice and helpful discussions which have been an invaluable contribution. The consideration and assistance given to the writer and this study by Drs. J. Kellett, T. Tanaka, R. Drake, M. Waters and I. Worseley have been greatly appreciated. Special thanks also to Dr. Y. Imai for his contributions in the measurement of EGF.

The mutual support and positive encouragement of the following graduate students has also been a source of inspiration: S. Kasper, C. Shellenberg, M. Levy, C. Leung. The writer is appreciative as well for the cooperation and technical assistance of Gail Drake, Helle Cosby and Bill Sloane.

The financial assistance of the Medical Research Council (Canada) and the National Cancer Institute (Canada) made this research project possible.

Finally, I would like to thank my wife and best friend, Helen, without whose understanding, quiet encouragement and patience this study would not have been possible.

ABSTRACT

The endogenous production of growth factors by neoplastic cells in culture has been proposed as a mechanism by which neoplastic cells may gain a selective growth advantage over their normal counterparts *in vivo*.

A novel growth factor has been identified in extracts of human mammary tumors obtained at biopsy. The human mammary tumor derived growth factor (h.MTGF) was found to stimulate the growth of 3 non-transformed cells in culture.

The partial purification and characterisation of this h.MTGF was monitored using the stimulation of growth in rabbit fetal chondrocytes maintained in F-10 medium supplemented with 10% FBS. The h.MTGF was found to have a molecular weight of 10-30,000 daltons and an isoelectric point of 8-9.3. The h.MTGF was trypsin sensitive, acid labile, and sensitive to denaturing agents, 8 M urea and 6 M guanidine-HCl.

These properties distinguish the h.MTGF from the other major classes of growth factors such as epidermal growth factor, platelet derived growth factor and the somatomedin family of growth factors.

The h.MTGF was 10% as active as a commercial preparation of bovine pituitary Fibroblast Growth Factor and had a comparable activity with two partially purified growth factors derived from human brain.

The conditions for optimal extraction have been determined. Purification using ion exchange chromatography and isoelectric focusing result in a high yield of recovery and an estimated purification of 100 fold from the crude extract.

The presence of a second family of acid-stable polypeptide growth factors was identified in the human mammary tumor extract. These growth factors were found to promote the growth of a human mammary carcinoma cell line. Gel filtration on Sephadex G-100 in 1 M acetic acid showed the growth promoting activity to elute with proteins of molecular weight ~80,000, 4,000-30,000 and ~4,000.

It is postulated that these growth factors play a role in the fibrovascular changes of malignant tumors and promote the autonomy of neoplastic cell proliferation.

CONTENTS

LIST OF ABBREVIATIONS

Growth factors:

EGF	Epidermal growth factor
FGF	Fibroblast growth factor
IGF	Insulin-like growth factor
MTE	Mammary tumor extract
MTGF	Mammary tumor growth factor
NGF	Nerve growth factor
PDGF	Platelet derived growth factor
SGF	Sarcoma growth factor

Peptide hormones:

GH	Growth hormone
PRL	Prolactin

Prefix to hormones and growth factors:

b	bovine
h	human
m	mouse

Others:

BS	Bovine serum
BSA	Bovine serum albumin
CaBP	Cancer basic protein
cm	centimetre
CPM	counts per minute
CM52	Carboxymethyl cellulose 52
C-50	Carboxymethyl sephadex C-50
CR	Collaborative Research
Cyt.C	Cytochrome C
^{o}C	degree centigrade
DMBA	7,12-dimethylbenzanthracene
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
ER	Estrogen receptor
FBS	Fetal bovine serum
g	gram
GIBCO	Grand Island Biological Company
hr	hour
IEF	Isoelectric focusing
IP	intra-peritoneal
INS	Insulin
^{125}I	^{125}-Iodine
kg	kilogram
L	liter
LKB	gamma counter
M	Molar
mg	milligram
ml	milliliter
nM	millimolar

min	minute
mol.wt.	molecular weight
MBP	Myelin basic protein
MMI	Macrophage migration inhibition
MSA	Multiplication stimulating activity
MuSV	Murine sarcoma virus
ng	nanogram
NIH	National Insitute of Health
NRK	Normal rat kidney
NEN	New England Nuclear
OCS	Organic counting scintillator
ovalb	ovalbumin
pg	picogram
P	probability
pI	isoelectric point
PAG	Polyacrylamide gel
PBS	Phosphate buffer saline
PA	Plasminogen activator
RIA	radioimmunoassay
RFC	Rabbit fetal chondrocytes
SD	standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate Polyacrylamide gel electrophoresis
SV-40	Simian virus 40
T	Trypsin
TI	Trypsin inhibitor
µg	microgram
µl	microliter
uCi	microcurie
v/v	volume/volume
Vo	void volume
w/v	weight/volume
xg	gravitational force

LIST OF FIGURES

LIST OF FIGURES (CONT'D)

LIST OF TABLES

LIST OF PLATES

SECTION I

INTRODUCTION

The regulation of cell growth

Cell growth, as defined by an increase in cell size and cell number and its regulation, involves a complex interaction of genetic, hormonal, biochemical and nutritional factors.

Growth factors - historical perspectives

The experimental observations on the effects of endocrine gland ablation (hypophysectomy, ovariectomy, adrenalectomy, etc.) coupled with hormone replacement therapy (tissue extracts and purified hormones) has led to the concept that trophic hormones are involved in the regulation of mammalian cell proliferation *in vivo*.

The important role played by growth hormone secreted by the pituitary gland, in addition to the hormones secreted by the thyroid, gonads and pancreas in the regulation of normal skeletal growth and development has long been recognised. The practical difficulties inherent in *in vivo* experiments has limited the understanding of trophic hormone-dependent growth. This led investigators to search for alternative experimental approaches to examine the problem. Target cells in culture might offer a useful approach, but a growing realisation emerged that some trophic hormones, most notably growth hormone, lack a direct mitogenic activity *in vitro*. This point of view has led to the proposal that "secondary growth

factors" may mediate the action of some trophic hormones *in vivo*.

In parallel with these advances, cell biologists recognised that most animal cells in tissue culture required, in addition to the nutrient media, an embryo extract or a serum supplement. Consequently, a number of investigators have focused their attention on the isolation of these trophic factors present in serum and a variety of tissue extracts.

The combined efforts of cell biologists and endocrinologists towards the isolation and characterisation of these growth factors has led to some fruitful approaches and modest advances. With the widespread use of tissue culture techniques, interest in this field has rapidly expanded.

The term growth factor has been used to describe those factors which stimulate DNA synthesis and cell proliferation in culture, the formation of an extracellular ground substance and prolongation of cell survival. Due to the growing realisation that many of these growth factors are closely related, if not identical, I have limited my discussion to some of the major and particularly more recent and germane advances. The reader is referred to the extensive reviews and monographs that consider growth factors more generally and comprehensively. Consequently, attention is focused here on the growth factors deemed pertinent to the experimental work carried out in this thesis.

The clinical relevance of the investigations in this area can readily be appreciated when one realises that the major cause of death in western societies is attributable to diseases such as atherosclerosis and cancer, both of which involve abnormal cell proliferation.

Growth factors present in serum

(i) The Somatomedins

The stimulation of skeletal growth at the epiphysial plate *in vivo* is influenced by growth hormone (65).

In 1957 Salmon and Daughaday (135) demonstrated that the incorporation of radioactive sulphate into chondroitin sulphate of cartilage *in vitro*, was not directly stimulated by GH. The serum from hypophysectomised rats also had no sulphate incorporation activity. However, the serum from hypophysectomised rats treated with GH *in vivo*, stimulated the uptake of sulphate into cartilage fragments. From these findings it was hypothesised that GH mediated its effects on skeletal growth *in vivo* through the generation of 'sulphation factors' in the circulation.

Later studies showed that the serum factors, under the regulation of GH *in vivo*, stimulated DNA synthesis in cartilage (35). Further studies revealed that crude extracts of serum and plasma, containing significant

sulphation activity, were also mitogenic for Hela cells in culture (136), and had an insulin-like effect on muscle and adipose tissue (51, 137).

The designated term somatomedin was therefore introduced by Daughaday et al in 1972 (36) for substances fulfilling the following four criteria: their concentration in serum is regulated by growth hormone, they stimulate sulphate incorporation into the proteoglycans of cartilage, they have mitogenic activity in fibroblasts, and have insulin-like effects on adipose and muscle tissue.

There are currently four groups of factors which have been purified which fulfill these criteria, somatomedin A (73,158), somatomedin C (162), non-suppressible insulin-like activity-soluble (NSILAs) (52) now designated insulin-like growth factors (IGFs) I and II (129) and multiplication stimulating activity (MSA) (40,41, 119,108) Table 1.

Although the somatomedins were originally proposed to be under the regulatory influence of growth hormone, further studies have shown that the somatomedin levels are influenced by prolactin (5, 50), insulin (34,114) thyroid hormone (18) estrogen (115,171) glucocorticoids (43,160) and nutritional factors (116,117).

Utilising a variety of techniques (bioassay, radioimmunoassay, radioreceptor assay) the somatomedin levels are however, generally lower in hypopituitary dwarfs

Table 1

Somatomedin family

Name	source	mol. wt.	isoelectric point	reference
Somatomedin A_1	h.plasma	~7000	neutral	73, 158
Somatomedin A_2	h.plasma	~7000	neutral	54
Somatomedin C	h.plasma	7600	basic	162
Insulin-like growth factor I	h.plasma	7649	basic	129
Insulin-like growth factor II	h.plasma	7471	neutral	129
Multiplication stimulating activity	rat serum, liver cell culture med.	~10000	neutral	40 41 119 108

compared to normal subjects and higher in acromegalic subjects (117). Experimental studies have shown that administration of somatomedin-A to hypophysectomised rats failed to increase the tibial width (53). The use of impure preparations of somatomedin have been reported to increase the body weight and length of Snell-dwarf mice (159). The use of highly purified preparations of somatomedin will be required to confirm these findings, and provide the final proof of the hypothesis proposed by Salmon and Daughaday (135)

(ii) Relationship between the somatomedins and insulin

The importance of insulin for the growth of cells in culture was first reported by Gey and Thalhimer (56) and postulated by Young, 1940 (173) to mediate the effects of growth hormone in vivo. The mitogenic effect of insulin for a variety of cultured cells has been described using high, unphysiological concentrations of insulin (63).

The early demonstration that purified preparations of somatomedin C competitively inhibited insulin from binding to its cell membrane receptor (78) supported the notion that the somatomedins and insulin were related. The somatomedins however, exhibit relatively weak insulin-like anabolic effects, and are about 150 times more potent than insulin in stimulating the growth of

cultured fibroblasts (174). These findings suggested that the mitogenic effects of the somatomedins were not mediated via the insulin receptors. Later studies demonstrated that the somatomedins interacted with specific, high affinity receptors which were distinct from the insulin receptors (107, 162).

The use of specific radioimmunoassays have shown that somatomedin C and IGF I behave almost identically (161). Analysis of the primary structure of somatomedin C has revealed a considerable, but not complete identity, with IGF I (145). Furthermore, the primary structure of IGF I and II have shown a closer relationship to proinsulin than to insulin (127, 128, 129). The amino-acid composition of somatomedin A_1 and A_2 and MSA, however, do not suggest a structural relationship to insulin and IGF I and II (54).

These findings have led to the proposal that certain families of growth factors and hormones, with homologous sequences, may have evolved through a process of natural selection, from a common ancestral gene (12).

(iii) Direct mitogenic effect of growth hormone

The hypothesis that the action of growth hormone *in vivo* was mediated via the somatomedins was due to the lack of a direct mitogenic response to growth hormone *in vitro*. However, further studies have shown

that growth hormone can directly stimulate the proliferation of a variety of cells in culture, such as, rabbit arterial medial cells (93), erythroid stem cells (58) and a rat lymphoma (Nb2) cell line (147).

(iv) Platelet derived growth factor

It is generally accepted that the growth of virtually all types of cells in culture requires the presence of a serum supplement (63) .

Using normal chicken fibroblasts, Balk (6) showed that chicken plasma was less effective in the stimulation of cell division when compared to serum, and postulated that growth promoting effect was either derived from inactive plasma precursors or from platelets during the clotting process. Later studies revealed that the mitogenic activity lacking in plasma could be restored by the addition of platelet extract (92, 132).

Purification of platelet derived growth factor (PDGF) has been described by Antoniades et al (1979) (2) and Heldin et al (1979) (75). The PDGF was reported to have an isoelectric point of 9.8 and a molecular weight of 13-16,000 daltons, as judged by gel filtration in 1 M acetic acid and sodium dodecyl gel electrophoresis under reducing conditions. The yields however, were extremely low, approximately 28 µg was purified from 500 units of clinically outdated platelets, or 250

liters of blood. An improved purification scheme for PDGF has recently been reported by Heldin et al (1981) (74) with a yield of 500 µg from 600 liters of blood. These authors reported that the molecular weight of PDGF as determined by sedimentation-equilibrium analysis was approximately 33,000. In the presence of reducing agents the biological activity of PDGF is irreversibly destroyed and converted to sub-units of 17,000 and 14,000 daltons. Unreduced PDGF is stable under denaturing conditions such as 6 M urea and 4 M guanidine-HCl.

The PDGF is mitogenic for a variety of cells in culture such as monkey smooth muscle cells (132), 3T3 cells (92) human glial cells and fibroblasts (169, 170).

It has been suggested that PDGF may promote tissue repair, such as wound healing. It has further been proposed by Ross and Glomset that denudation of the vascular endothelium causes platelet aggregation at the site of exposed collagen and the release of PDGF (133). The resultant proliferation of smooth muscle cells thereby contributes to the atherosclerotic plaque.

Growth factors purified from tissues

The growth factors purified from tissue which have received considerable attention include nerve growth

factor (NGF), epidermal growth factor (EGF) and fibroblast growth factor (FGF).

(i) Nerve growth factor

The promotion of nerve outgrowth and subsequent innervation of mouse sarcomas 180 implanted into the body wall of a chick embryo, was first demonstrated by Bueker (1948) (17), and later by Levi-Montalcini and Hamburger (1951) (96). The substance responsible was later named nerve growth factor (NGF) and significant amounts have been isolated from the mouse submaxillary gland (Cohen, 1960) (30).

The NGF can be isolated from the mouse submaxillary gland at neutral pH as part of a high molecular weight complex called 7SNGF (163,164). The complex contains 3 sub-units designated α, β, and γ. The NGF activity is associated with the B sub-unit composed of 2 non covalently linked chains of 13,000 daltons (1).

NGF in vitro elicits a diverse pleiotypic response in cultured neurons. These include stimulation of RNA synthesis, protein synthesis, lipid synthesis, and enhanced transport of glucose and nucleotides (98, 99). The pleiotypic effect of NGF and de novo synthesis of neurotubules are proposed to be the mechanism for neurite extension.

These insulin-like properties of NGF are further strenthened by a 21% homology in the amino-acid sequence

of NGF and pro-insulin, and similarities in the secondary and tertiary structure between NGF and the insulin monomer (99). These findings might therefore suggest that NGF and insulin evolved from an ancestral gene.

Maximal survival and morphological differentiation of neonatal sympathetic neurons in culture has been reported with NGF at concentrations as low as 5 ng ml^{-1}. Withdrawal of NGF leads to rapid neuronal and cell body degeneration and finally to cell death (69). When injected in vivo, NGF causes extensive hypertrophy of the sympathetic ganglia in newborn mice. Conversely, the injection of antiserum to NGF in neonatal mice causes near complete repression of the developing sympathetic nervous system, and destruction of the vertebral ganglia (99).

There are conflicting reports regarding the decrease of circulating levels of immunoreactive NGF following removal of the salivary gland (77,110). Detection of NGF in other tissues such as spleen, heart, kidney, skeletal muscle and granulation tissue have been reported (76, 97). These findings might suggest a role for NGF on the cell metabolism in extraneuronal cells.

(ii) Epidermal growth factor

Mouse epidermal growth factor (m-EGF) was isolated by Cohen, 1962 (31) on the basis that a crude extract

of mouse submaxillary gland stimulated premature incisor eruption and eyelid opening in new born mice. EGF has been characterised as a single chain polypeptide with a molecular weight of 6,100 and an isoelectric point 4.6 (21). EGF has been shown to elicit a mitogenic response in a variety of cell and organ culture systems (22). The amino-acid sequence of m-EGF has been shown to have an 80% homology with human urogastrone (32). The ability of m-EGF to inhibit gastric secretion and urogastrone to prematurely open the eyelids of new born mice, has confirmed their high degree of biological identity. (70). Specific cell surface receptors have been demonstrated for EGF in a variety of target cells in culture and crude membrane preparations (22).

The demonstration of cell surface receptors for EGF and the subsequent stimulation of DNA has permitted studies regarding possible mechanisms which mediate the EGF induced stimulation of growth. The binding and rapid internalization of EGF has been shown in cultured cells using a variety of labelling techniques (33). The preparation of a fluorescent derivative of EGF has permitted the direct visualisation of binding to cultured cells. (71). The binding of EGF appears initially to be diffuse over the entire border of the cell surface. At 37^{o}C there is a rapid clustering of the cell surface receptor-EGF complex, which after approximately

10 - 30 min is internalised into endocytotic vesicles which subsequently fuse with lysosomes (139).

The use of photoaffinity agents have been described which permit the monitoring of degradation of ^{125}I-EGF-receptor complex by lysosomes (49). Studies using this technique reveal that the photoaffinity labelled ^{125}I-EGF upon binding to cell surface receptors has a molecular weight of 190,000 daltons. Subsequently, the internalised complex is degraded and can be monitored by the appearance of lower molecular weight radiolabelled species within 30 min after binding. However, the continued presence of optimal concentrations of EGF for periods of up to 6 hr have been shown to be required for the transmission of the hormonal signal leading to the initiation of DNA synthesis (49). These findings have led to the hypothesis that a continued EGF-induced internalisation of the receptor is an obligatory event in the transmission of the hormonal signal.

Subsequent to the binding of EGF in membrane preparations, there is a rapid phosphorylation of membrane proteins of molecular weight approximately 170,000 and 150,000 daltons (20). Upon continued incubation, additional membrane components are labelled, with molecular weight approximately 70,000 and 28,000 daltons. The relationship between the phosphorylation of membrane components and the subsequent events leading to the initiation of DNA synthesis is presently unclear.

(iii) Fibroblast growth factor

The presence of mitogens in partially purified preparations of thyroid-stimulating hormone was first described by Holley and Kiernan, 1968 (79) using 3T3 cells as the target cells in culture. These findings were later confirmed by Armelin (4) using leuteinising hormone and shown subsequently by Clark (28) to be due to impurities in the hormone preparations. Gospodarowicz (60) later isolated the mitogen from b.pituitary glands using the initiation of DNA synthesis in serum-starved cultures of Balb/c3T3 cells and called it fibroblast growth factor (FGF). The purification of an additional FGF from b.brain was reported, based initially on the observation that the pituitary and brain contained 1 to 2 orders of magnitude greater levels of growth promoting activity compared to a variety of other tissue extracts tested (64). The pituitary FGF has a molecular weight of 13,000 daltons and isoelectric point of 9.5 (61). The purification of FGF from b.brain revealed two biologically active factors, FGF_1 and FGF_2. (65). The brain FGFs have a similar biological effect on cultured cells compared to pituitary FGF but were reported to be chemically unrelated (65).

More recent studies by Westall have revealed the identity of at least 3 forms of bovine brain FGF which were claimed to be fragments of myelin basic protein (MBP)

corresponding to amino acid residues 44-166, 44-153, and 91-153 (168). The intact molecule of myelin basic protein was, however, biologically inactive. Two independent reports have subsequently shown that the biological activity present in bovine (150) and human (87) brain can be separated from MBP. The relationship between the pituitary FGF and brain FGF described by Gospodarowicz is therefore unclear.

The mitogenic properties of FGF have been described by many investigators (66) and FGF has been shown to stimulate a variety of mesodermal cells, whilst lacking activity for endodermal and ectodermal derived cells in vitro (64). FGF in vitro has been shown to stimulate a variety of events, including precursor uptake and synthesis of m RNA and t RNA and protein (64). More recently, Gospodarowicz (68) has shown that vascular endothelial cells of bovine and human origin, do not proliferate in plasma when cells are maintained on plastic dishes. Addition of FGF can induce proliferation under these conditions. However, when the endothelial cells are maintained on a naturally produced extracellular matrix, these cells proliferate equally well in plasma or serum and no longer require FGF.

These findings might indicate that FGF is required by some cells for the formation of an extracellular matrix, thereby rendering the cells responsive to mitogenic

factors present in the plasma. The author speculates that factors such as FGF may therefore be more important during early fetal development. The correct formation of an extracellular scaffold upon which epithelial cells are maintained might render these cells responsive to other growth factors and hormones in plasma.

SECTION II

GROWTH FACTORS AND NORMAL CELL GROWTH

The major growth factors which have been identified have been isolated using a variety of investigative approaches. The isolation of some of these factors has permitted a closer examination regarding their mechanism of action and putative role as growth controlling factors for normal and neoplastic cell growth in culture.

The Balb/c3T3 cell line has been used in the study of growth factors and the regulation of cell proliferation. The concept of 'dual control' of normal cell growth by growth factors was introduced by Pledger et al (120) and Vogel et al (166). This concept has been further studied using purified growth factor preparations. Cells rendered quiescent, in the G0/G1 phase of the cell cycle, become "competent" to synthesise DNA by the addition of PDGF and FGF. However, "progression" through the G1 phase requires the presence of growth hormone dependent factors such as somatomedin C (142).

Further studies have shown an increase in the somatomedin C receptor binding activity in density inhibited Balb/c3T3 rendered competent to divide by PDGF and FGF (29). These findings support the hypothesis that PDGF and plasma factors act in a sequential manner to initiate

DNA synthesis. Growth factor induced modulation of receptors for other growth factors is a recent development concerning the regulation of cell division. These findings might suggest that growth factors related to FGF and PDGF in their biological action may play a role in the initial sequences of events which render cells competent to divide in response to growth factors/hormones normally found in the plasma.

GROWTH FACTORS AND AUTONOMOUS CELL GROWTH

It has been proposed by Todaro et al (151) that the autonomy of tumor growth may result in part from the ability of malignant cells to synthesise and secrete polypeptide growth factors which interact with receptors on the tumor cell membrane and perpetuate their autonomous proliferation. Normal cells are therefore likely to be dependent upon the continued supply of exogenous growth factors.

The general strategy used for the identification of these tumor derived growth factors has been to examine for the presence of growth factor receptors on the surface of a variety of untransformed cells in culture. The loss of receptor binding on the surface of tumor cells, following transformation by oncogenic viruses or naturally occurring tumor cells of similar origin provides an indication that the abnormal cell is producing the putative growth factor endogenously leading to down regulation or occupancy of the receptor. Using this approach, Todaro and co-workers have identified 3 major classes of growth factors produced by tumor cells in culture.

Production of epidermal growth factor-like polypeptides

The first family of growth factors identified were secreted into the medium of murine sarcoma virus (MuSV)-transformed mouse 3T3 cells and MuSV-transformed

normal rat kidney cells (NRK). These factors compete at the membrane receptor level for ^{125}I-EGF and therefore are believed to be related to the EGF family of growth factors (112). Fractionation of the medium in which the transformed cells were maintained (conditioned medium) in the presence of 1 M acetic acid, revealed 3 peaks of activity, eluting with proteins of molecular weight approximately 20-27,000, 12,000 and 7,000 daltons. The 12,000 molecular weight growth factor, sarcoma growth factor (SGF), was shown to induce colony formation of normal rat fibroblast in soft agar. The SGF factor also induced a morphological phenotype of the normal fibroblast which was indistinguishable from the genetically transformed fibroblast. This factor was found to be immunologically distinct from EGF. Further experiments using the temperature sensitive Moloney sarcoma virus mutant (6M2), showed that transformed NRK cells when maintained at the permissive temperature (32^{o}C) exhibit the transformed phenotype. These transformants grow well in soft agar, lose their ability to bind ^{125}I-EGF and release SGFs into the culture medium (152). When shifted to the non-permissive temperature (39^{o}C) the 6M2 transformed cells, acquire the normal morphology, lose their ability to grow in soft agar, express the cell surface EGF receptor, and do not produce SGFs. When incubated at the non-permissive temperature in the

presence of the SGF (harvested from the medium at the permissive temperature) these cells lose their normal behavior and can be induced to growth in soft agar (through the interaction of the SGF with the EGF receptor). Hence the production of the SGF correlates with the expression of the transformed phenotype and the absence or presence of the expression of the EGF receptors.

Production of multiplication stimulating activity-like polypeptides

A variety of human cells in culture were screened for receptors to the MSA family of polypeptides. Normal fibroblast and some tumor cell lines were shown to exhibit ^{125}I-MSA binding. Two tumor cell lines, a fibrosarcoma and an osteosarcoma, failed to bind ^{125}I-MSA. These cells were therefore potential candidates for the production of endogenous MSA-like polypeptide growth factors. The serum free conditioned medium from the human fibrosarcoma cell line was then chromatographed under dissociating conditions (1 M acetic acid). (153) MSA-competing activity on normal fibroblasts was detected with proteins of apparent molecular weights 7,000, 10,000, 15-18,000, and several peaks at 24,000.

These results are analagous to the original description of MSA produced by the Buffalo rat liver conditioned

medium described by Dulak and Temin (40). MSA-receptors were reported in the fibrosarcoma cell line where the cells are maintained at low density. As the fibrosarcoma cells grow to high density there is a loss of MSA binding. These findings were interpreted as evidence that the endogenous production of MSA was preventing the externally supplied ligand from competing at the cell surface receptor. These findings are compatible with the general notion that cells in tissue culture are capable of conditioning their medium when maintained at high density.

Production of nerve growth factor-like polypeptides

In a study using 6 human melanoma cell lines, 5 of the cells exhibited binding of ^{125}I-NGF. The cell line which failed to bind NGF (A375) was subsequently shown by immunofluorescence in serum free medium, to have NGF on its cell surface. The 5 cell lines which contained NGF receptor failed to show fluorescence on their surface membrane when incubated in serum free medium. These findings would suggest that the A375 cell line was producing a NGF that was capable of cross-reacting with antibodies raised against the β-sub-unit of mouse NGF. Antibodies against m-EGF showed no cross-reactivity.

The general model proposed by Todaro predicts that the endogenous production of growth factors by cells

which respond to them would provide a system of endogenous growth promotion. These findings provide a mechanism by which tumor cells may gain a selective growth advantage over their normal counterparts.

FIBROBLAST GROWTH FACTOR AND AUTONOMOUS CELL GROWTH

Rudland has shown that following transformation of Balb/c3T3 cells with a temperature sensitive mutant of polyoma virus, clones exhibited temperature-sensitive properties. At the non-permissive temperature (37^{o}C), these cells exhibit their normal phenotype and respond to cationic fibroblast growth factor (134). When shifted to the permissive temperature (32^{o}C) these cells exhibit the transformed phenotype and no longer respond to FGF. These findings were reported in 1974 and are analagous to the experimental findings of Todaro (153) presented in this thesis. However, the effects described by Gospodarowicz were not complemented by receptor studies.

Stiles et al (143) have shown that Balb/c3T3 cells proliferate slowly in medium supplemented with plasma. These cells can be induced to proliferate rapidly following transformation by SV-40 (oncogenic DNA virus) or by treatment with the cationic platelet derived growth factor. Bourne and Rosengurt (15) have reported the isolation of a cationic polypeptide growth factor of molecular weight 18,000 daltons from the conditioned

medium of SV-40 transformed BHK cells. This factor was shown to stimulate DNA synthesis in 3T3 cells. These findings might suggest therefore that as yet unidentified cationic growth factors may play a role in the autonomous proliferation of transformed cells.

SECTION III

GROWTH OF MAMMARY TUMORS

Growth factors for mammary epithelial cells

Independent reports of novel mitogens for mammary epithelial cells have been described from a number of sources and include platelets, serum, pituitary, kidney, uterus, and brain. Sirbasku et al (140) have identified growth factors from the kidney, brain, uterus and platelets for a rat mammary tumor cell line. The identity of these mitogens were derived from the paradoxical findings that a hormone reponsive transplantable rat mammary tumor cell line failed to proliferate in culture in response to the hormone combination of prolactin and estrogen which were necessary for optimal tumor formation in the host animals. These authors speculate that the peripheral source of mammary tumor mitogens may occur through absorption by platelets from the environment e.g. kidney, brain, uterus, etc. in a manner analagous to that described by Davey and Luscher (37) and Maupin (106). The mechanism of release of platelet mitogens *in vivo* for mammary tumor cells may occur due to the presence of platelet aggregation sites on the surface of the tumor cells as described by Gasic et al (55). This phenomenon may also be enhanced by certain hormones such as estrogen which facilitate platelet aggregation *in vivo* and *in vitro* (42) and might account in part, for the

widely accepted etiological role of estrogen in the genesis of mammary neoplasms.

Evidence that the growth of a human mammary tumor cell line (T-47D) might be stimulated by the putative estrogen-induced growth factor, or as yet other novel mitogens, has been proposed by Leung and Shiu (95). These authors reported the stimulation of growth of the T-47D cells in estrogenised athymic nude mice bearing a GH and Prl secreting pituitary tumor (GH_3). Since the growth of the T-47D cells was not stimulated by either estrogen or the GH_3 tumor cell implant alone, the authors speculate that the GH_3 pituitary tumor might produce a novel factor(s) under the influence of estrogen which stimulates the growth of the mammary tumor.

Another source of mitogenic activity for mammary tumor cells initially described in the pituitary has subsequently been identified as phospoethanolamine (84). The hormone responsive cell line when tested in 0.2% FBS was shown to be responsive to prolactin, insulin and hydrocortisone in combination, with enhanced stimulation following the addition of phospoethanolamine. Since phospoethanolamine had no stimulating effect by itself, the mechanism of synergism between these hormones and phospoethanolamine remains to be determined. Whilst there have been a great many mitogens isolated from the serum, a unique serum mitogen has been identified which

stimulates cultured mammary epithelial cells (124). At high concentrations this mitogen exhibits biological cross-reactivity but lacks immuno cross-reactivity with another serum mitogen, multiplication-stimulating activity (MSA). Based on a variety of physiological, biological or immunological properties, this mitogen was found to be different from other mitogens such as EGF, NGF, FGF, MSA, the somatomedins A and C, and the platelet derived growth factor of Antoniades. From these studies, a diverse variety of novel mitogens have been identified providing further insight into the complex nature of hormonal involvement in the regulation of mammary tumor cell proliferation.

Growth factors from mammary epithelial cells

It has been postulated that the proliferation of normal cells in the vicinity of tumor cells is mediated by the elaboration of growth promoting substances released by the tumor cell population. Evidence in support of this concept was originally obtained from histological examination of solid tumors (3). Later experimental studies by Ludford and Barlow, 1944 (102) reported that in con-joint culture, mammary tumors had a pronounced stimulating effect on fibroblast growth. Nair and De Ome described the presence of growth promoting factors in extracts of spontaneous mouse

mammary tumors using the stimulation of DNA synthesis in mouse embryo cells as the bioassay (111). More recently mitogenic activity was found in the conditioned medium of 3 human mammary tumor cell lines, which stimulated DNA synthesis in Balb/c3T3 cells (81). A transplantable mouse mammary tumor cell line has been reported to produce a family of somatomedin-like polypeptides when cultured in vitro. The polypeptides released into the cultured medium were reported to range in molecular weight from 7,000 - 12,000 daltons (91).

Although the production of growth factors is not unique to tumor tissue and tumor cell lines, it is an interesting speculation that growth factors produced by tumor cells may play an important role in the control of tumor growth in vivo.

Growth factors from normal mammary tissue and milk

Klagsbrun (90) identified and partially characterised a mitogen (mol. wt. approximately 18,000, pI 4.4-4.7) from human milk which stimulated DNA synthesis and cellular proliferation in cultured fibroblasts. Activity was also detected in bovine colostrum. A possible speculated role of this milk derived mitogen might be to stimulate the growth of the gastrointestinal tract of the newborn. More recent experiments have shown that the principal

mitogen present in human milk can be neutralised by antibodies to m-EGF (23).

Another putative milk-derived mitogen was partially isolated from tissue-culture-grade ∝ lactalbumin hydrolysate (∝ lactalbumin being a specific milk protein). This mitogen was shown to stimulate DNA synthesis and plasminogen activator (elevated levels normally associated with transformed or malignant cells) in cultured 3T3 cells (27). Swanson and Moore (146) reported the establishment of 3 human mammary tumor cell lines from a total of 254 breast biopsies. To account for this poor success rate, these workers hypothesised that specific growth factors may be contained in the same tissue from which the cell type was being cultured and necessary for its survival. Utilising bovine mammary gland as a possible source of this mitogen, two cell lines were successfully established in the presence of this extract to ensure its continued growth in culture. This putative mitogen was reported to be labile in low pH and considered to be different from Klagsbrun's milk growth factor.

Summary

A diverse source of growth factors have been identified and characterised. The use of purified growth factors and their radioligand derivatives has increased our knowledge and understanding concerning their mechanism of action. The mutual dependency of these factors controlling the rate of normal cellular division has provided new investigational approaches into the study of regulatory events governing cell growth. The identification and purification of 'novel' growth factors might therefore lead to a greater understanding of the regulatory factors governing normal and neoplastic cell proliferation.

SECTION IV

AIM OF INVESTIGATION

1) To determine the presence of growth factors in solid human mammary tumors.
2. To compare the physical and biological characteristics of these growth factors with factors isolated from the human brain, pituitary gland, and other growth factors reported in the literature.
3. To establish a purification scheme for the isolation of
   i) human mammary tumor derived growth factor(s).
   ii) human brain derived growth factor(s).

SECTION V

MATERIALS AND METHODS

Materials

a) Human tissue

Brain - Human brains were obtained 12-24 hours post-mortem and stored at -20°C. There was no preselection of brains on the basis of age, sex, cause of death or otherwise.

Mammary tumors - Mammary tumors were collected following surgical biopsy and specimens frozen at -70°C.

b) Chemicals

Carboxymethyl-sephadex C-50 (CM-Sephadex) Sephadex G-75 and Sephadex G-75 superfine, Sephadex IEF, Blue Dextran and isoelectric focusing and SDS electrophoresis calibrating kits were obtained from Pharmacia Fine Chemicals. Whatman carboxymethyl cellulose CM-52 was obtained from either Reeve Angel or Mandel Scientific Co. Ltd. ^{3}H-thymidine and ^{125}I were either from New England Nuclear (NEN) or Amersham. Toluene (Fisher) and omnifluor (NEN) was used initially for scintillation counting and subsequently replaced by a pre-prepared complete organic counting scintillator (OCS) from Amersham Co. Trypsin (soybean) and ∝-chymotrypsin (b.pancreas) were obtained from Calbiochem. Trypsin inhibitor (soybean) BSA, ovalbumin, cyctochrome C and uridine were obtained from Sigma Chemical Co. Spectrapor dialysis tubing was obtained from Spectrum Medical Industries. Coomassie Brilliant Blue R-250,

2-Mercaptoethanol, SDS, TRIS Ammonium persulphate, Acrylamide, and Bis-acrylamide were obtained from Bio-Rad Laboratories.

All the standards laboratory reagents were obtained from Fisher.

Tissue Culture supplies

All culture plastic ware (Falcon or Corning) was obtained from Fisher. Tissue culture media (DMEM and Ham F 10) including sera antibiotics (Penicillin 10,000 u/ml-Streptomycin, 10,000 µg/ml) trypin-EDTA, L-glutamine (200 mM) were purchased from GIBCO. Collagenase type II was obtained from Worthington.

Bovine pituitary growth factor , nerve growth factor, epidermal growth factor, and multiplication stimulation activity were obtained from Collaborative Research or KOR Biochemicals. Somatomedin C was kindly donated by Dr. M. Bala, University of Calgary.

R.I.A. Reagents

Human myelin basic protein was kindly donated by Dr. Marion Kies, N.I.H. Human myelin basic protein antiserum was kindly donated by Dr. John Palfreyman, University of Glasgow.

Preparation of rabbit-antiserum myelin basic protein

New Zealand white rabbits were injected with 10 µg

of h.myelin basic protein in Freund's complete adjuvant intradermally at multiple sites every 1 - 2 weeks. Antisera was obtained after 4-5 months. Dilution of the antisera was in the range of 5-8,000.

Radioimmunoassay of Myelin Basic Protein

Radioimmunoassay for myelin basic protein (MBP) was according to the method of Palfreyman et al (1978) with the following modifications. Deterioration of the tracer was reduced by the addition of phenylmethylsulfonylfluoride (0.2 mg/ml) and bacitracin (100 units/ml) to the buffer solutions. Non-specific binding of tracer was reduced when the buffer contained 1% Triton X-100.

Samples were pre-incubated for 24 hr with anti-human myelin basic protein (1 in 6000 dilution). The tracer was then added and the samples incubated for a further 24 hr. Sheep antiserum to rabbit gamma globulin (0.1 ml) was then added and the samples incubated for 24 hr, at which time the tubes were centrifuged, the supernatants decanted and the precipitate counted in an LKB Gamma Counter. Bovine pituitary FGF, EGF, NGF and MSA did not react in this assay.

Discontinuous SDS gel electrophoresis

Sodium dodecyl sulphate slab gels were prepared according to the method described by Weber and Osborne 1969, (167). The stacking gel (4%, 5 x 140 mm) and resolving gel (15%, 95 x 140 mm) were prepared from stock solutions of acrylamide (30% w/v) and N,N^{1}-methylene bisacrylamide (0.8% w/v). Gels (0.75 mm thickness) were cast using a BIO-Rad vertical slab gel unit.

Samples (10-25 µl) were prepared by dissolving in a cocktail containing Tris-HCl (0.0725 M, pH 7.0) 2% SDS, 10% glycerol 5% β-mercaptoethanol and 0.01% bromophenol blue (tracking dye). Samples were boiled for 5 min. at 100^{o}C prior to use. Electrophoresis was carried out at a constant power of 20 m A/gel until the bromophenol blue migrated to within 5 mm of the bottom of the gel (approximately 6 hr.). Gels were stained for 1 hr. at room temperature in 0.3% coomassie brilliant blue 250 in 10% acetic acid and 45% ethanol. Destaining of the gels was carried out with 3 changes of a 7% acetic acid, 20% ethanol solution.

Isoelectric focusing

Horizontal slab gel isoelectric focuing was carried out on a LKB Multiphore unit (Model No. 2117) using pre-prepared polyacrylamide gels (LKB) or sephadex IEF gel (Pharmacia).

Polyacrylamide Gels

Isoelectric focusing was performed using a LKB ampholine PAG plate (gel concentration of 5%, cross-linking 3%, ampholine 2.4%, dimensions 245 x 110 x 1 mm and a focusing range pH 3.5 - 9.5).

Samples (15 µl) were applied to paper wicks to the gel at a distance of 1.5 cm from the anode. The anode electrolyte was 1 M NaOH. Focusing was carried out at a constant power of 4 watts for 3 hours using a LKB constant power supply. At the completion of electrofocusing the gel was removed and sliced into appropriate sections. One section was fixed in a solution containing 57.5 g TCA, 17.25 g sulphosalicylic acid in 500 ml d.H_2O, and then stained using 0.1% Coomassie Brilliant Blue R-250 in destaining solution (8% acetic acid 25% ethanol in d.H_2O).

The section of gel used for bioassay was cut into 20 or 21 consecutive sections and placed in a tube containing either buffer or d.H_2O, and eluted overnight in a shaking water bath at 4^{o}C. Aliquots of each eluate was assayed for growth factor activity and myelin basic protein by R.I.A.

Gel segments to which sample material had not been applied were also prepared from another gel section and eluted in d.H_2O for the determination of pH and bioactivity.

Sephadex

Approximately 7.5 g of Sephadex IEF gel was swollen at room temperature in 120 ml of d.H_2O containing either 100 - 200 mg of protein and 6 ml of pharmalyte carrier ampholine, pH interval 3 - 10. The cathode consisted of paper wicks soaked in 1 M diethylamine and the anode wicks soaked in 0.1 M H_2SO_4. A solution of cytochrome C was spotted on the gel surface at the anode. Isoelectric focusing was carried out at a constant power of 4 watts for 24 hr. at 20°C after which the gel was divided into 30 equal segments. The gel from each segment was resuspended in 5 ml of d.H_2O and centrifuged at 1000 x g for 30 min. An aliquot of the supernatant from each fraction was diluted in 200 mM Hepes buffer pH 7.4, containing 0.1% BSA for the determination of growth factor activity. The absorbance at 280 nm and pH of each fraction were recorded.

Gel Chromatography

Ion Exchange Chromatography

Carboxmethyl-sephadex C-50 was first swollen and prepared according to the manufacturer's specifications and a column (3 x 10 cm) was equilibrated with 0.1 M sodium phosphate buffer pH 6.0 at 4°C. Absorbed particles were eluted in 0.1 M sodium phosphate with a stepwise increase of 0.1 M and 0.5 M NaCl.

Whatman CM52 ion exchange (pre-swollen) was equilibrated in ammonium formate (0.2 M, pH 6.0) or distilled water. Elution of the absorbed proteins was carried out stepwise or by gradient elution using ammonium formate.

Sephadex gel filtration

All Sephadex gels were swollen, packed and flow rates adjusted according to the recommendations outlined by Pharmacia. Blue dextran and calibrated proteins were chromatographed separately.

Estrogen receptor (E.R.)

Tris-HCl (10 mM) pH 7.4 containing 1.5 mM EDTA, 10% Glycerol and 1.5 mM DTT (added just prior to the use of the buffer).

Stability studies

Chemical

Samples to be tested were incubated overnight at $4^{o}C$ in either 0.1 N acetic acid (pH 3.0), 1 N acetic acid (pH 2.1), 0.5 or 2 M NaCl 8 M urea, 4 M guanadine HCl or 1% mercaptoethanol. Samples were either dialysed or diluted in medium containing 0.1% BSA and tested for biological activity.

Heat treatment

Samples were dissolved in buffer and heated to 56°, 70° or 100°C for 5 or 30 minutes. The samples were cooled rapidly and tested for biological activity.

Proteolytic Enzyme Treatment:

Samples were dissolved in 0.01 M phosphate buffered saline pH 7.4 (PBS) and were incubated with 100 ug of trypsin or chymotrypsin for 4 or 18 hours at 20°C. Trypsin inhibitor was then added and the samples were assayed for growth factor activity.

Protein determination

Protein concentration was determined by a) the ratio of absorbance at 280/260 nm, or, b) by the method of Lowry (100) using B.S.A. as a standard.

Radioreceptor assay for m-EGF

The radioreceptor assay for m-EGF was used as described by Imai et al (83). Mouse EGF was purified by the method of Savage and Cohen (138). EGF was labeled with ^{125}I by the method of Hunter and Greenwood (82) Briefly, ^{125}I-EGF (0.2 ng) was incubated with 100 µl of mouse liver particulate fraction in 50 mM Tris-HCl buffer containing 10 mM $MgCl_2$ and 0.1% BSA in a total volume of

0.5 ml. Assays were performed in duplicate at 22^{o}C for 120 min. Native m-EGF was used as a competitive inhibitor over a concentration range from 0.1-100 ng ml^{-1}. At the end of the incubation period, assay tubes were diluted with 2 ml of ice cold assay buffer and bound and free ^{125}I-labelled hormone were separated by centrifugation at 2000 g for 20 min. at 4^{o}C. The supernatant was removed by aspiration, and the precipitate was counted in an LKB Gamma Counter.

Cell Culture

All cultures were maintained at 37°C in a humidified 95% air 5% CO_2 incubator (Hotpack, Waterloo, Ontario).

Cell Lines

Balb/c3T3 clone C_3 (obtained from R.P.C. Shiu) and T-47D (human mammary carcinoma cell line, obtained from Mason Research Institute, Rockville, Maryland)

Stock cultures were maintained in T-75 flasks containing Dulbecco's Modified Eagles Medium (DME) (GIBCO) containing 100 units of penicillin and 100 µg streptomycin ml^{-1}, and supplemented with 10% FBS, glucose 140 mg ml^{-1} and porcine insulin 1 µg ml^{-1}, (growth medium). The growth medium was changed 2-3 times weekly and the cells passaged at confluency.

Primary cultures - Rabbit fetal chondrocytes

Chondrocytes were obtained from rabbit fetuses obtained during the 4th week of gestation. The fetal knee cartilage was dissected free of muscle and connective tissue and chopped into small pieces (approximately 1-2 mm cubes) and digested in F-10 medium containing 10% FBS and 0.25% collagenase (type II) for 2 hr at

37°C in an agitating water bath. Undigested fragments were removed by passage through a sterile wire gauze and the cells were harvested by centrifugation at 500 x g for 3 minutes. The cells were resuspended in F-10 medium supplemented with 10% FBS (growth medium) and approximately 1 x 10^6 cells were inoculated into T-75 flasks, primary culture. When the cells reached confluency, they were trypsinized, centrifuged as before and resuspended in growth medium + 10% DMSO. Approximately 1 x 10^6 cells in 1 ml of medium was transferred to storage vials and allowed to freeze slowly at -80°C in a styrofoam container for 24 hr before being transferred to liquid nitrogen. When cells were required for the bioassay, 1 vial was quickly thawed at 37°C and the contents transferred to a T-75 flask containing 15 ml of growth medium, and the cells were allowed to grow to confluency (secondary culture). These cells were trypsinized and sub-cultured for the bioassay.

DNA synthesis:

Balb/c3T3

Confluent cultures of Balb/c3T3 cells were dispersed by 0.25% trypsin. Trypsinization was terminated by DME medium containing 10% bovine serum (BS). Approximately

1-2 x 10^4 cells were plated in 35 mm dishes in 2 ml of medium containing 10% BS. Twenty-four hours later the medium was aspirated and replaced with medium containing 1% BS. Between 24-72 hours later the cells were arrested (as evidenced by no further increase in cell number) and test samples were added in 100 ul of buffer at pH 7.4. Eight hours later 1 uCi ^{3}H-thymidine (New England Nuclear) (NEN) and 1.4 µg thymidine in 100 µl of medium was added to each dish and incubated for 20 hr, after which the medium was aspirated and replaced by 2 ml of 10% TCA at 4°C.

Rabbit fetal chondrocytes

Confluent secondary cultures of rabbit fetal chondrocytes (RFC) were trypsinized and harvested in growth medium and approximately 4-5 x 10^4 cells were plated in 35 mm dishes in 2 ml of medium. Twenty-four hours later the medium was aspirated and replaced with serum free medium. Twenty-four hours later test samples were added in 10 mM Tris buffer at pH 7.4 containing 0.1% BSA. Twelve hours later 2 µ C of ^{3}H-thymidine was added to each dish and incubated for 12 hours, after which the medium was aspirated and replaced with 2 ml of 10% TCA at 4°C.

Collection of TCA precipitates

The Balb/c3T3 cells + RFC were scraped from the

dish and collected on Whatman glass fibres (GF/C) and rinsed with 3 x 2 ml of 10% TCA at 4°C. The fibres were dried overnight and placed in 10 ml of toluene containing omnifluor (NEN) and counted in a liquid scintillation counter (NEN).

Later studies showed that following aspiration of the medium, cells could be removed from the dishes by exposure to 0.5 ml of 0.5% SDS in $d.H_2O$ for 5 min. The SDS solubilised material was then transferred to 3 ml of cold TCA and the precipitate collected on glass fibres as described. An organic counting scintillator (OCS, Amersham) was used in place of the toluene/omnifluor cocktail. The revised protocol reduced the time required to process samples and improve duplicates without loss of incorporated ^{3}H-thymidine.

Cell growth

Rabbit fetal chondroctyes

Confluent cultures of chondrocytes were obtained from a frozen stock of cells as already described. Initially cells ($2-4 \times 10^4$) were inoculated into 60 mm dishes in 3 ml of growth medium (F-10 + 10% FBS). On day 2, medium was replaced with fresh medium and samples were added to replicate cultures on day 2, 4 and 6. On day 8, cells were trypsinized and counted in a coulter counter.

This protocol was modified at a later stage to allow for a more rapid and less costly assay. In brief, the chondrocytes (2×10^4 cells) were inoculated into 35 mm dishes in 2 ml of growth medium, day 0. Medium was replenished on day 1 and samples to be tested were added on day 1 and 3 and cell number determined on day 5 as described.

Samples to be tested were either prepared in F-10 medium containing 0.1% BSA or 10% FBS or in buffer containing 0.1% BSA.

T-47D

Approximately $1-2 \times 10^4$ cells were inoculated in growth medium into 35 mm dishes, day 0. On day 1, the medium was aspirated and the cultures washed in serum free medium and incubated in medium containing 0.1% BS or FBS. Samples to be tested were added in buffer every other day and cell number determined after 8 - 14 days.

SECTION VI

RESULTS

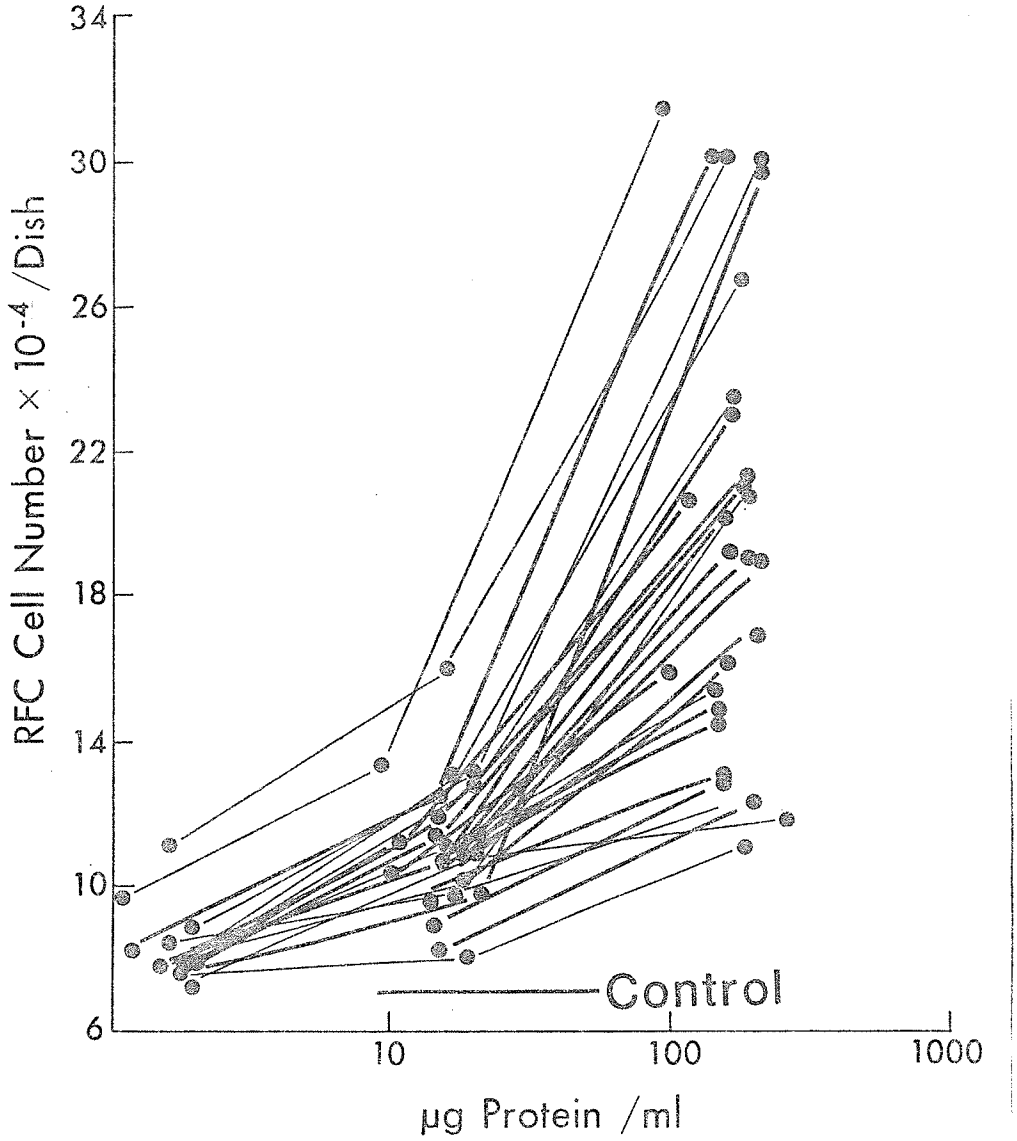
Characterisation and partial purification of growth factors in extracts of human breast cancer

Preliminary investigations

Initial experiments utilising the Balb/c3T3 and chondrocyte bioassay system were performed to screen for mitogenic activity in crude extracts of human breast cancer routinely performed in this laboratory for the quantitation of estrogen receptors. Fig. 1 shows the stimulation of growth of RFC when exposed to increasing amounts of tumor protein. Stimulation of cell growth by the tumor extracts was demonstrable over the range of 1-100 µg of protein/ml. There was no apparent correlation between the content of estrogen and progesterone receptors in the breast tumors, the histological type or patient age with the magnitude of the growth response. Approximately 75% of the tumors tested stimulated a 2-4 fold increase in cell growth.

Six tumor extracts were also tested for growth factor activity using Balb/c3T3. Three of the six tumors tested stimulated DNA synthesis in quiescent cultures of Balb/c3T3 cells, whilst two of the tumors tested showed less activity and one tumor extract was inactive , Fig. 2.

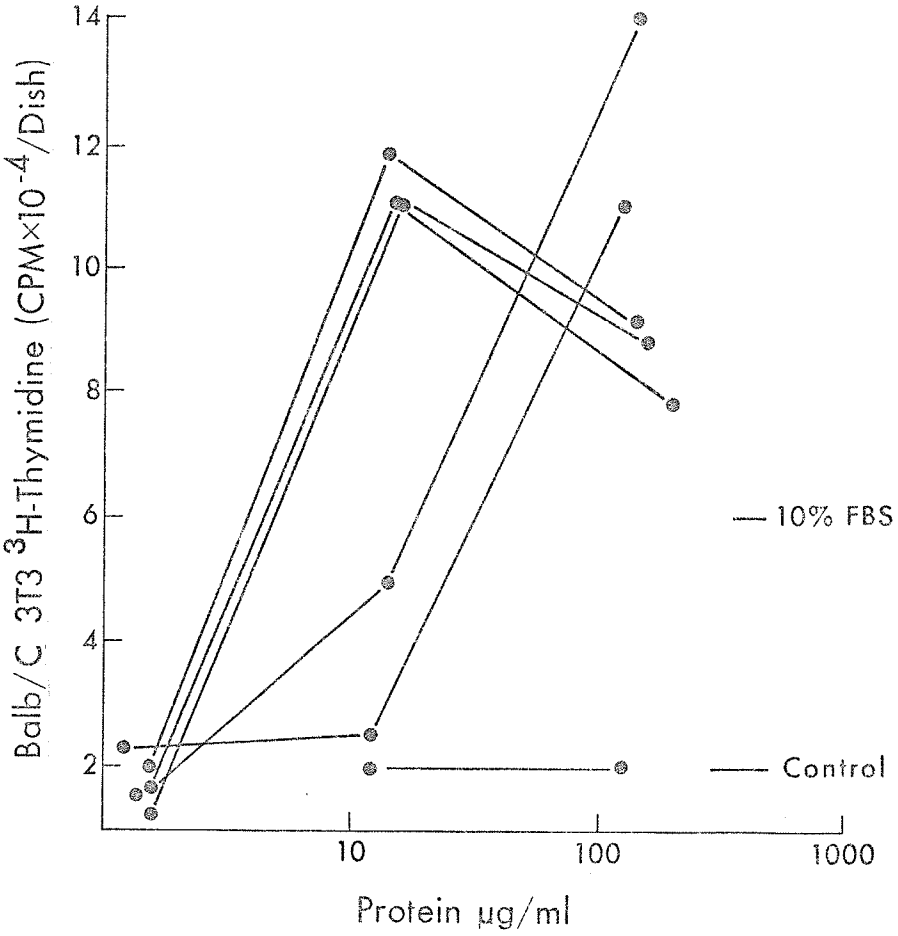
Figure 1



Stimulation of rabbit fetal chondrocytes by crude extracts prepared from a variety of human breast carcinoma specimens

Supernatants from 28 human breast carcinoma extracts were assayed for bioactivity at 3 dilutions, as described in the methods.

Figure 2



Stimulation of DNA synthesis in Balb/c3T3 cells by increasing concentration of h.mammary tumor extract.

The stimulation of DNA synthesis in Balb/c3T3 cells was determined in response to 6 randomly chosen h.mammary tumor extracts. Values represent mean of duplicate cultures assayed at 3 protein concentrations.

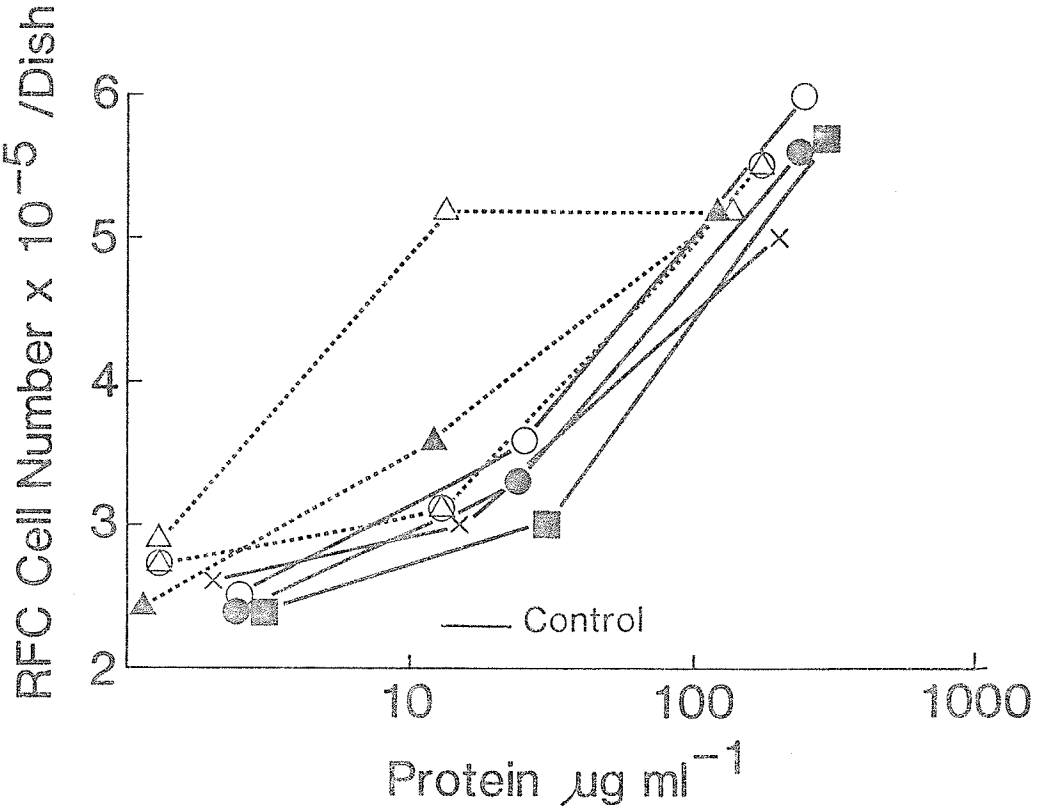
Comparison of the biological potency of the h.breast cancer extract with a h.pituitary extract

Growth factors from the b.pituitary gland (60) and h.pituitary gland (86) have been reported. It was of interest, therefore, to compare the growth factor activity of the h.breast cancer extracts with extracts prepared from the h.pituitary gland.

Three of the most potent h.mammary tumor extracts were compared to extracts from four h.pituitary glands prepared under the same conditions. Two of the h.mammary tumor extracts were found to contain comparable levels of growth promoting activity and one tumor was significantly more active when compared to the extracts of h.pituitary glands Fig. 3. These data demonstrate the presence of growth factor activity in human breast cancers. The level of growth factor activity in positive tumor samples is therefore comparable to that observed in h.pituitary glands.

Significant stimulation of RFC growth was observed in 25% of the breast cancer specimens listed, when as little as 2 µg of extracted protein was added to the culture medium in the presence of 10% FBS. Due to the range of potency in growth factor activity and the extremely high levels present in some tumors tested (exceeding that detected in h.pituitary gland extracts) we have hypothesised that the growth factors might be

Figure 3



Stimulation of RFC in response to extracts prepared from individual h.mammary tumors and h.pituitary glands

Growth factor activity present in extracts prepared from 3 tumor mammary tumors were compared to extracts prepared from 4 pituitary glands. All tissues were homogenised at pH 7.4 in E.R. homogenisation buffer (see methods) and the 100,000 g supernatants assayed on the growth of rabbit fetal chondrocytes as described.

h.mammary tumor #1, #2, #3 .....

h. pituitary gland #1, #2, #3, #4 ——

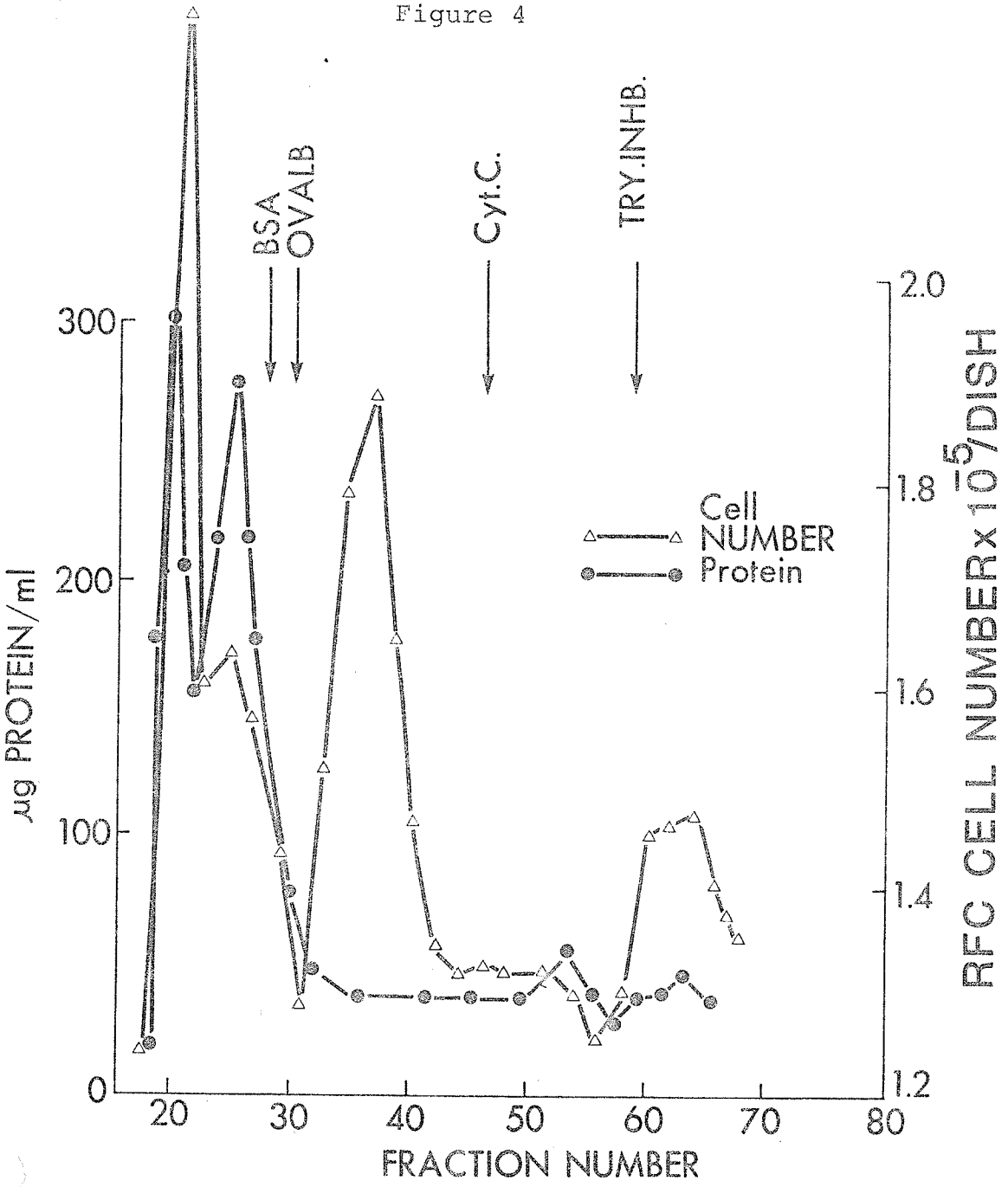
produced ectopically by some breast tumors.

The ectopic production of growth factors by tumors proposed by Todaro (153) and the known ectopic production of hormones by some tumors (126) would support this claim.

Molecular weight determination of the h.mammary tumor derived growth factor (h.MTGF) using Sephadex G-100

When the supernatant from a single mammary tumor extract was chromatographed on Sephadex G-100 Fig.4 2 major peaks of activity were observed. The activity associated with peak I eluted with proteins of molecular weight ~150,000 and a shoulder of peak I corresponding to molecular weight of ~80,000 daltons. The second major peak of activity eluted at a position corresponding to a molecular weight of 20,000 daltons. Further characterisation of the h.MTGF was carried out using a pool of 100 tumor extracts. Following dialysis of the pooled supernatant and lyophilization, approximately 180 mg of tumor protein was chromatographed using Sephadex G-100, and bioactivity was monitored by the stimulation of DNA synthesis in quiescent RFC cultures, Fig. 5. Two peaks of activity were again demonstrated and peak II corresponding to proteins of molecular weight 12-30,000 were pooled, dialysed, lyophilized and fractionated using polyacrylamide slab-gel isoelectric focusing.

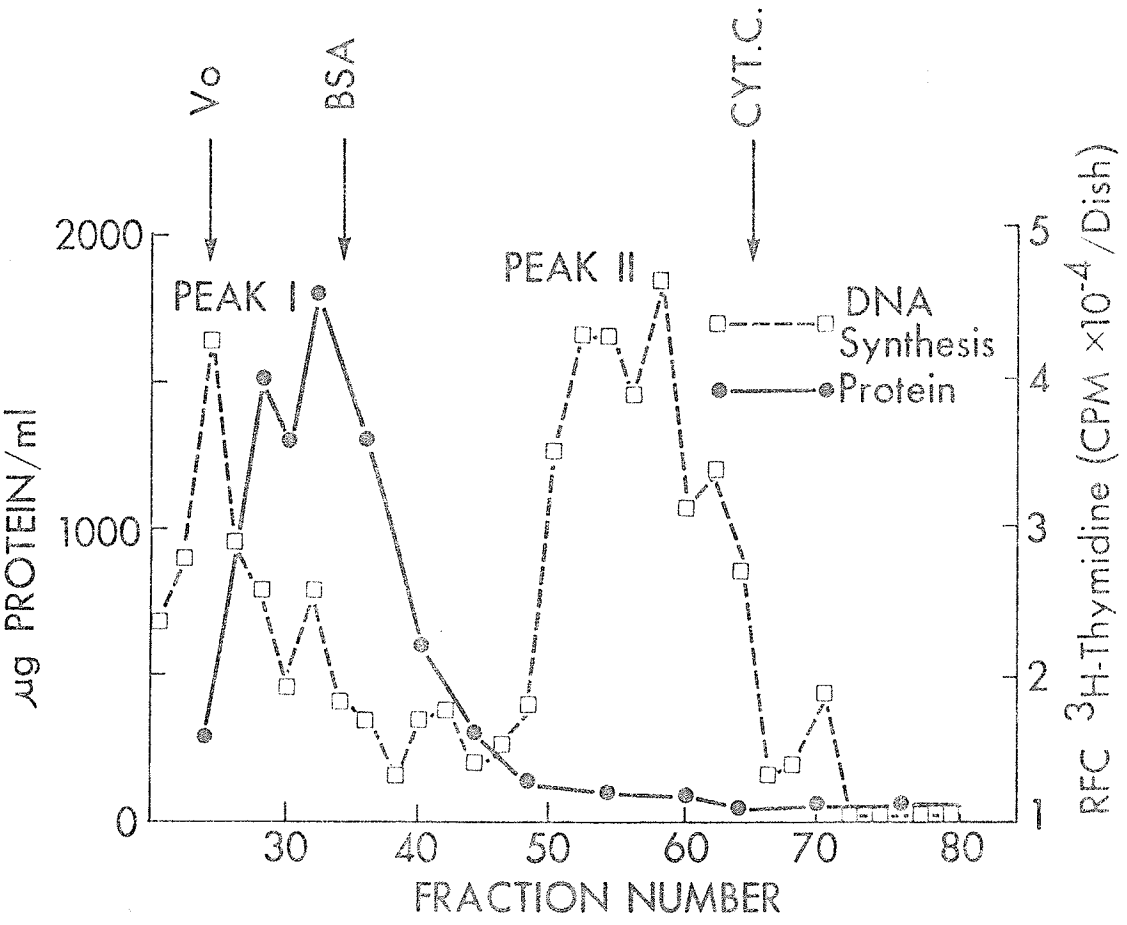
Figure 4



Sephadex G-100 chromatography of a single h.mammary tumor extract

The supernatant from a single tumor extract (approx. 10 mg protein) was chromatographed using a Sephadex G-100 column (70 x 1.5 cm) equilibrated in the estrogen receptor assay (ERA) homogenisation buffer. Fractions (2 ml) were collected and aliquots (100 µl) were tested for their ability to stimulate the growth of rabbit fetal chondrocytes.

Figure 5



Sephadex G-100 chromatography of a pool of h.mammary tumor supernatants

The supernatant prepared from approximately 100 h.mammary tumors was pooled, dialysed and lyophilized. Approximately 180 mg of protein was applied to a Sephadex G-100 column (3.3 x 64 cm) equilibrated in 10 mM Tris buffer pH 7.4. Aliquots of the eluted fractions were adjusted to a protein concentration of 50 μg ml^{-1} and 100 μl of this was added to duplicate cultures of rabbit fetal chondrocytes and the stimulation of DNA synthesis determined as described in the methods.

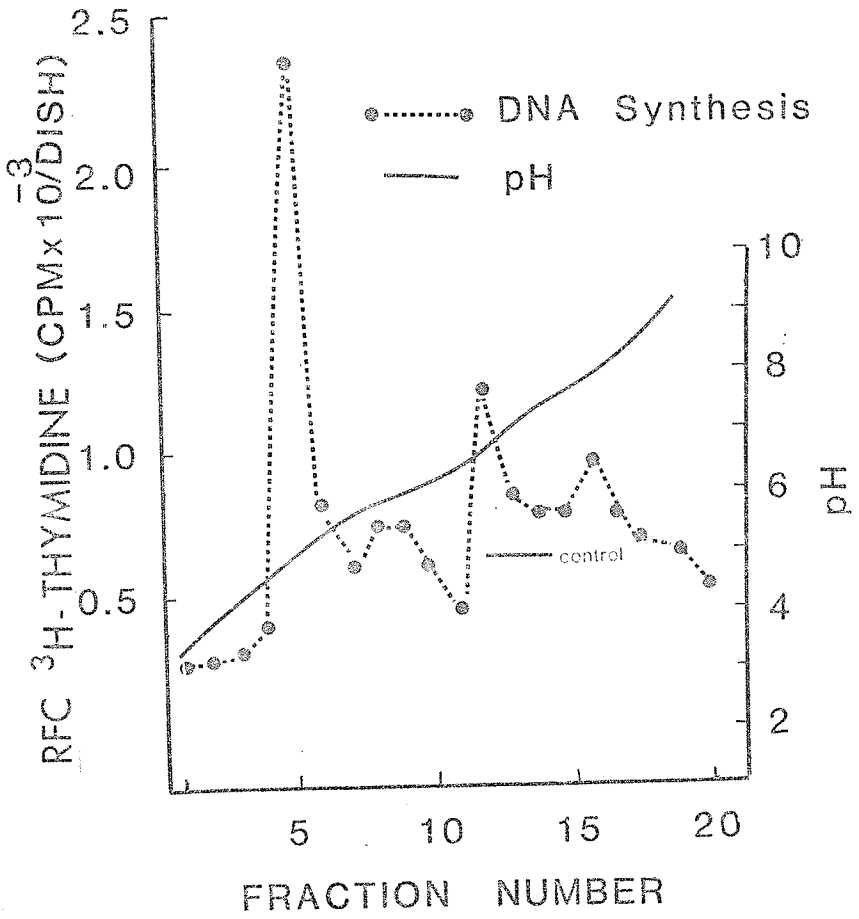
Isoelectric focusing of Peak II

Approximately 250 µg of peak II protein was focused with a pH interval from 3.5 - 10. Bioactivity was detected in gel eluants corresponding to a pH 5, 6.5 and 8, when the stimulation of DNA synthesis in RFC cultures was used as the assay Fig. 6.

The characterisation of the h.MTGF using Sephadex G-100 gel chromatography and slab-gel isoelectric focusing revealed the presence of a heterogeneous population of growth factors in the tumor extract. In order to obtain a sufficient quantity of the h.MTGF for the purpose of purification of one of these mitogens, a pool of the h.mammary tumor supernatant was fractionated using ammonium sulphate $(NH_4)_2So_4$. Fig. 7 shows the dose response curves for fractions corresponding to 25%, 25-50%, 50-75%, 75-100% and saturated $(NH_4)_2So_4$. The fractions corresponding to 50-75% and 75-100% saturation of $(NH_4)_2So_4$ contained most of the bioactivity present in the original extract, but did not significantly increase the specific activity of the growth factor when compared to the original tumor supernatant. The specific activity of the most active preparations was in fact 5-10,000 times less when compared to a commercial preparation of b.pituitary FGF.

Since the amount of h.mammary tumor tissue obtained as a biopsy was usually less than 1 g and potentially

Figure 6



Isoelectric focusing of Peak II

Isoelectric focusing of Peak II (250 µg obtained by gel filtration of the crude extract of h.mammary tumors on Sephadex G-100) was carried out using polyacrylamide gels in the range of pH 3.5 - 10. The stimulation of DNA synthesis in rabbit fetal chondrocytes using using rabbit fetal chondrocytes as described.

limiting, a more efficient method of extraction of the h.MTGF was investigated. Earlier studies by Gospodarowicz (62) showed that extraction of FGF from the b.brain in 0.15 M $(NH_4)_2So_4$ was optimal at a pH of 4.5. Utilising h.brain we had shown that significantly more bioactivity was recovered from h.brain homogenates when the extraction was performed at pH 4.5 as compared to pH 7.0. An experiment was therefore carried out to determine the pH optimum of extraction of the h.MTGF in 0.15 M $(NH_4)_2So_4$.

Figure 7:
Ammonium sulphate fractionation of a pool of h.mammary tumor supernatant

The 100,000 g supernatant from 400 individual h.mammary tumor specimens was pooled (500 ml) and 50 ml of this was used for the $(NH_4)_2$ So_4 fractionation at pH 7.4. By a combination of sequential additions of $(NH_4)_2$ So_4 (stirring at 4^oC, 2 hr) followed by centrifugation (10,000 g 20 min), precipitated fractions were obtained corresponding to 0-25%, 25-50%, 50-75%, 75-100% saturation with $(NH_4)_2$ So_4. The starting material, the final supernatant, and each fraction (re-dissolved in $d.H_2O$) were dialysed (mol. weight cut off 3,500) extensively against 10 mM Tris pH 7.4 and the dialysates were assayed for their ability to stimulate cell proliferation in rabbit fetal chondrocytes. Values represent the mean of duplicate cultures assayed at 5 protein concentrations.

CR b.pituitary FGF ●———●,

h.mammary tumor pool (starting material) ▲———▲,

$(NH_4)_2$ So_4 fractions: 0-25% o———o,

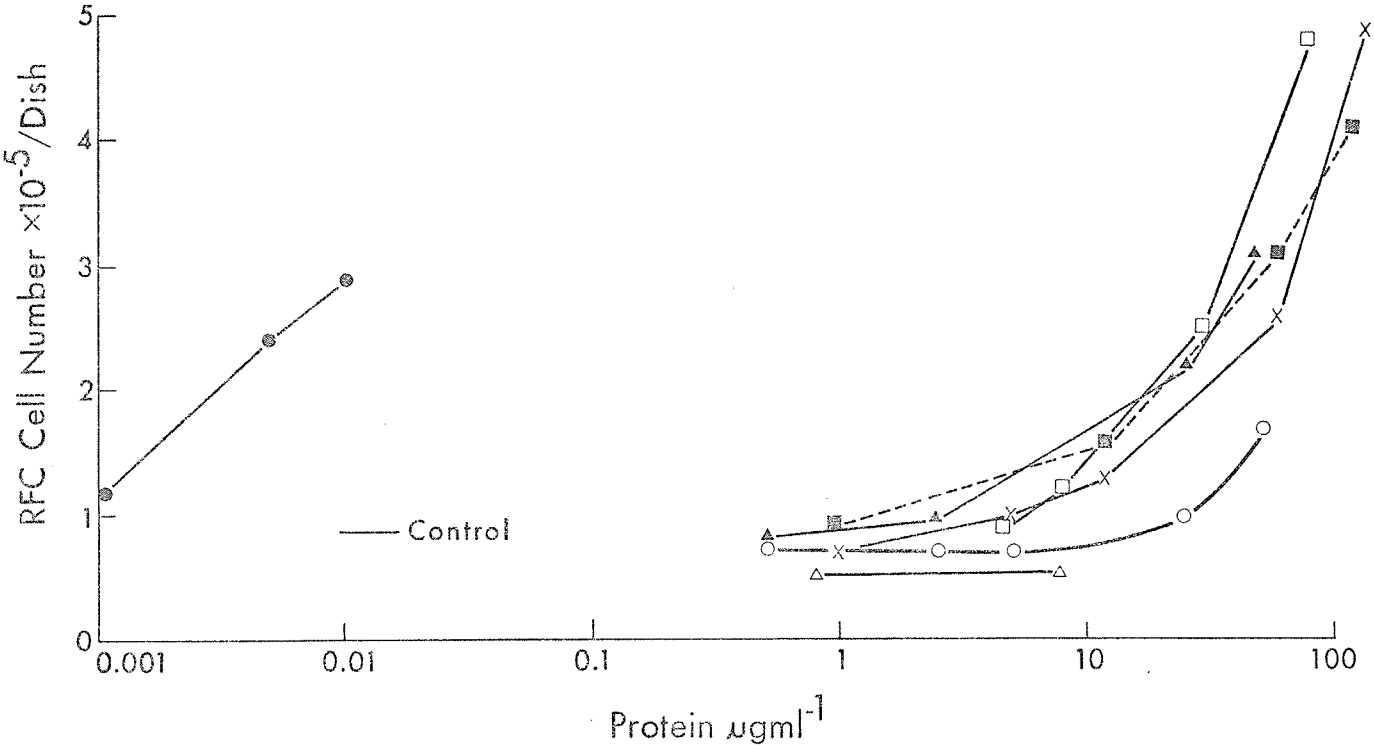
25-50% x———x,

50-75% □———□,

75-100% ■———■,

100% supernatant Δ———Δ.

Figure 7


RFC Cell Number ×10-5/Dish
5
4
3
2
1
0
Control
0.001
0.01
0.1
1
10
100
Protein μgml-1

Extraction of h.MTE at different pH

The specific activity (activity per unit protein) of the mammary tumor factor was approximately 10 times greater when the extraction was carried out at pH 3 and 4 in the presence of 0.15 M $(NH_4)_2So_4$ compared to the supernatant prepared in the routine procedure for the determination of the estrogen receptor Fig. 8 . Extractions performed at pH 10.5 and 13.5 yielded significantly less active material, due perhaps to the ineffectiveness of extraction of growth factors, or to the destruction of the biologically active material, or to the increased extraction of growth inhibitors (101). To test these possibilities an additional extraction was carried out to determine if:

i) the relatively inactive extract prepared at pH 10 could be "activated" when adjusted with HCl to pH 3.5,

ii) the biological activity in the extract prepared at pH 3.5 was stable when adjusted with NaOH to pH 10, and

iii) if the residues obtained after centrifugation of the homogenates contained growth inhibitors.

Comparison of stability of bioactivity recovered during extraction at pH 3.5 and 10

Significant bioactivity from the homogenate was recovered from the extract exposed to pH 3.5 with little or no activity recovered from the homogenate extracted at pH 10

Figure 8:

The effect of pH and $(NH_4)_2$ So_4 on the extraction of the h.mammary tumor factor

The residual tissue from 6 h.breast tumors (previously shown to contain significant bioactivity) was sliced and pooled to yield 7 g of tissue. Of this, 5.6. g was homogenised in 10 volumes of 0.15 M $(NH_4)_2$ So_4 and aliquoted into 8 equal volumes. The pH of each was adjusted (using NaOH or HCl) to either 3, 4, 5, 6, 7, 8, 10.5 or 13.5. The remaining tissue was homogenised in the buffer routinely used for the determination of estrogen receptors. All homogenates were mixed by using a shaking water bath at $4^{o}C$ for 2 hr. The samples were then centrifuged (100,000 g, 1 hr) and the supernatants dialysed against 2 x 4 litres 10 mm Tris buffer pH 7.4 at $4^{o}C$. Each extraction was assayed at 3 dilutions on the growth of rabbit fetal chondrocytes. The bioactivity of the h.brain B_3 fraction is shown for comparison.

Figure 8

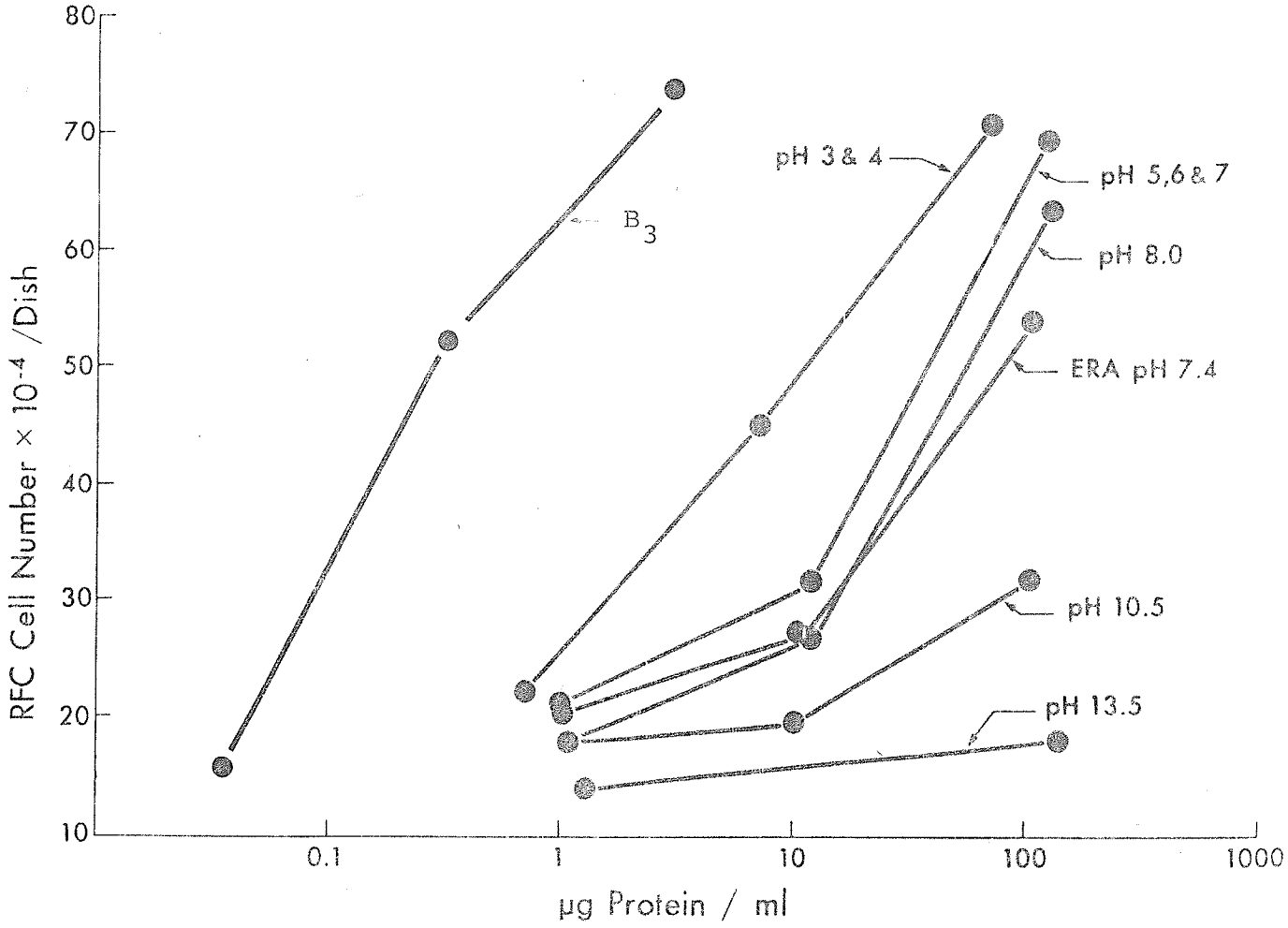

80
70
60
50
40
30
20
10
RFC Cell Number × 10-4 /Dish
0.1
1
10
100
1000
µg Protein / ml
B3
pH 3 & 4
pH 5,6 & 7
pH 8.0
ERA pH 7.4
pH 10.5
pH 13.5

Fig. 9 as shown in the previous experiment. However, when an aliquot of the pH 10 supernatant was adjusted to pH 3.5 for 1 hr significant bioactivity was demonstrated equivalent to that obtained for the original extraction at pH 3.5. The precipitate which formed at pH 3.5 showed a slight inhibitory effect on cell growth after resolubilisation at pH 10. When an aliquot of the supernatant from the extraction at pH 3.5 was adjusted to pH 10 - no loss of activity was demonstrable. Thus, pH 10 had no destructive action of the bioactivity. However, the effect of pH 3.5 in promoting the increased biological potency of the tumor extract may be due to the removal/inactivation of certain growth inhibitors at this low pH, and/or the activation of growth factors by physical and/or chemical modification or inactive precursors.

Figure 9:
Effect of pH on the extraction and stability of the mammary tumor factor using rabbit fetal chondrocytes

Four breast tumors (2.9 g) previously shown to contain significant levels of growth factor activity were homogenised in 30ml of 0.15 M $(NH_4)_2$ So_4 pH 7.0. The homogenate was divided into two equal volumes ~16 ml and the pH adjusted with HCl to pH 3.5 or with NaOH to pH 10. Following a 1 hr incubation at 4°C in a shaking water bath the homogenates were centrifuged at 100,000 g 4°C for 1 hr. The supernatants from each extraction were divided into two equal volumes. One half of the supernatants prepared at pH 3.5 was adjusted to pH 10 and one half of the supernatant prepared at pH 10 was adjusted to pH 3.5. Both supernatants were again incubated and centrifuged as before. All supernatants were treated in a comparable manner. The precipitates from each of the centrifugations were resuspended in 0.15 M $(NH_4)_2$ So_4 pH 10 and centrifuged. All supernatants were then dialysed against 3 x 4 litres of H_20 for 24 hr at 4°C. Samples were assayed in duplicate between 3 to 5 doses. The h.brain B_3 fraction is shown for comparison of bioactivity.

Stimulation

Extraction carried out at pH 3.5, o———o, and adjusted to pH 10, ●———●

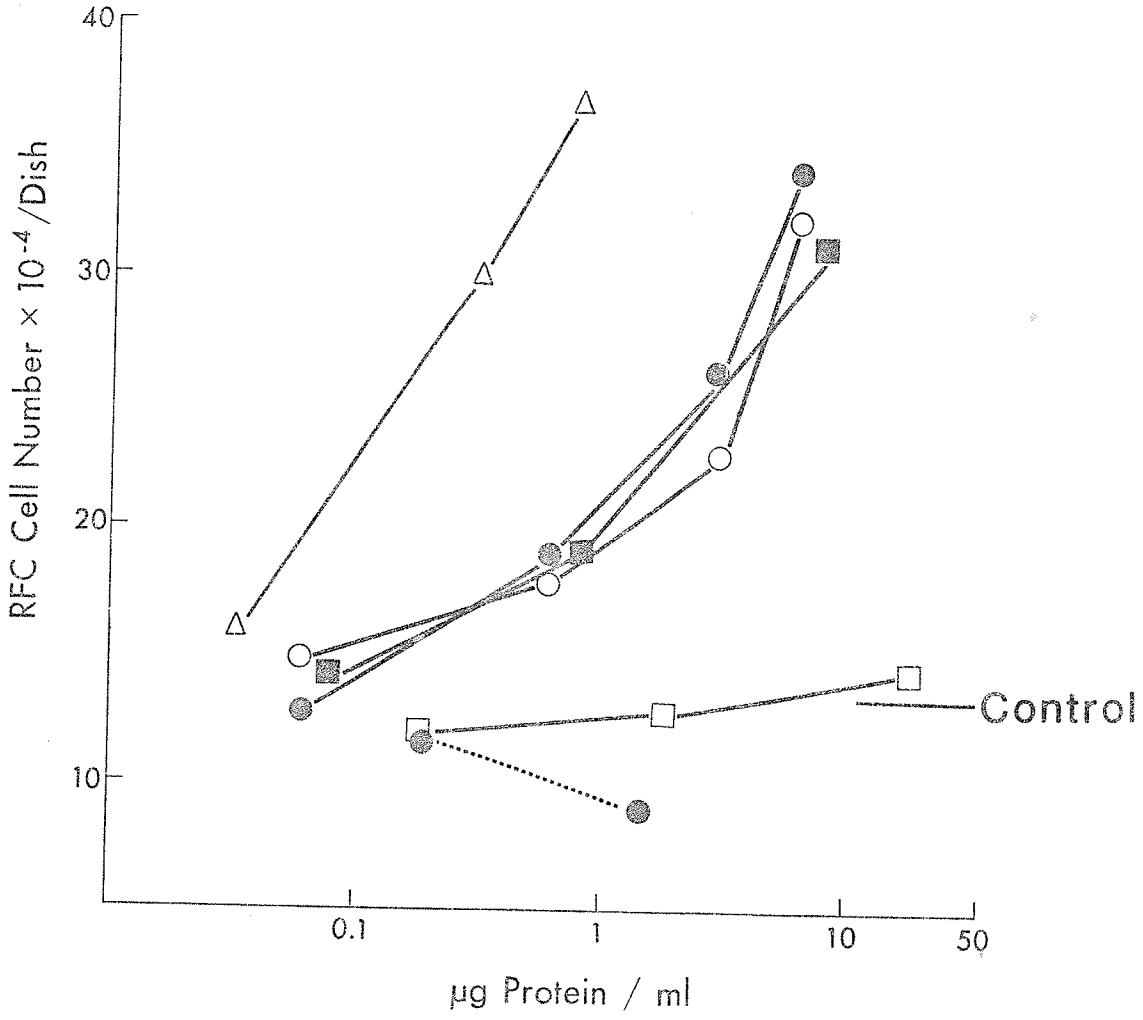
Extraction carried out at pH 10, □———□, and adjusted to pH 3.5, ■———■

Human brain B_3, Δ———Δ

Inhibition

Precipitate formed at pH 3.5 - resolubilised at pH 10, ●-----●

Figure 9


40
30
20
10
RFC Cell Number × 10-4/Dish
0.1
1
10
50
µg Protein / ml
Control

Preparative isoelectric focusing of a crude extract of h.breast cancer

Extraction of the breast cancer at pH 3.5 was shown to significantly increase the amount of growth factor activity per unit protein extracted from h.breast cancer specimens. Preparative isoelectric focusing of the tumor extract was undertaken to determine the isoelectric point of the growth factor(s) extracted at pH 3.5.

Fig. 10 shows the protein and pH profile of a typical extraction after isoelectric focusing. Bio-activity of the gel eluants was measured using the stimulation of DNA synthesis and cell growth assay of RFC cultures. In both bioassays, a major peak of activity was present corresponding to a pH interval of 8.6 - 9.5. The peak tubes stimulated a 3 and 7 fold increase in cell growth and ^{3}H-thymidine incorporation respectively. In gel segments corresponding to a pH of 6-8.5 cell growth and thymidine incorporation was stimulated 2 and 4 fold above control cultures respectively. The stimulation of cell growth was not demonstrated in gel segments below pH 6.0, however, the stimulation of thymidine incorporation was measured in the pH range of 4.5-6.0.

The presence of carrier ampholytes in the tumor extract was shown to exhibit an inhibitory influence on cell growth, and inclusion of these contaminants

Figure 10:

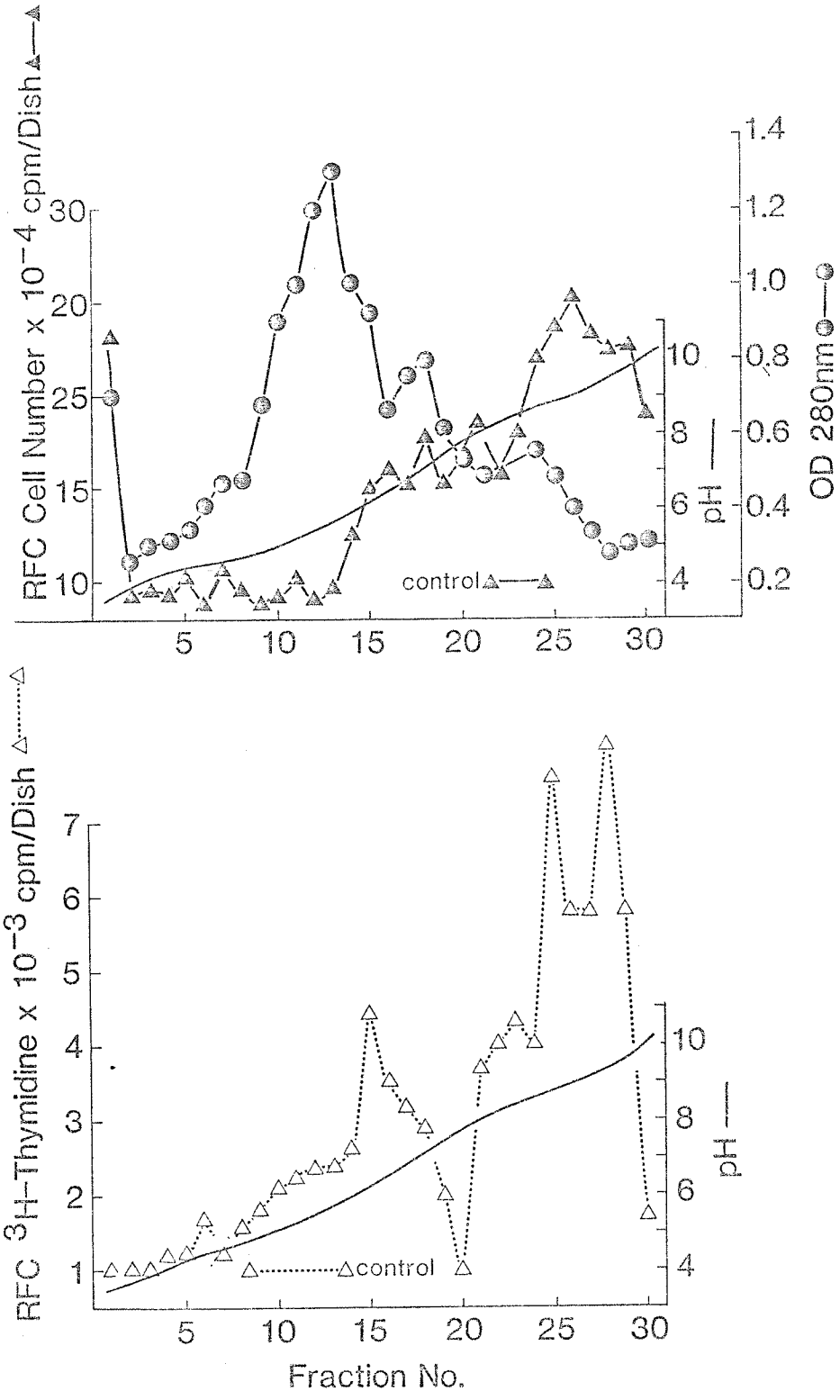
Isoelectric focusing of human mammary tumor extract

Approximately 200 mg of protein extracted from 30 g of h.mammary tumors (37 chosen at random) was focused using Sephadex IEF in a flat bed system as described in the methods. At the completion of isoelectric focusing the gel was resuspended as described and 100 µl aliquots from each fraction were diluted to 1 ml using 200 mM Hepes buffer pH 7.4 containing 0.1% BSA and 100 µl of this was added to duplicate cultures of rabbit fetal chondrocytes as described.

Panel A - Stimulation of cell growth

Panel B - Stimulation of DNA synthesis

Figure 10


RFC Cell Number x 10^{-4} cpm/Dish
OD 280nm
pH
control
RFC ^{3}H-Thymidine x 10^{-3} cpm/Dish
Fraction No.

prior to purification was avoided. Since the majority of bioactivity was, however, recovered in the alkaline region of the IEFgel, chromatography of the crude h.mammary tumor extract using a cation exchange was investigated.

Purification of the h.MTGF using Whatman CM52

Fig. 11 shows the stimulation of cell growth of RFC in response to fractions obtained from chromatography of the h.MTGF on Whatman CM52 ion exchange. The unabsorbed fractions and fractions eluting at 0.2 M ammonium formate contained little or no bioactivity, and represented over 90% of the applied protein (Table 2). The most active fraction eluted from the column at 0.4 M ammonium formate (CM4) and represented 38% of the applied activity, and a 100 fold increase in the specific activity. The yield of CM4 was 2.0 mg protein, or 35 mg/kg tissue. The fractions which eluted at 0.3 M and 1 M ammonium formate represented less than 15% of the applied activity. Fraction CM4 was 8 times more active when compared to the h.brain B_3 fraction. The CM4 fraction represented a significant increase in the bioactivity of the h.MTGF when compared to the crude extract. Further characterisation of the CM4 fraction was carried out to determine the isoelectric point and mol. wt. using Sephadex G-100 and SDS PAGE.

Figure 11:

Chromatography of the h.mammary tumor extract using Whatman CM52 ion exchange

Approximately 60 g of h.mammary tumor tissue (46 tumors) was sliced into small pieces (approximately 2-3 mm^3) and homogenised in 0.15 M $(NH_4)_2$ So_4 pH 7.0. The pH of the homogenate was adjusted to pH 3.5 by the addition of 6MHCl. The homogenate was then stirred for 2 hr at 4^oC and then centrifuged 30 min. at 30,000 g. The supernatant was dialysed against distilled H_20 (3 x 16 litres) for 18 hr at 4^oC using spectrapor 1 mol. weight cut off 6-8000. The retentate (540 ml) was gravity fed through a column of Whatman CM52 ion exchange (1.8 x 2.00 cm) pH 6.0. The absorbed proteins were then sequentially eluted with 0.2, 0.3, 0.4, and 1 M ammonium formate (pH 6.0). At each step, the elution was discontinued when the absorbance at 280 nm fell below 0.01. The eluates were lyophylized and redissolved in dH_20. The absorbance at 280 nm was recorded and the biological activity of each fraction determined using the rabbit fetal chondrocytes.

Figure 11

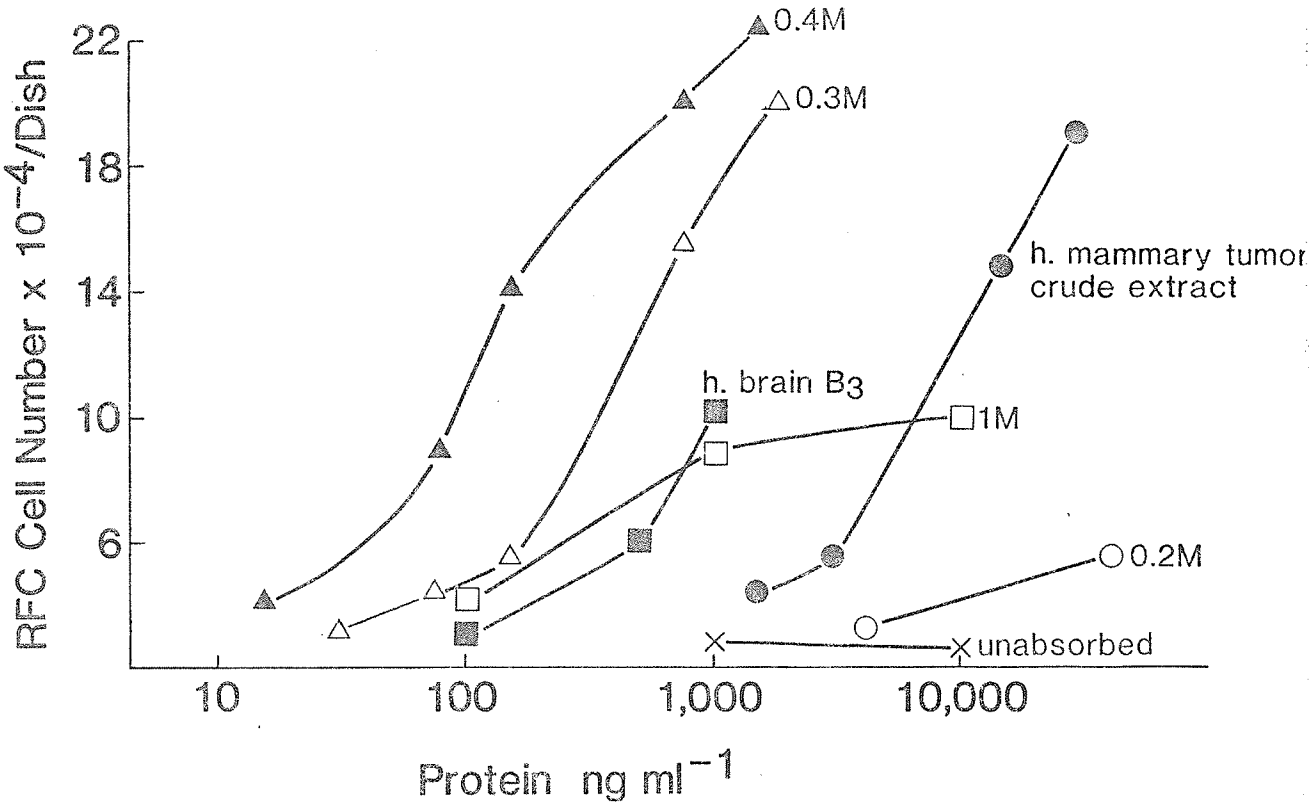

RFC Cell Number x 10^{-4}/Dish
22
18
14
10
6
10
100
1,000
10,000
Protein ng ml^{-1}
0.4M
0.3M
h. mammary tumor
crude extract
h. brain B_3
1M
0.2M
unabsorbed

Table 2

Summary of purification steps on CM52 of the h.mammary tumor extract

	Protein	Activity	Total activity	Activity recovered
	mg	U/mg		%
crude extract	650	1	650	100
CM52 unabsorbed	240	ND	-	-
0.2	350	ND	-	-
0.3	3.5	22	77	12
0.4	2	100	200	31
1.0	2	5	10	1.5

ND - not detectable. Activity is expressed relative to CM4 100 ng ≡ 0.01U

Isoelectric focusing of CM4

Isoelectric focusing of CM4 (200 µg) was performed using analytical polyacrylamide slab-gels, in the range of pH 3.5 - 10. A single broad peak of activity was detected in gel eluants corresponding to a pH interval of 8.0 - 9.3, with the peak activity in two gel segments corresponding to a pI 8.8 - 9.3 (Plate 1). The isoelectric point of the peak was confirmed by comparison of its migration between the lentil lectin basic band, pI 8.65 and trypsinogen, pI 9.3. The staining pattern of the gel in the area corresponding to the activity revealed a faint diffuse band. A major protein band was observed at pH 10, but the gel eluants in this region were inactive in the assay. Since the h.mammary tumor derived growth factor is stable at pH 10.0, Fig. 9, it is unlikely that the biological activity was destroyed at this pH. The major growth promoting activity present in the crude mammary tumor extract (demonstrated using preparative IEF, with a pI of 8.5-9.5, Fig.10), could therefore be selectively concentrated using CM52. Further purification of the CM4 fraction might therefore be achieved utilising analytical isoelectric focusing over a narrower range of pH 8.5 - 10.5.

Plate 1:

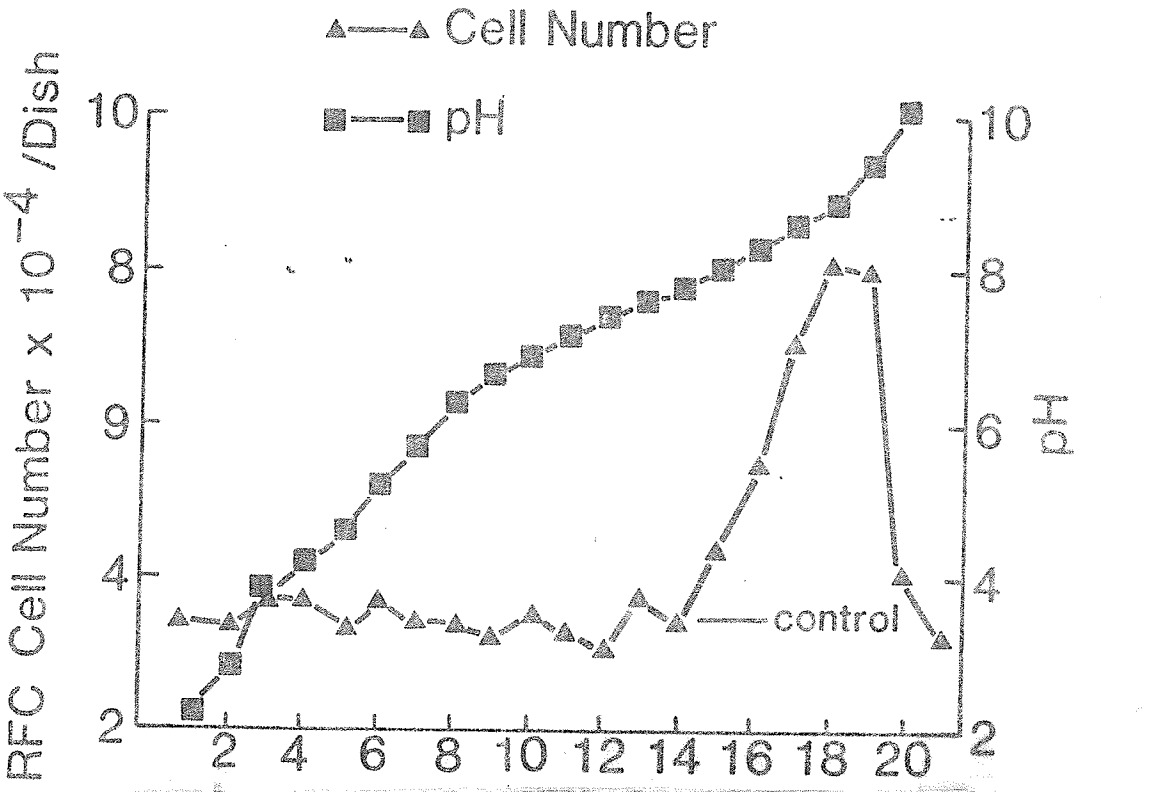
Isoelectric focusing of human mammary tumor fraction CM4

Approximately 100 µg protein of the h.mammary tumor fraction CM4 was subjected to isoelectric focusing using LKB polyacrylamide gels as described in the methods. After completion of electrofocusing, the gel was sliced into 21 segments and each segment eluted in distilled water overnight at $4^{o}C$. The pH of each segment eluate was determined and an aliquot (100 µl) diluted with 100 µl of Hepes buffer (200 mM pH 7.4) and 800 µl of F10 containing 10% FBS. Aliquots (100 µl) were then tested for growth promoting activity using rabbit fetal chondrocytes.

Lane 1. 200 µg of h.mammary tumor fraction CM4

Lane 2. Calibrated proteins standards

Plate 1



	pI(24°C)
amyloglucosidase	3.5
soybean trypsin inhibitor	4.55
B-lactoglobulin A	5.20
b. carbonic anhydrase B	5.85
human carbonic anhydrase	6.55
horse myoglobin-acidic band	
horse myoglobin-basic band	7.35
lentil lectin-acidic band	8.15
lentil lectin-middle band	8.45
lentil lectin-basic band	8.65
trypsinogen	9.3

Molecular weight determination of CM4 fraction

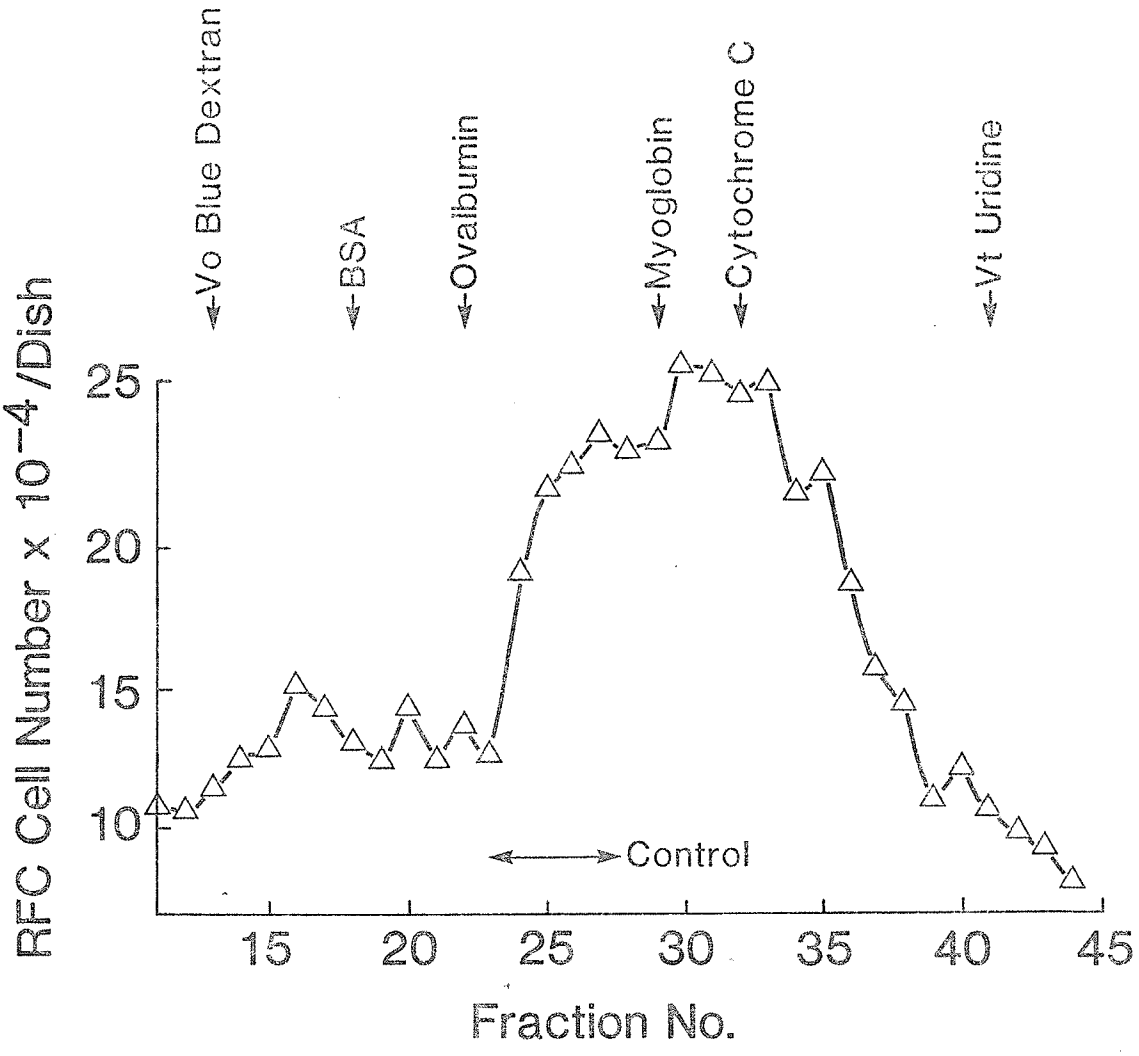
Sephadex G-100

Approximately 700 µg of the CM4 fraction was chromatographed using Sephadex G-100. The majority of bioactivity eluted in a broad peak with proteins of molecular weight 10-30,000 daltons Fig. 12 . A small peak of activity eluted between the void volume and the BSA marker.

SDS-Polyacrylamide electrophoresis

Various fractions during the purification of the h.mammary tumor derived growth factor were analysed using SDS-PAGE (Plate 2). The CM4 fraction (lane 3) revealed several bands. The gel eluants corresponding to the peak activity of the CM4 after isoelectric focusing (pI 8.8-9.3) were lyophilised and run in lane 2 for comparison. One very faint staining band corresponding to a molecular weight of 80,000 daltons, and a diffuse band between 15-30,000 daltons was observed. The staining pattern of proteins in these regions of the gel is consistent with the molecular weight estimate (80,000 and 10-30,000 daltons) obtained by gel filtration of the CM4 fraction on Sephadex G-100 Fig.12.

Figure 12



Gel Filtration of CM4 using Sephadex G-100

Approximately 700 µg of CM4 was chromatographed using a Sephadex G-100 column (70 x 1.5 cm) equilibrated in 10 mM Tris, pH 7.4. Fractions (3 ml) were collected and aliquots (100 µl) were collected and tested for their ability to stimulate the growth of rabbit fetal chondrocytes.

Plate 2:

Sodium dodecyl sulfate gel electrophoresis of the partially purified h.mammary tumor and h.brain derived growth factors

The electrophoretic mobility of various fractions obtained during purification of the h.mammary tumor and h.brain derived mitogens were compared on 15% SDS PAGE as described in the methods.

Lane 1 and 8 Molecular weight standards

h.mammary tumor derived growth factors

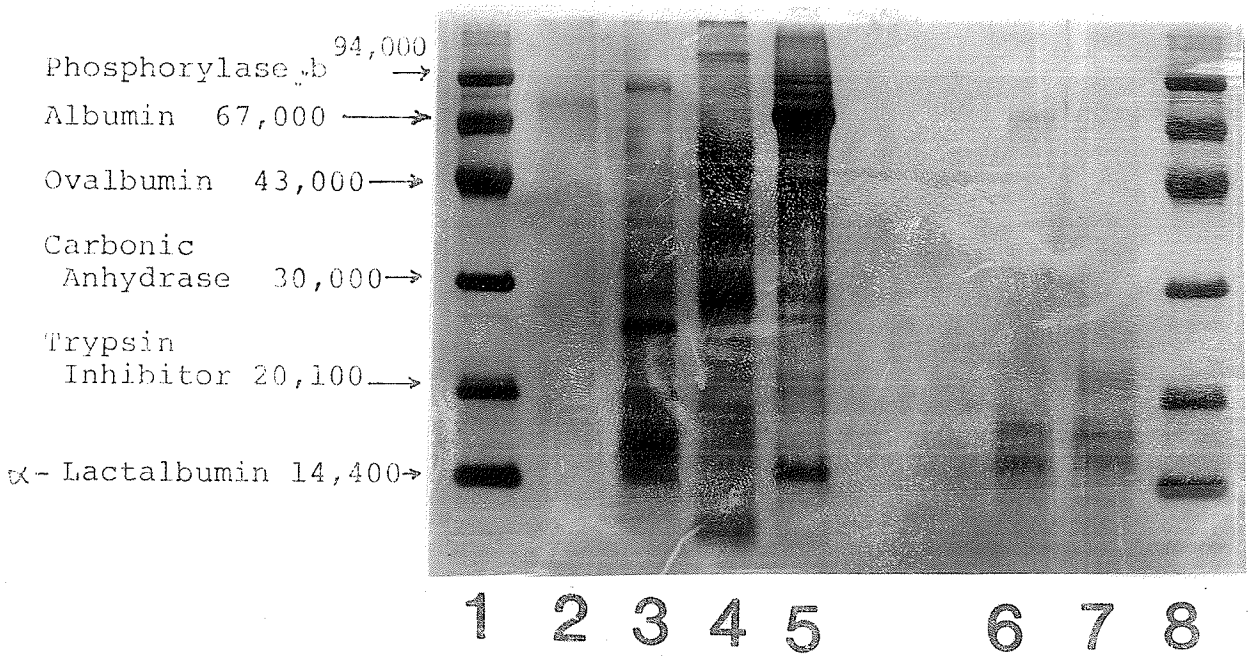
	Lane 2	IEF peak of CM4	
	Lane 3	CM4	50 µg
crude tumor homogenate	Lane 4	acid extraction	50 µg
	Lane 5	neutral extraction	50 µg

h.brain derived growth factors*

Lane 6	C4-IVC	20 µg
Lane 7	C4-IVD	20 µg

*Protein fractions from the Whatman CM52 ion exchange chromatography (Section II).

Plate 2

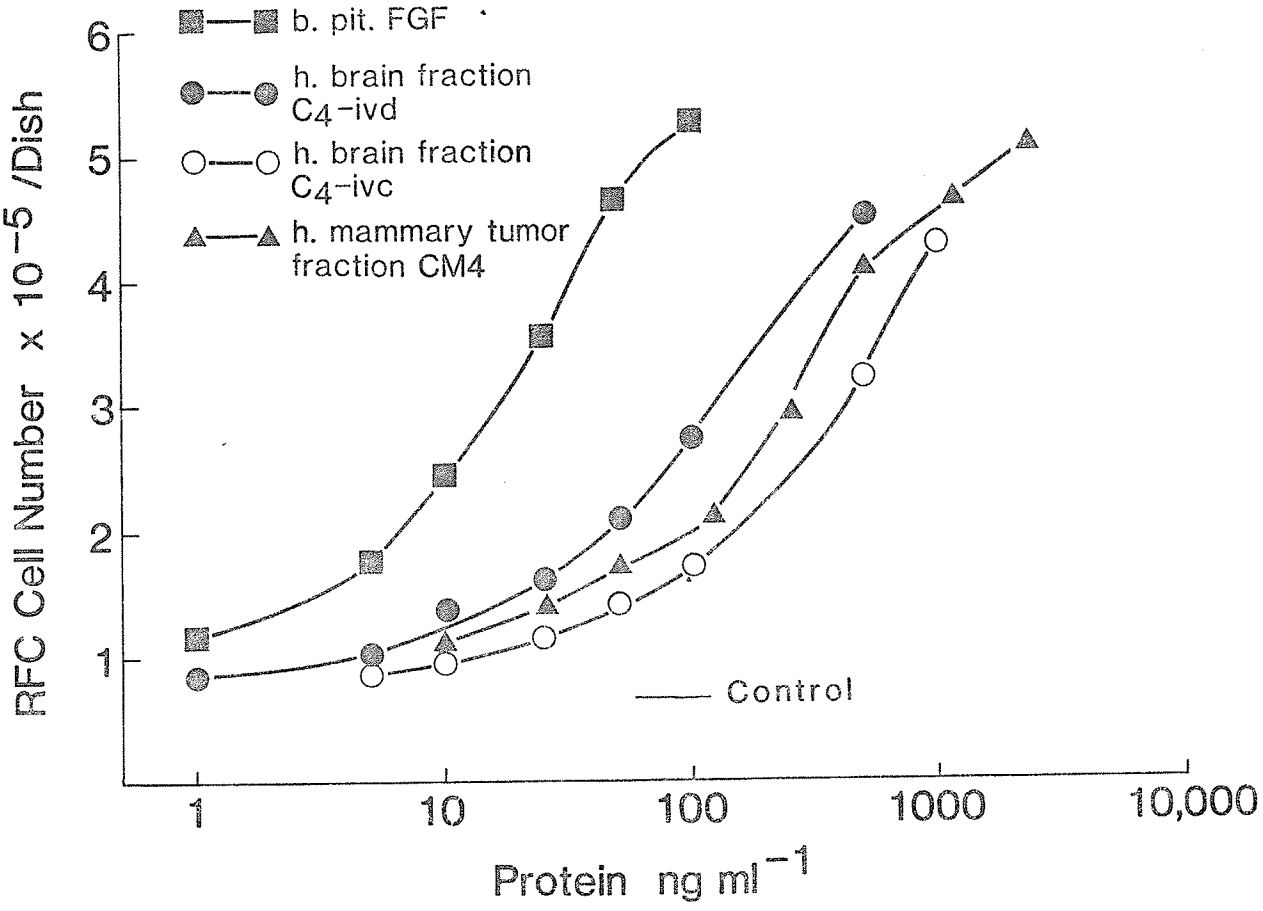

Phosphorylase b 94,000
Albumin 67,000
Ovalbumin 43,000
Carbonic Anhydrase 30,000
Trypsin Inhibitor 20,100
α-Lactalbumin 14,400
1 2 3 4 5 6 7 8

Comparison of the biological activity of the h.mammary tumor growth factor and the h.brain derived growth factors with b.pituitary FGF

The biological potency of the h.mammary tumor derived growth factor CM4 and the h.brain derived growth factors C4-IVc and C4-IVd were compared with a freshly prepared commercial preparation of b.pituitary FGF Fig.13 . The h.brain growth factors (C4-IVc and C4-IVd, Section II) were partially purified from h.brains by ammonium sulphate precipitation, ion exchange and gel permeation chromatography. These factors have a molecular weight of 10-15,000 daltons and represent the protein fractions following the last step in their purification on Whatman CM52 ion exchange.

The partially purified human mammary tumor growth factor CM4 was 12 times less active compared to purified b.pituitary FGF. Stimulation of cell growth by the CM4 fraction was demonstrated as low as 25 ng ml^{-1}. The bioactivity of the CM4 fraction was twice as active when compared to the h.brain derived growth factors C4-IVc, and 50% as active compared to the C4-IVd factor.

Figure 13



Comparison of biological potency of growth factor preparations on the proliferation of rabbit fetal chondrocytes

The bioactivity of 2 partially purified h.brain derived growth factors C4-IVc and C4-IVd (see Fig. 25, page 108). were compared to the potency of CM4 fraction obtained from h.mammary tumor tissue. Bovine pituitary FGF was used as a standard and each point represents the mean of duplicate cultures of rabbit fetal chondrocytes exposed to an increasing dose of the various growth factors.

The Table below compares the relative yields of the h.brain and h.mammary tumor derived growth factors using b.pituitary FGF as a reference standard. The yield of growth factor from the h.mammary tumor was 300-800 times greater when compared to the h.brain derived growth factors. Due to the number of purification steps involved in the isolation of h.brain growth factors, it is conceivable that the low yield of activity is due to a substantial loss of growth factors during the lengthy purification procedure, or alternatively the amount of mitogens purified is a reasonable estimate of their abundance in the h.brain.

Comparison of the yields of growth factors from h.mammary tumors and h.brain

Growth Factor	specific activity U/mg	Yield mg/kg wet weight	Total activity U/kg
b.pit. FGF	4000	-	
h.mammary tumor CM4	260	33	8500
h.brain C4-IVc	150	0.057	9
h. brain C4-IVd	430	0.043	18

Table 3

Effect of CM4 on the proliferation of human foreskin fibroblasts and Balb/c3T3 cells

The CM4 fraction stimulated a dose dependent increase in the growth of human foreskin fibroblasts and Balb/c3T3 cells maintained in medium supplemented with 0.5% FBS. The CM4 was approximately 10% as active as b.pituitary FGF. These findings indicate that the partially purified h.MTGF can stimulate other cells of mesodermal origin. (Table 4).

Effect of various growth factors on the stimulation of RFC proliferation

Fig. 14 shows the effect of growth factors on the stimulation of RFC growth in cultures maintained in the presence of 10% FBS as described in the methods. Of the mitogens tested, m-EGF and b.pituitary FGF were found to stimulate cell growth in a dose dependent manner as shown. Other growth factors such as NGF, MSA and insulin had no effect on the stimulation of cell growth, when tested over the same concentration. Furthermore, pituitary hormones and growth factors such as h.GF, h.Prl, h.FSH, h.LH, vasopressin, oxytocin and phosphoethanolamine when tested over the range of 5-500 ng ml^{-1} were also found to be ineffective in stimulating cell growth (S. Kasper, 1981).

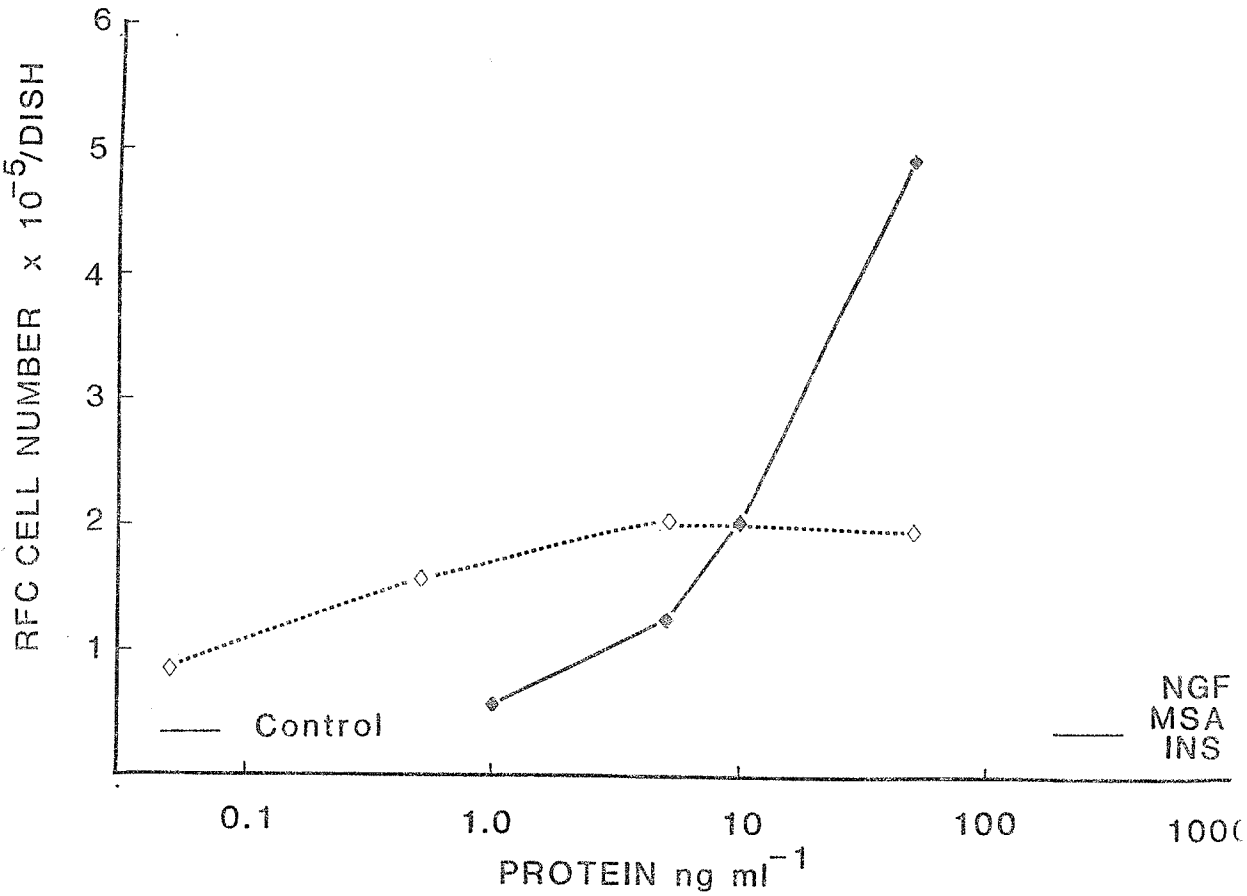
Table 4

Effect of CM4 on the proliferation of human foreskin fibroblasts and Balb/c3T3 cells

	Cell number x 10^4/dish	
	Human foreskin fibroblasts	Balb/c3T3
conditions		
CONTROL	2.5	5.2
CM4 ng ml^{-1}		
1	2.5	5.3
10	2.4	6.2
100	3.3	6.9
1000	4.4	8.1
b.pituitary FGF ng ml^{-1}		
0.5	2.3	4.8
5	2.9	6.7
50	3.8	7.9
10% FBS	8.8	16.0

Cells were plated at 2 x 10^4 cells/dish in DMEM containing 10% FBS, day 0. Twenty four hours later the medium was changed to serum free and replenished with fresh medium containing 0.5% FBS, day 1. Samples were added on day 1 and 3 and cell number determined on day 5. Values represent the mean of duplicate cultures.

Figure 14



Effect of various growth factors on the stimulation of RFC proliferation

Rabbit fetal chondrocytes were maintained in F-10 medium supplemented with 10% FBS as described, and exposed to increasing concentrations of b.pituitary FGF ◆——◆, m-EGF ◇-----◇.

Other characteristics of the h.MTGF

The effect of heat, protease digestion and acetic acid on the h.MTGF

The h.MTGF was found to be stable when incubated at 24°C overnight or at 56°C for 30 minutes. However, heating the h.MTGF at 100°C for 30 minutes, or incubation under very acidic conditions (1 M acetic acid, pH 2.1) destroyed the biological activity. Incubation of the h.MTGF with trypsin and chymotrypsin abolished 80% of the growth promoting activity, Table 5 . The data suggests that a protein is an essential component of the biological activity which is subject to denaturation when heated to 100°C, and labile at a pH of < 2.5.

Stability of the h.MTGF under various chemical conditions

When the h.MTGF was exposed to the denaturing conditions of 8 M urea only 20% of the initial activity remained, whereas exposure to 6 M guanidine hydrochloride completely abolished the bioactivity, Fig.15. Incubation of h.MTGF with 2 M NaCl, or 0.1 M acetic acid was without effect on the biological activity. Incubation with 1% β-mercaptoethanol resulted in a 50% increase in bioactivity, suggesting that disulphide bonds are not required for biological activity.

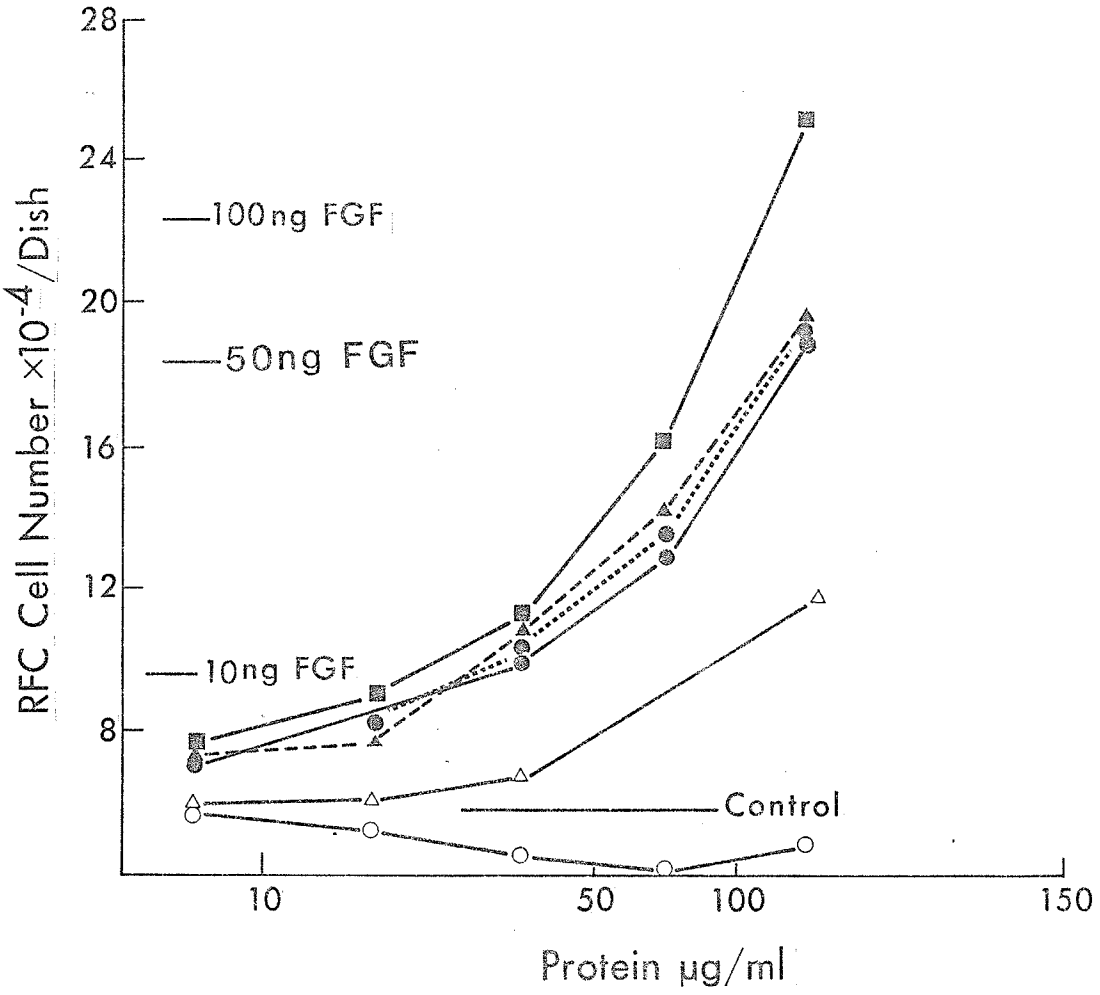
Table 5

Treatment	Rabbit fetal chondrocytes cell number x 10^{-4}/dish ± S.D.
CONTROL	5.5 ± 0.96
h.MTF	15.7 ± 1.9
h.MTF + (T + CH) +TI	7.4 ± 0.5
h.MTF + (T + CH + TI)	12.9 ± 0.5
T + CH + TI	5.9 ± 1.0
h.MTF 56°C	15.9 ± 1.1
100°C	8.0 ± 0.1
1 M acetic acid	4.4 ± 0.1

Effect of enzyme digestion, temperature and acetic acid on the growth factor activity of the h.mammary tumor extract for rabbit fetal chondrocytes

Approximately 1.8 mg of h.mammary tumor extract was incubated with trypsin (T) 300 µg and chymotrypsin (CH) 300 ug overnight at room temperature. The incubation was terminated by the addition of trypsin inhibitor (TI) 600 ug. Control samples were incubated in the presence of T + CH + TI. Additional samples were incubated at 56°C or 100°C in a water bath for 30 minutes, or in the presence of acetic acid (1 M pH 2.1) overnight at 4°C followed by dialysis. All samples were assayed at 55 µg ml^{-1} for the ability to stimulate cell growth using rabbit fetal chondrocytes.

Figure 15



Stability of the human mammary tumor extract

The supernatant from a variety of h.mammary tumors was pooled and the 5 ml aliquots were incubated overnight at 4°C in the presence or absence of a variety of chemicals, followed by dialysis (mol. wt. cut off 3,500) against 2 x 4 litres of 10 mM Tris buffer pH 7.4. The concentrations quoted are final concentrations and the values represent the mean of duplicate culture using rabbit fetal chondrocytes.

No treatment ▲-----▲,
urea 8 M △——△,
guanidine hydrochloride 6 M o——o,
acetic acid 0.1 M ●——●,
Mercaptoethanol 1% ■——■,
sodium chloride 2 M ●-----●

Standards are bovine pituitary FGF.

Comparison of the effect of the h.mammary tumor extract on the proliferation of a variety of h.mammary carcinoma cell lines

The h.mammary tumor extract (h.MTE) was prepared from a pool of tumor supernatants prepared for the routine determination of the estrogen receptor content as described.

Table 6 shows the effect of the h.MTE on the proliferation of 4 h.mammary tumor cell lines. The h.MTE when assayed at 20 µg protein ml^{-1} culture medium, in the presence of 0.1% FBS, stimulated an increase in cell growth in 3 of the 4 lines tested, compared to control cultures maintained in the presence of 0.1% FBS alone. With respect to the MDA-MB-231 cell line, significant cell loss was observed in control cultures maintained in serum free medium, and 0.1% FCS. However, in the presence of 1% FCS, the h.MTE stimulated cell growth 2.5 fold. The h.MTE did not stimulate cell growth in any of the four cell lines tested when cultures were maintained in the presence of 10% FBS during the culture period.

Effect of dialysis, heat, proteases, and acetic acid on the growth-promoting activity of the h.MTE on human breast cancer cell line T-47D

Incubation of the h.MTE in the presence of 1 M acetic acid overnight at $4^{o}C$, followed by dialysis was without

Table 6

Conditions		CELL LINE			
		Final cell number x 10^4/dish			
		T-47D	HS-0578T	MCF-7	MDA MB 231
FBS	h.MTE				
NONE	-	NT	7.7	3.4	0.6
	+	NT	13.1	19.0	-
0.1%	-	7.8	12.2	24.9	0.4
	+	21.9	17.1	23.1	2.7
1.0%	-	NT	23.1	41.6	8.2
	+	NT	21.7	31.2	19.2
10%	-	59.5	28.3	36.6	31.1
	+	55.5	27.4	37.5	33.8

Comparison of the effect of h.mammary tumor extract on the proliferation of a variety of h.mammary carcinoma cell lines

Stock cultures of h.mammary carcinoma cell lines were maintained as described. Approximately 1 x 10^4 cells were inoculated in 35 mm dishes in the presence of 10% FBS, day 0. On day 1 cells were washed in serum free media and the media replaced by fresh serum free medium. Fetal bovine serum (FBS) was added to the culture dishes to give a final concentration of 0.1, 1.0 and 10% FBS. These cells were then incubated in the presence (+) or absence (-) of 20 $\mu g\ ml^{-1}$ of a pool of h.mammary supernatants prepared from solid tumors as described for the determination of the estrogen receptor. Media was changed every other day and fresh FBS and h.mammary tumor supernatant added. Cell number was determined on day 9 of duplicate culture.

effect on the growth factor activity for T-47D cells, Table 7. Dialysed samples of the extract had similar potency to undialysed control extracts. Heating of the h.MTE (100^{o}C for 30') and incubation of the extract with proteases abolished 70% and 90% of the biological activity.

These data would suggest that the growth factor for T-47D cells is a thermolabile, acid stable protein. In contrast, however, when the tumor extract was tested on the RFC cultures (see Table 7, page 84) the biological activity was found to be unstable following treatment with 1 M acetic acid. These data suggest therefore, the presence of a different population of growth factors in the h.MTE.

The growth of these cells was found to be almost maximally stimulated by the presence of 1.0% FBS and the cells proliferate for a few days even in 0.1% FBS. Hence in the presence of of 0.1% FBS, the continued growth of these cells often obscured the effect of the added mitogen, and necessitated long culture periods in order to observe a net difference in growth.

When the serum supplement was changed to 10% bovine serum, the growth of T-47D slowed dramatically compared to cultures maintained in the presence of 10% FBS. Further experiments revealed that the T-47D cells could be maintained in a non-proliferative state in 0.1% BS for up to 15 days in culture, provided the culture medium was supplemented with glucose and insulin and changed every other day.

Table 7

Expt. I

Treatment		T-47D cell number x 10^5/dish mean ± SD
	control	1.0 ± 0.1
h.MTE :	dialysed	1.7 ± 0.1
	non-dialysed	1.7 ± 0.2
	100°C 30 min.	1.2 ± 0.04
	1 M acetic acid	1.7 ± 0.2

Expt. II

	control	2.5 ± 0.1
h.MTE		4.5 ± 0.3
h.MTE	+ (T) + TI	3.1 ± 0.1
h.MTE	+ (T + TI)	4.7 ± 0.2
	(T + TI)	2.7 ± 0.2

Effect of various treatments on the growth factor activity of h.MTE for T-47D cells

Samples of the h.MTE were either incubated at 100°C for 30 min. and rapidly cooled on ice or incubated in 1 M acetic acid overnight at 4°C, followed by dialysis. Additional samples (400 µg protein) were incubated with 250 µg trypsin (T) at room temperature for 3 hr. The incubation was terminated by the addition of 600 µg of trypsin inhibitor (TI).*

All samples were assayed at 10 µg protein ml^{-1} for the stimulation of T-47D cell growth. Values represent the mean ±SD of triplicate cultures maintained in the presence of 0.1% FBS for 9 days.

* Trypsin and trypsin inhibitor were also pre-mixed (T + TI) and subsequently incubated in the presence or absence of the h.MTE. These samples were included as a control for the effect of either trypsin or trypsin inhibitor.

Effect of hormones and growth factors on the growth of T-47D cells

Mouse EGF and b.pituitary FGF did not have a significant effect on the stimulation of growth of T-47D cells Fig. 16 . Insulin (0.5 - 5.0 $\mu g\ ml^{-1}$) and MSA (10 - 100 $ng\ ml^{-1}$) had a slight mitogenic effect stimulating cell growth by 50%, compared to control cultures. The mammary tumor extract however, stimulated a dose dependent increase in cell growth over the concentration range from 0.2 - 250 μg protein ml^{-1}. The sample of dialysed h.milk tested was stimulatory at 25 and 250 μg protein ml^{-1} and was approximately 100 times less active on a protein weight basis compared to the tumor extract.

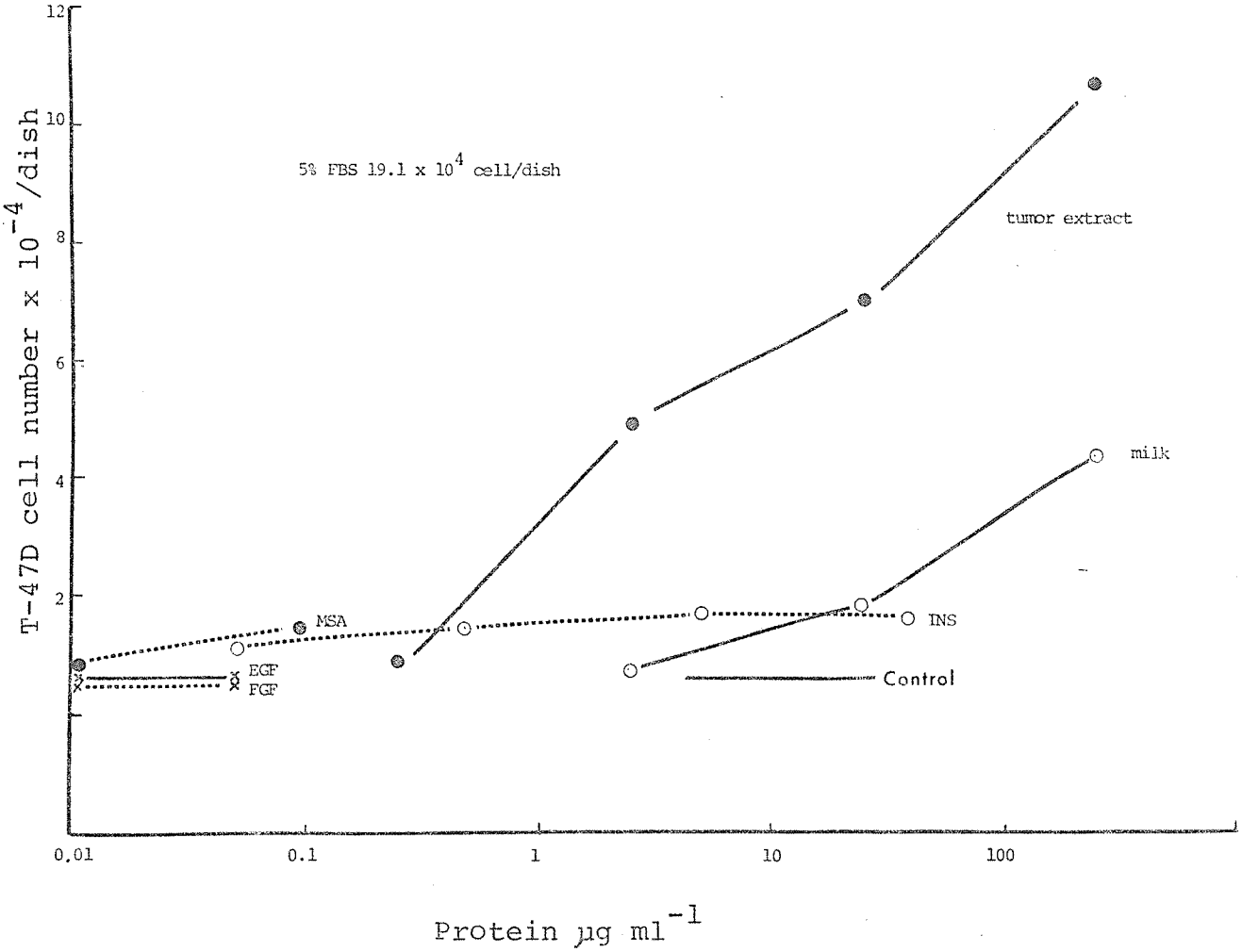
Under serum-free conditions the growth of T-47D cells are responsive to a combination of Transferrin (25 $\mu g\ ml^{-1}$), EGF (10 $ng\ ml^{-1}$), T3 (1 $ng\ ml^{-1}$), Insulin (0.1 $\mu g\ ml^{-1}$) and hydrocortisone (0.1 $\mu g\ ml^{-1}$). Human prolactin (1 $\mu g\ ml^{-1}$) and h.growth hormone (1 $\mu g\ ml^{-1}$) do not further increase the growth of these cells (R.P.C. Shiu personal communication). The cells are also unresponsive to 17β-estradiol, $10^{-8}M$, testosterone, $10^{-8}M$, dexamethasone, $10^{-7}M$ and progesterone $10^{-7}M$. T_3, $5 \times 10^{-7}M$ had a slight stimulatory effect.

Figure 16:

Effect of various growth factors, h.MTE and h.milk on the growth of T-47D cells.

Cultures of T-47D cells were maintained in medium containing 0.1% BS for 7 days as described, in the presence of MSA ●------●, Insulin o------o, h.milk o———o, h.MTE ●———●, EGF x———x, or FGF x------x. Media and growth factors were replenished every other day. Values represent the mean of duplicate cultures.

Figure 16


12
10
8
6
4
2
T-47D cell number x 10^{-4}/dish
5% FBS 19.1 x 10^4 cell/dish
tumor extract
milk
MSA
INS
EGF
FGF
Control
0.01
0.1
1
10
100
Protein µg ml^{-1}

Characterisation of the h.MTE on the growth of the T-47D cell line

The growth of T-47D cells during a 15 day culture period in the presence or absence of 20 $\mu g\ ml^{-1}$ of the h.MTE extract is shown in Fig. 17 . In the presence of 0.1% BS, the h.MTE stimulated an 8 fold increase in cell number compared to control cultures. In the presence of 10% BS however, the h.MTE stimulated a slight increase (40%) in cell number on day 15 of culture. The final cell number of cultures maintained in the presence of 10% BS alone, was the same as cultures maintained in 0.1% BS alone. Hence high concentrations, i.e. 10% BS, did not stimulate the growth of T-47D cells. Furthermore, the presence of 10% BS inhibited the mitogenic effect of the h.MTE. The final cell number of T-47D cells cultured in 10% BS in the presence of the h.MTE was 7.1×10^4 cells/dish. However, in the presence of 0.1% BS and the h.MTE, the final cell number was 41×10^4 cell/dish. When the T-47D cells were cultured in the presence of 10% FBS, the final cell number (day 15) was 1.4×10^6 cells/dish, which was further increased to 2.1×10^6 cells/dish in the presence of h.MTE. This observed difference in cell number, due to the presence of the extract, was not evident on day 11 of culture.

These data might suggest that in the presence of an adequate serum supplement, the growth of T-47D at low density is governed by factors present in the h.MTE and FBS. In contrast, at high density the growth of T-47D might be further regulated by factors present in the tumor extract.

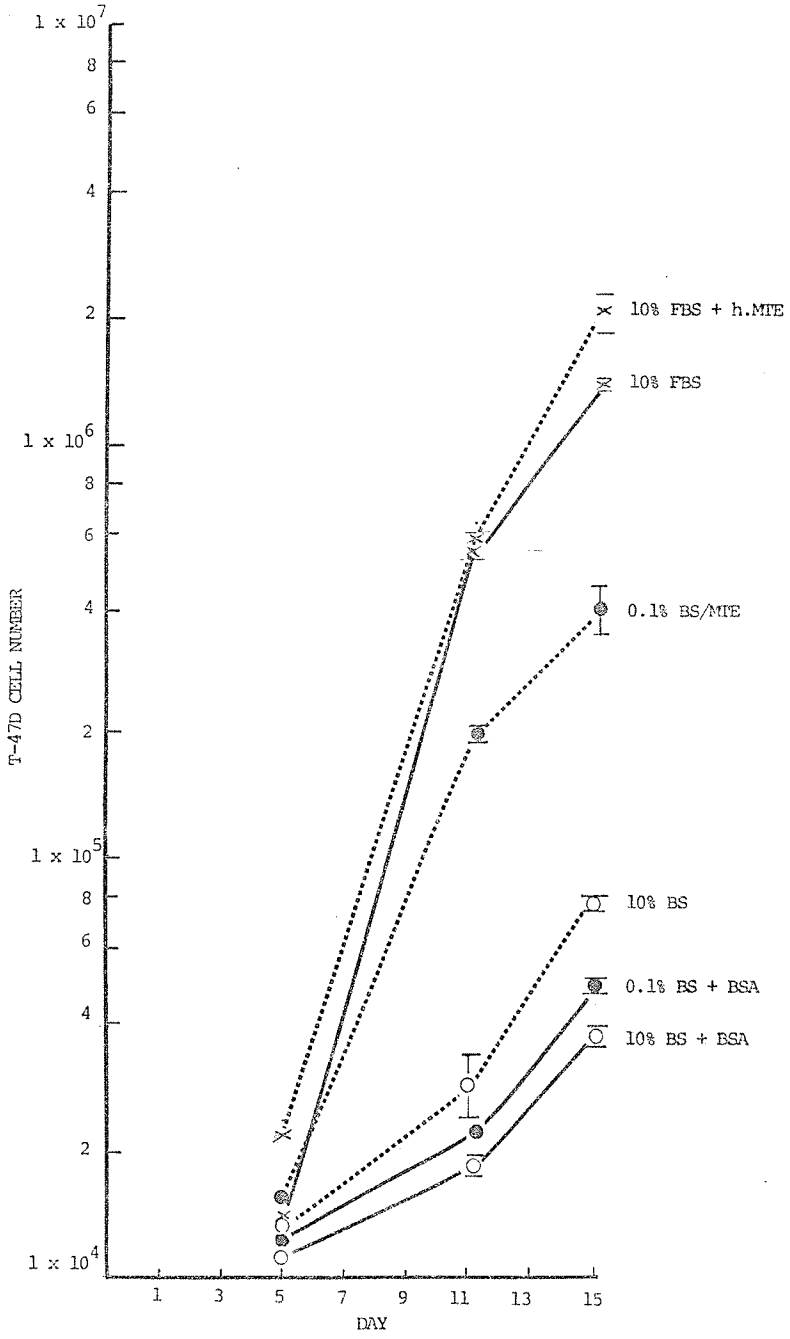
Figure 17:

Stimulation of T-47D cell growth in response to the h.MTE

Approximately 1 x 10^4 cells were plated in 10% FBS as described, day 0. On day 1, the cells were washed in serum free medium and replaced with fresh medium containing 0.1% BS + BSA (20 µg ml^{-1}) ●———●, 0.1% BS + h.MTE (20 µg ml^{-1}) ●-----●, 10% BS + BSA o———o, 10% BS + h.MTE o-----o, 10% FBS x———x or 10% FBS and h.MTE x-----x.

The medium was changed every other day followed by the addition of h.MTE. The cell number of triplicate cultures was determined on day 5, 11 and 15. Values represent the mean ± SD.

Figure 17


1 x 10^7
8
6
4
2
1 x 10^6
8
6
4
2
1 x 10^5
8
6
4
2
1 x 10^4
T-47D CELL NUMBER
1
3
5
7
9
11
13
15
DAY
10% FBS + h.MIE
10% FBS
0.1% BS/MIE
10% BS
0.1% BS + BSA
10% BS + BSA

Further characterisation of the h.mammary tumor derived growth factor for T-47D using Sephadex G-100

The h.mammary tumor extract was chromatographed using Sephadex G-100 under non-dissociating conditions (10 mM Tris, pH 7.4) and dissociating conditions (1 M acetic acid. Fig. 18 panel A, shows the elution profile under non-dissociating conditions. Two peaks of growth promoting activity for T-47D cells were detected. Peak I eluted with proteins of molecular weight 80,000 daltons and Peak II eluted at the bed volume molecular weight 4,000 daltons. When the chromatography was carried out using dissociating conditions (1 M acetic acid) 3 peaks of activity were detected, Fig. 18 panel B. Peak I and Peak II eluted at the same position as described above. The third peak of activity eluted with proteins of molecular weight 4,000-30,000 daltons. The appearance of a third peak of activity in the presence of 1 M acetic acid could be due to, a) destruction of certain growth inhibitors which co-migrate within the molecular weight range of Peak III, or b) the dissociation of growth factors from Peak I.

These data reveal the presence of a heterogeneous population of growth factors for T-47D cells in the h.MTE. On the basis of stability in 1 M acetic acid, these growth factors appear to be different from the acid labile growth factors present in the h.MTE which stimulate the growth of mesodermal derived cells such as chondrocytes.

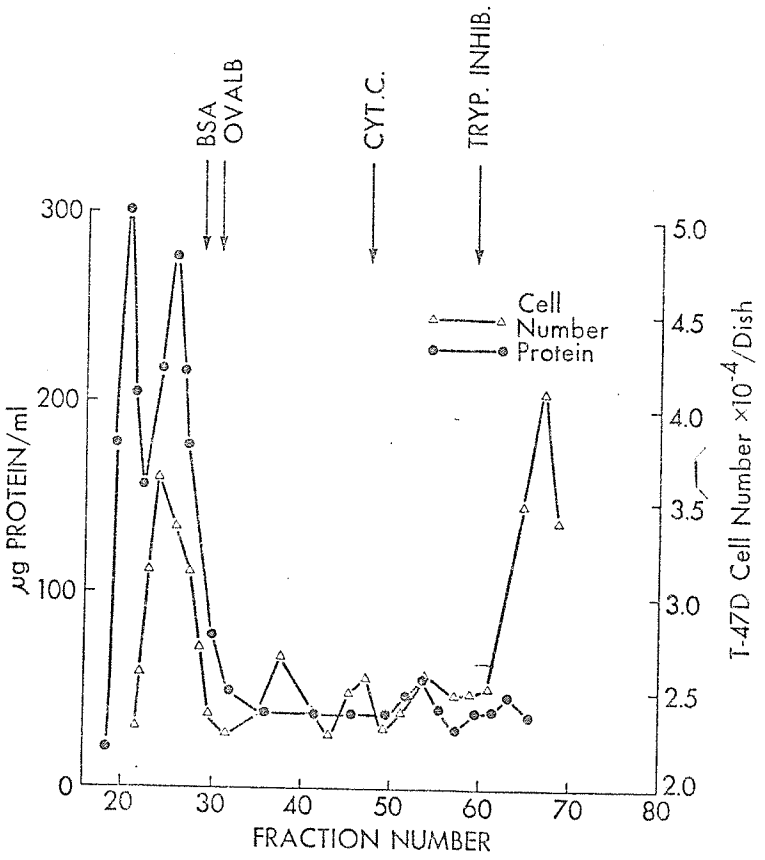
Figure 18:

Gel chromatography of the h.MTE on Sephadex G-100 under dissociating and non-dissociating conditions

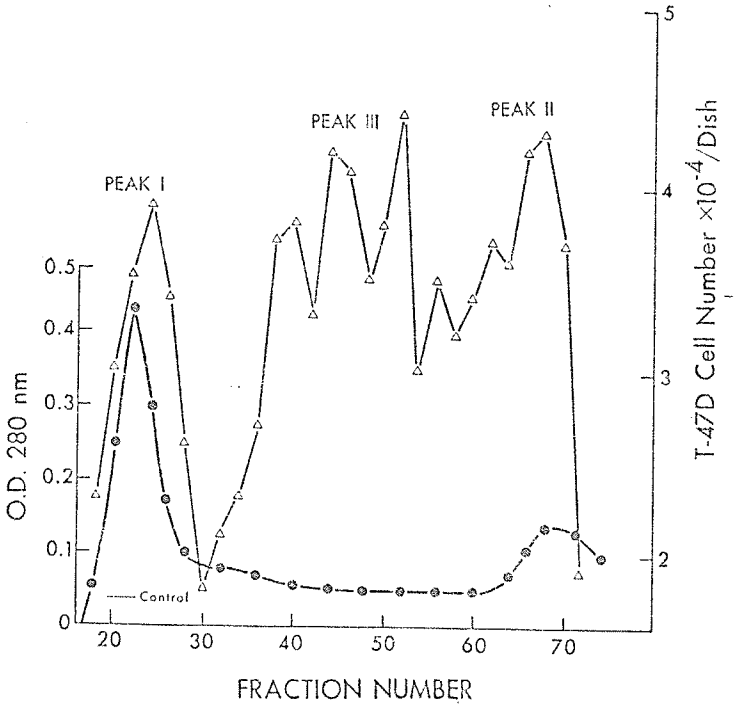
The h.MTE (approximately 5 mg protein) was chromatographed using a Sephadex G-100 column (70 x 1.5 cm) equilibrated in estrogen receptor assay homogenisation buffer (pH 7.4) upper panel, or 1 M acetic acid (pH 2.1) lower panel. Fractions (2 ml) were collected and aliquots were assayed on the stimulation of growth using T-47D cells. Fractions containing 1 M acetic acid were neutralised with 1 M NaOH and diluted before assay.

Figure 18

A


BSA
OVALB
CYT.C.
TRYP. INHIB.
Cell Number
Protein
µg PROTEIN/ml
T-47D Cell Number ×10⁻⁴/Dish
FRACTION NUMBER

B


PEAK I
PEAK III
PEAK II
Control
O.D. 280 nm
T-47D Cell Number ×10⁻⁴/Dish
FRACTION NUMBER

Further indirect evidence in support of this claim was obtained by comparing the biological activity of some of the various fractions derived during the purification of h.brain derived growth factors presented in this thesis.

C-50 Sephadex ion exchange of h.brain extract

Briefly, the initial extraction of the h.brain derived growth factors and the initial purification steps were carried out according to the method of Gospodarowicz (65). Chromatography of the crude brain extract on C-50 Sephadex ion exchange resulted in 3 protein fractions, B_1, B_2 and B_3. The B_3 fraction was 70 fold more active than the crude extract, in stimulating DNA synthesis of Balb/c3T3 and rabbit fetal chondrocytes. The fractions B_1 and B_2 which represented the bulk of the applied protein were less active than the original extract Fig. 20 .

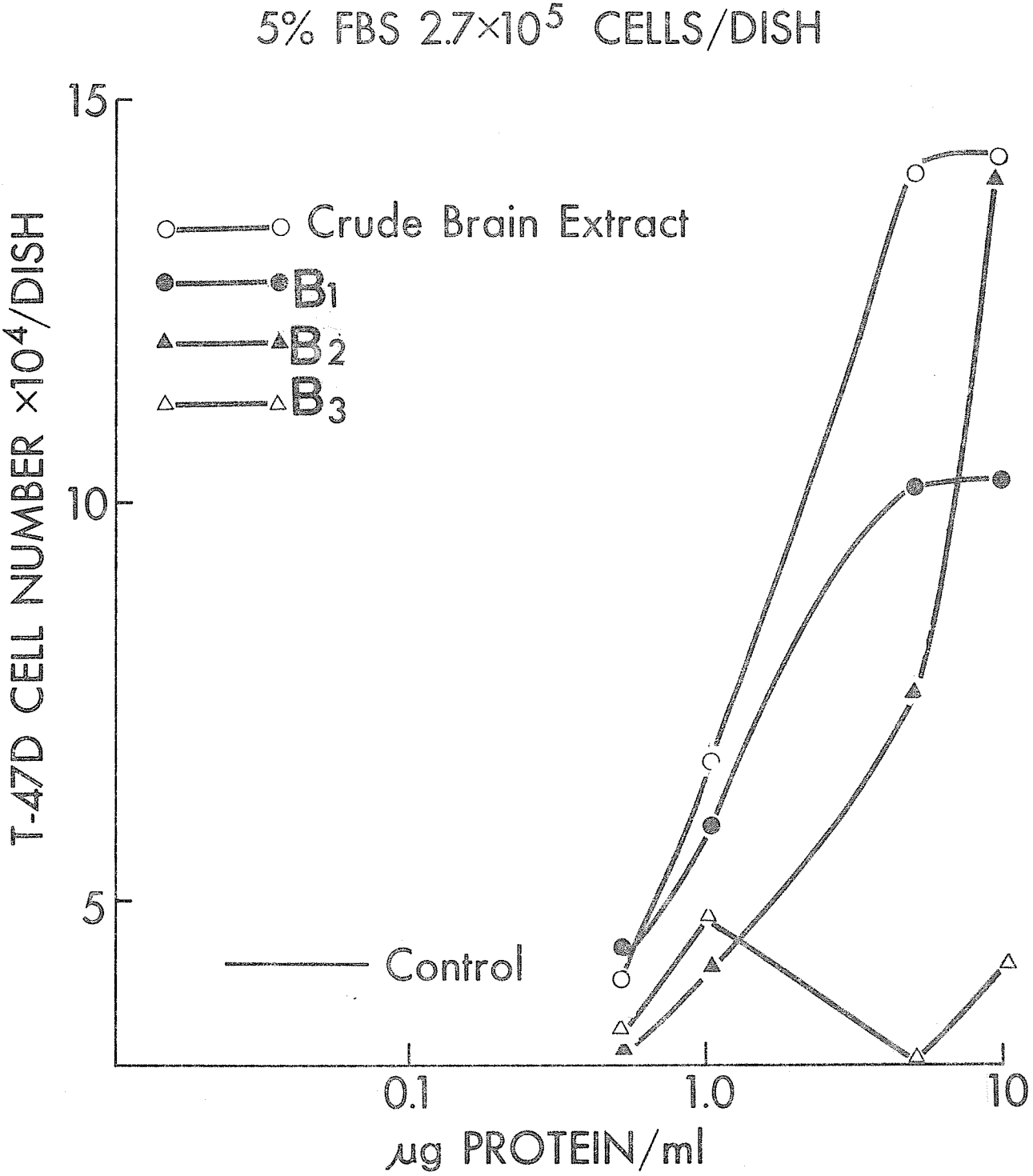
Conversely Fig. 19 shows the dose response curve of the effect of the crude extract of human brain and the protein fractions B_1, B_2 and B_3 on the proliferation of T-47D cells. The crude extract of h.brain was shown to stimulate cell growth at 1 $\mu g\ ml^{-1}$ and result in a 4 fold increase in cell number at 10 $\mu g\ ml^{-1}$. The unabsorbed protein fraction B_1 and the proteins eluting from the column at 0.15 M NaCl, fraction B_2, were also mitogenic over the dose range tested. The fraction B_3, however,

Figure 19:

Stimulation of T-47D cell growth in response to h.brain derived growth factors

A crude extract of h.brain was chromatographed using CM Sephadex C-50 ion exchange as described in Section I. The unabsorbed protein fraction B_1, and the fractions which eluted from the column with 0.15 M NaCl, B_2 and 0.5 M NaCl, B_3 were assayed for growth factor activity by monitoring the increase in cell number of T-47D cells as described. Values represent the mean of duplicate cultures.

Figure 19


5% FBS 2.7×10^5 CELLS/DISH
T-47D CELL NUMBER ×10^4/DISH
15
10
5
Crude Brain Extract
B1
B2
B3
Control
0.1
1.0
10
µg PROTEIN/ml

did not stimulate growth of the T-47D cells. The B_3 fraction was, however, found to be the most active fraction when the stimulation of RFC and Balb/c3T3 cells were used as the bioassay (see brain growth factors, Fig. 20.) Hence the h.brain growth factor for T-47D cells remained unabsorbed to the cation exchange resin at pH 6.0. These data suggest that the putative h.brain growth factor for T-47D cells is anionic (assuming it to be a protein) with a pI below 6. Recently, Maciag et al (104) have reported a growth factor for epidermal derived cells from b.brain, with an isoelectric point between pH 4 and pH 5. These findings were based on the observation that the removal of the brain extract from a serum-free hormonally defined medium, had a greater negative impact on cell replication of keratinocyte cultures than did the removal of m-EGF, hydrocortisone, T_3 or Cohn fraction IV.

Characterisation and purification of growth factors in h.brain and h.pituitary gland

The purification of a potent mitogen, fibroblast growth factor (FGF) from bovine pituitary (60) and brain (65) were based initially on the observation that these two tissues contained 1 to 2 orders of magnitude greater levels of growth promoting activity compared to a variety of other tissue extracts tested. More recent studies by Westall have revealed the identity of at least 3 forms of bovine brain FGF which were claimed to be fragments of myelin basic protein (MBP) corresponding to amino acid residues 44 - 166, 44 -153, and 91 - 153 (168). The intact molecule of myelin basic protein was, however, biologically inactive.

Utilising a purified preparation of FGF, Gospodarowicz has shown that FGF is a potent mitogen for a variety of mesodermal cells in culture including bovine and human endothelial cells, chondrocytes, granulosa cells etc.

The purification of additional mitogenic factors in the brain have also been reported. An endothelial cell growth factor was reported by Maciag et al (103) to be present in the brain and pituitary which had different physical and biological properties compared to FGF. More recent studies by Thomas et al (150) revealed that the FGF present in b.brain was distinct from myelin basic protein since the mitogenic activity could be recovered in the acidic region pI 4.8 - 5.7 following isoelectric focusing

in contradistinction to MBP fragments, pI approximately 10. In addition, the biological activity of b.brain FGF was not retained on a column of chicken anti-bovine MBP-Sepharose. The following results presented on the characterisation of human brain growth factor support the finding that brain FGF and MBP are unrelated, as reported by Kellett, et al (87).

Isolation of growth factors in human pituitary and human brain

Preliminary investigations were carried out to determine the presence of growth factor activity in crude extracts of freshly frozen h.pituitary glands and h.brain.

Isolation of human pituitary growth factors

Preliminary extractions of the h.pituitary were carried out at pH 8.5 in the presence of 0.1 M ammonium bicarbonate. Fig. 20 shows the effect of increasing doses of a crude pituitary extract on the initiation of DNA synthesis in quiescent Balb/c3T3 cells. The crude pituitary extract stimulated a dose dependent increase in DNA synthesis over the protein concentration range of 1-100 μg ml^{-1}. The crude pituitary extract was also shown to stimulate rabbit fetal chondrocyte growth using the assay system described in the methods. (S. Kasper, 1981). A significant increase (5 fold) in the specific activity of the pituitary extract was obtained when the method described by Gospodarowicz for the purification of b.pit. FGF was used. Further characterisation and purification of the pituitary derived growth factor has been carried out (S. Kasper, 1981). Briefly, the factor is non-dialysable, sensitive to heat (100^{o}C, 15 min.) 1 M acetic acid and protease digestion. It has an apparent

mol. wt. of 40-60,000 daltons when chromatographed on Sephadex G-100 and an isoelectric point of 7.9. These data would suggest the h.pituitary derived growth factor was protein in nature. The relatedness of this growth factor with other pituitary derived mitogens is presently unknown. Further purification of the human pituitary derived growth factor is being carried out. The development of specific radio-ligand assays (receptor and/or immunological) will prove useful in determining its relationship with other known pituitary growth factors and hormones.

Isolation of growth factors from human brain

The extraction of h.brains was carried out using the method described by Gospodarowicz for the isolation of b.brain growth factors (65). Biological activity was initially monitored using the stimulation of DNA synthesis in quiescent Balb/c3T3 cells. Subsequently, the use of RFC was used as the principal assay for monitoring the purification of h.brain derived growth factors. Following the report by Westall (168) that the amino-acid sequence of b.brain FGF corresponded to fragments of MBP, a radio-immunoassay was developed for h.MBP. Fractions obtained during the purification of h.brain growth factors were therefore tested for MBP immuno cross-reactivity, as described in the methods.

Ammonium Sulphate fractionation of crude homogenate

Unless otherwise indicated all operations were performed at 0-5°C. Frozen human brains (6 kg) were thawed, chopped into small fragments and homogenised using a Polytron homogeniser in a total of 12 liters of 0.15 M $(NH_4)_2$ So_4. This crude homogenate was adjusted to pH 4.5 with 1 N HCl and stirred for 2 hr. The homogenate was centrifuged at 10,000 x g for 40 min to remove cells and debris. The supernatant was adjusted to pH 6.5 to 7.0 by addition of 1 N NaOH. Two hundred grams of $(NH_4)_2$ SO_4 was added per liter and stirred for 40-80 minutes. The suspension was centrifuged at 10,000 x g for 20 min. This yielded a very small pellet of material which was discarded. The supernatant was passed through a cheesecloth to remove any residual lipid material and an additional 250 gm/l of $(NH_4)_2$ So_4 was added and stirred for 40-60 minutes. The suspension was centrifuged at 10,000 x g for 20 minutes. The supernatant was discarded and the pellet which contained 70 gm of protein, (Fraction A_1) was collected and dissolved in 1,000 ml of cold distilled water.

Fraction A_1 was dialysed against continuously running tap water for 18 - 24 hr. The dialysate was adjusted to a pH of 6.0 with formic acid and the precipitate which formed was removed by centrifugation for 10 min at 10,000 x g.

Sephadex C-50 chromatography of the crude brain extract

The clarified dialysate was applied to a column (3 x 20 cm) of carboxymethyl-Sephadex C-50 equilibrated with 0.1 M sodium phosphate buffer, pH 6.0. Six liters of 0.1 M sodium phosphate pH 6.0 was washed through this column over the next 12 hr. The unabsorbed fraction (B_1) contained 80% of the proteins. Elution of the column was terminated when the absorbance (O.D.) at 280 nm of the eluate was below 0.15. The column was then eluted with 2 liters of 0.1 M sodium phosphate buffer pH 6.0 containing 0.1 M NaCl. Elution of the column was again terminated when the O.D. at 280 nm was below 0.1, and this represented fraction B_2. The third fraction, B_3 was obtained by eluting the column with 0.1 M sodium phosphate buffer pH 6.0 containing 0.5 M NaCl. All fractions were dialysed extensively against d.H_2O at 4^{o}C (using Spectrapor No# 1, mol. wt. cut off 6-8,000), lyophilised and stored at -20^{o}C.

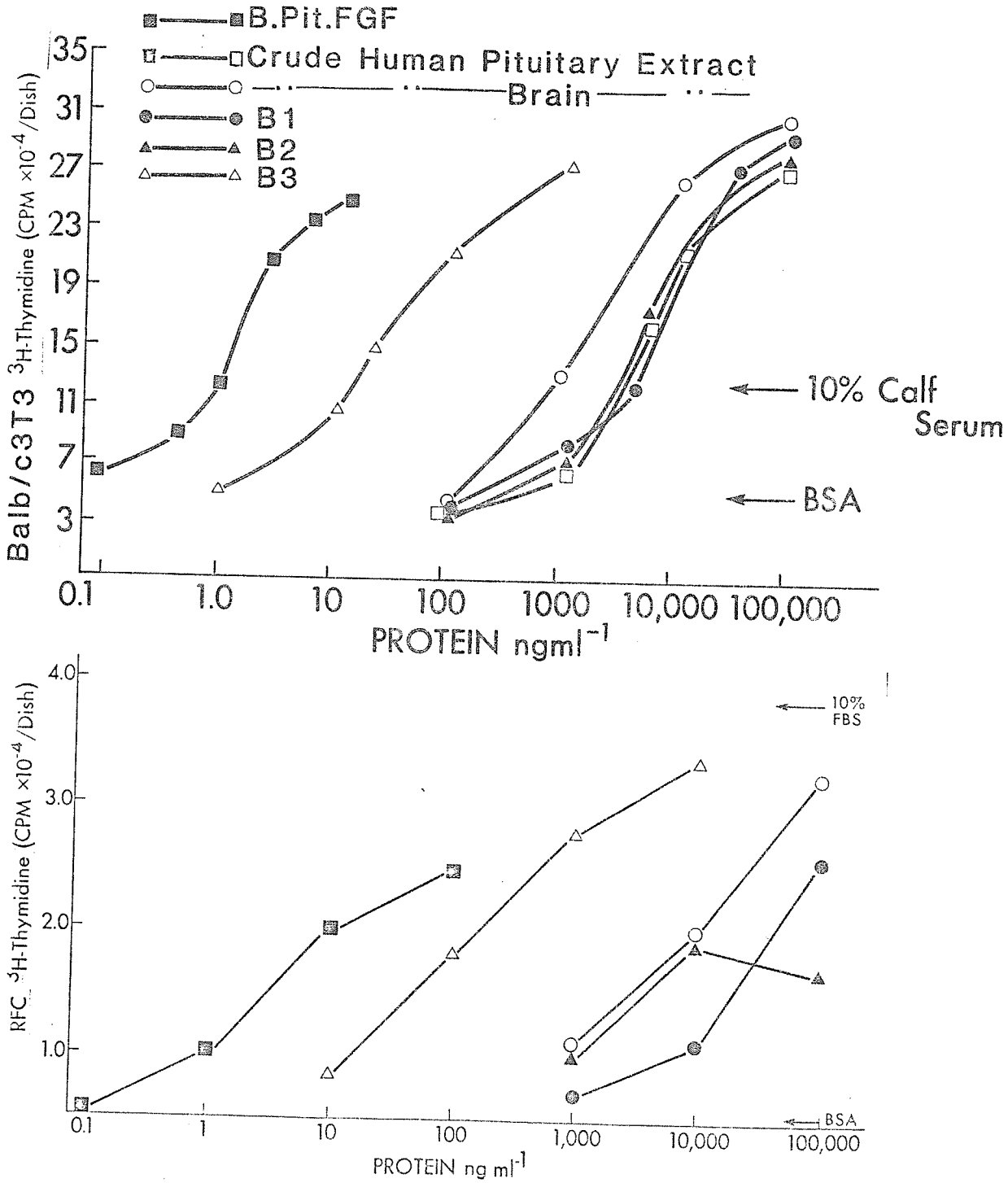
Fig. 20 shows the stimulation of DNA synthesis in quiescent cultures of RFC and Balb/c3T3 cells in response to increasing doses of B_1, B_2 and B_3. The crude extract of brain was mitogenic for each cell type over a protein concentration range of 0.1 - 100 μg ml^{-1} culture medium. Fractions B_1 and B_2 were less active than the original crude brain extract, and represented approximately 90% of the applied protein.

Figure 20:

Stimulation of DNA synthesis in RFC and Balb/c3T3 cells in response to brain and pituitary growth factors

The stimulation of DNA synthesis in quiescent cultures was performed as described in the methods. Chondrocytes and Balb/c3T3 cells were exposed to increased doses of a crude extract of h.brain and fractions obtained from C-50 ion exchange chromatography. B.pituitary FGF was used as a reference standard. Balb/c3T3 cells were also exposed to a crude extract of h.pituitary.

Figure 20


B.Pit.FGF
Crude Human Pituitary Extract
Brain
B1
B2
B3
Balb/c3T3 ³H-Thymidine (CPM ×10⁻⁴/Dish)
35
31
27
23
19
15
11
7
3
0.1
1.0
10
100
1000
10,000
100,000
PROTEIN ngml⁻¹
10% Calf Serum
BSA
RFC ³H-Thymidine (CPM ×10⁻⁴/Dish)
4.0
3.0
2.0
1.0
0.1
1
10
100
1,000
10,000
100,000
PROTEIN ng ml⁻¹
10% FBS
BSA

Fraction B_3 which eluted from the C-50 column with 0.5 M NaCl represented less than 0.5% of the applied protein. The B_3 was 70 times more active than the crude extract of brain, A_1. The yield however, at this stage was approximately 30% of the applied activity.

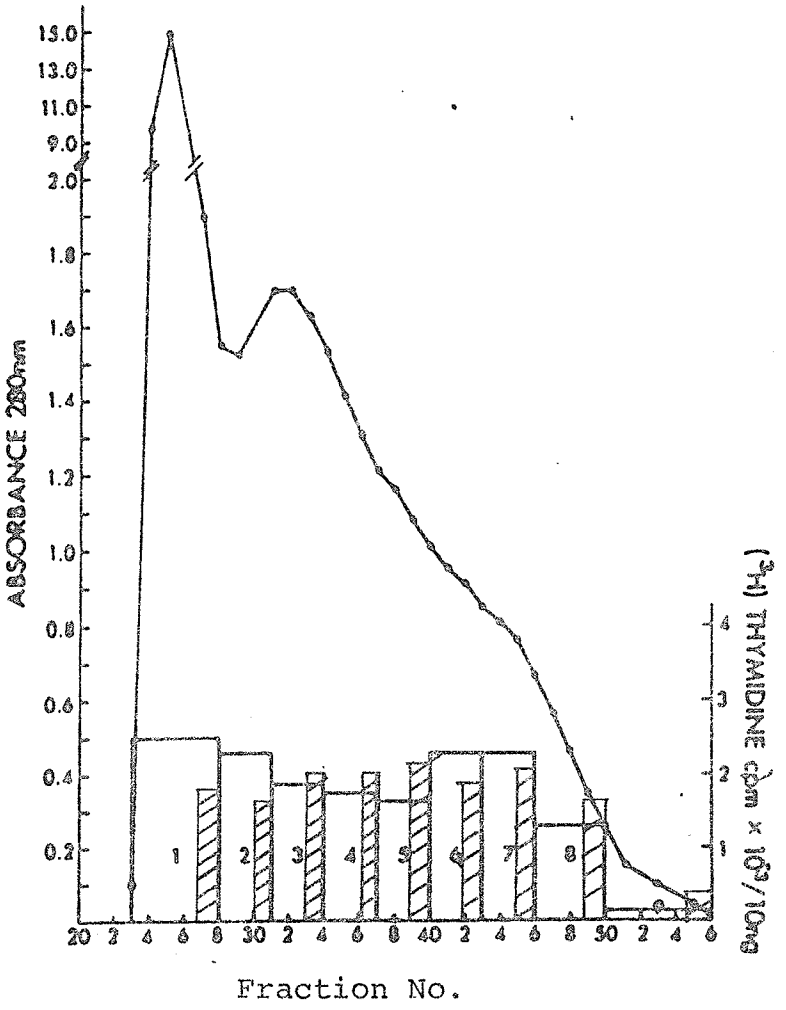
In order to obtain sufficient material for further purification and characterisation, 10 x 6 kg batches of h.brain were processed to the B_3 stage of purification.

Gel filtration on Sephadex G-75 of the h.brain derived growth factor

Gel filtration studies were carried out on Sephadex G-75 to determine the molecular weight of h.brain growth factor and establish further purification steps. Initial studies were carried out using 0.1 M ammonium bicarbonate pH 8.5 as described by Gospodarowicz for the purification of b.brain (65). Fig. 21 shows the protein elution profile of B_3 and the mitogenic activity of the pooled fractions. None of the pooled fractions showed an increase in bioactivity when compared to the applied material, B_3. Fractions were tested using quiescent cultures of RFC and Balb/c3T3 cells.

Elution of the B_3 fraction was then carried out at pH 4.5 using 0.1 M sodium acetate buffer pH 4.5 containing 0.5 M NaCl. Mitogenic activity was determined in pooled

Figure 21



Gel Filtration on Sephadex G-75 of fraction B_3 obtained from the carbomethyl-Sephadex C-50 ion exchange chromatography

One hundred and fifty milligrams of protein B_3 in 3 ml of 0.1 M ammonium bicarbonate (pH 8.5) was applied to a column of Sephadex G-75 (2.5 x 90 cm) equilibrated in the same buffer. Fractions (5.5 ml) were collected and pooled as shown. The histogram shows the relative activities of the different pooled fractions on the initiation of DNA synthesis in quiescent sparse cultures of RFC (open bars) and Balb/c3T3$_1$ (hatched bars). Fractions were tested at 10 ng ml^{-1}.

fraction by monitoring DNA synthesis in quiescent rabbit fetal chondrocytes. Proteins eluting between fraction 42 to 47 were the most active, Fraction C4 (Fig. 22 and resulted in a 4 fold purification when compared to the applied B_3 fraction.

Subsequently several batches of B_3 100 - 200 mg of protein were chromatographed on Sephadex G-75 and fractions 42 - 47 referred to as C4 corresponding to the active material were pooled. The yield of protein at this stage was 150 mg of C4/70kg h.brain.

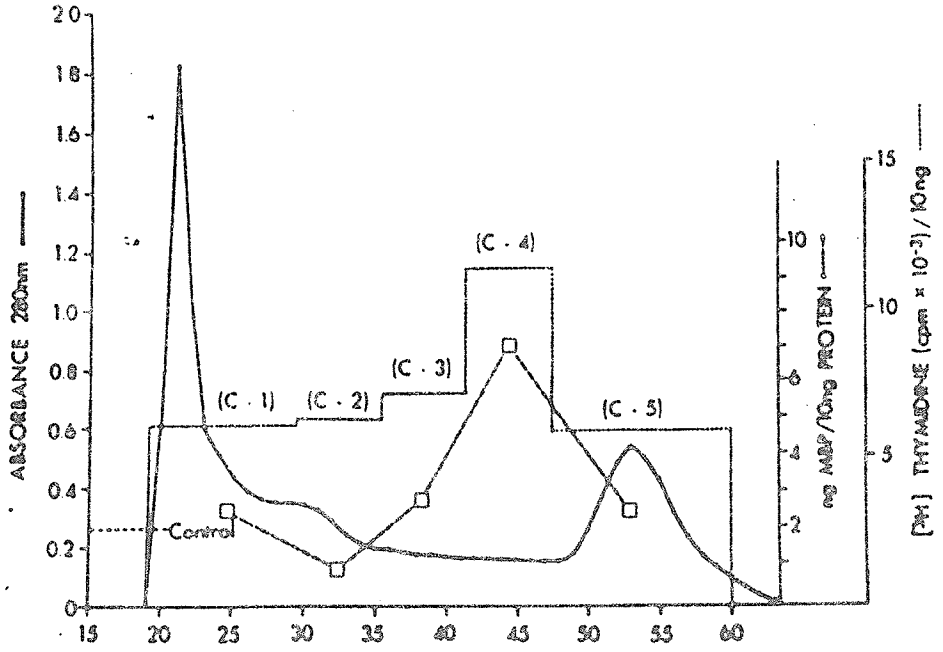
MBP crossreactivity

The pooled fractions from the Sephadex G-75 column were also tested for MBP crossreactivity. Significant crossreactivity was detected in fraction C4 which accounted for approximately 80% of the protein Fig. 23.

Purification of C4 on Sephadex G-75 superfine

Two 75 mg batches of the lyophilized C4 fraction was dissolved in 0.5 M sodium acetate buffer pH 4.5 containing 0.5 NaCl and chromatographed on a Sephadex G-75 superfine column (110 x 2 cm) equilibrated in the same buffer. The elution profile is shown in Fig. 24. The eluted fractions were tested for mitogenic activity monitoring DNA synthesis using rabbit fetal chondrocytes

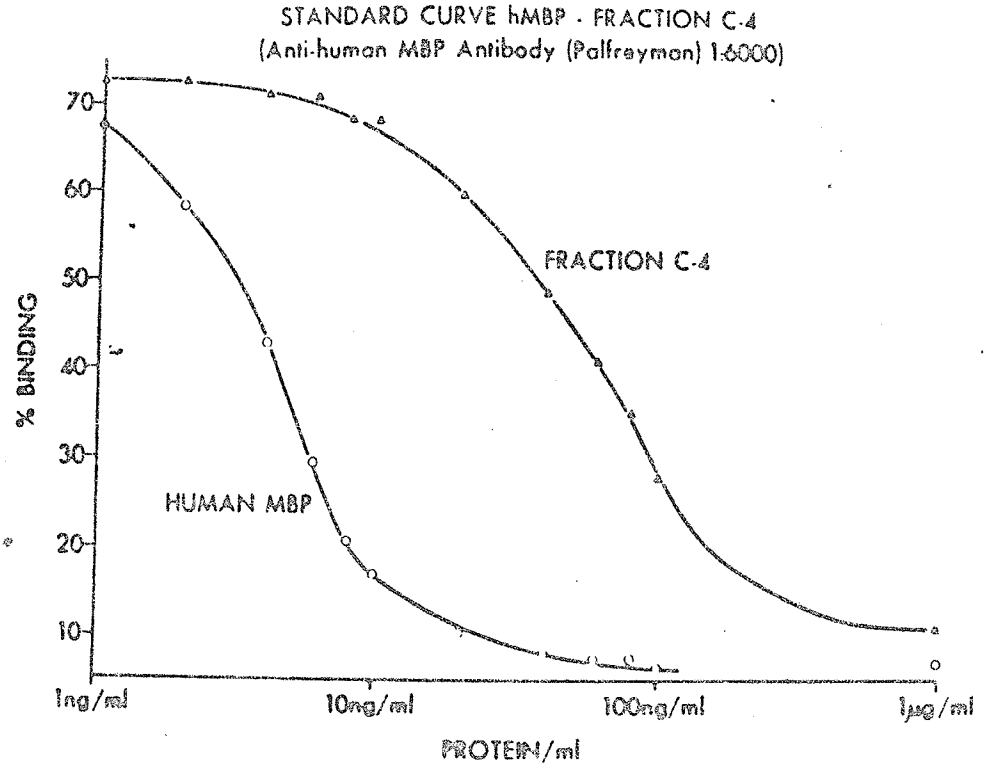
Figure 22



Gel filtration on Sephadex G-75 of fraction B_3 obtained from the carboxymethyl-Sephadex C-50 ion exchange chromatography

Two hundred milligrams of protein (B_3) in 3 ml of 0.1 M sodium acetate 0.5 M NaCl pH 4.5 was applied to a column of Sephadex G-75 (2.5 x 90 cm) equilibrated in the same buffer. Fractions (4.4. ml) were collected and pooled (C 1-5) dialysed and lyophilized. The histogram shows the relative activity of the different pooled fractions on the initiation of DNA synthesis in quiescent sparse cultures of RFC. Immuno cross-reactivity with h.MBP □———□ for each fraction is shown.

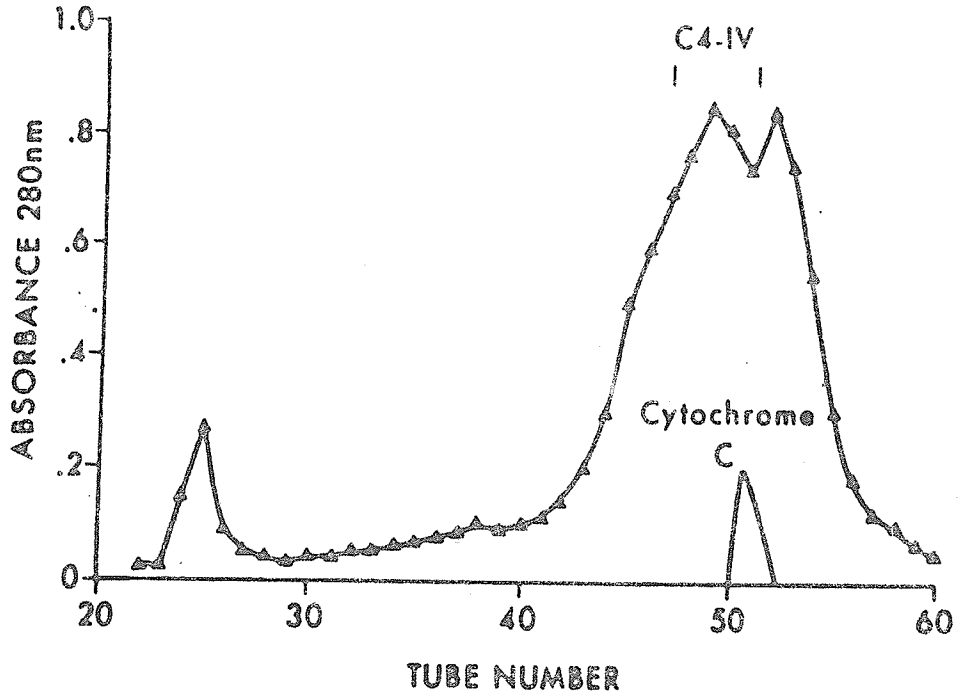
Figure 23



Immunocrossreactivity of fraction C4 with h.MBP

Fraction C4, obtained from the filtration of fraction B_3 on Sephadex G-75 was tested for cross-reactivity with anti-human MBP antibody (1:6000).

Figure 24



Gel filtration on Sephadex G-75 superfine of fraction C4

Seventy five milligrams of the fraction C4 (obtained from the previous filtration on G-75) in 2 ml of 0.1 M sodium acetate 0.5 M Na Cl pH 4.5 was applied to a column (100 x 2.0 cm) of Sephadex G-75 superfine equilibrated in the same buffer. Fractions (2 ml) were collected and tubes 47-50 (Fractions C4-IV) were pooled dialysed and lyophilized.

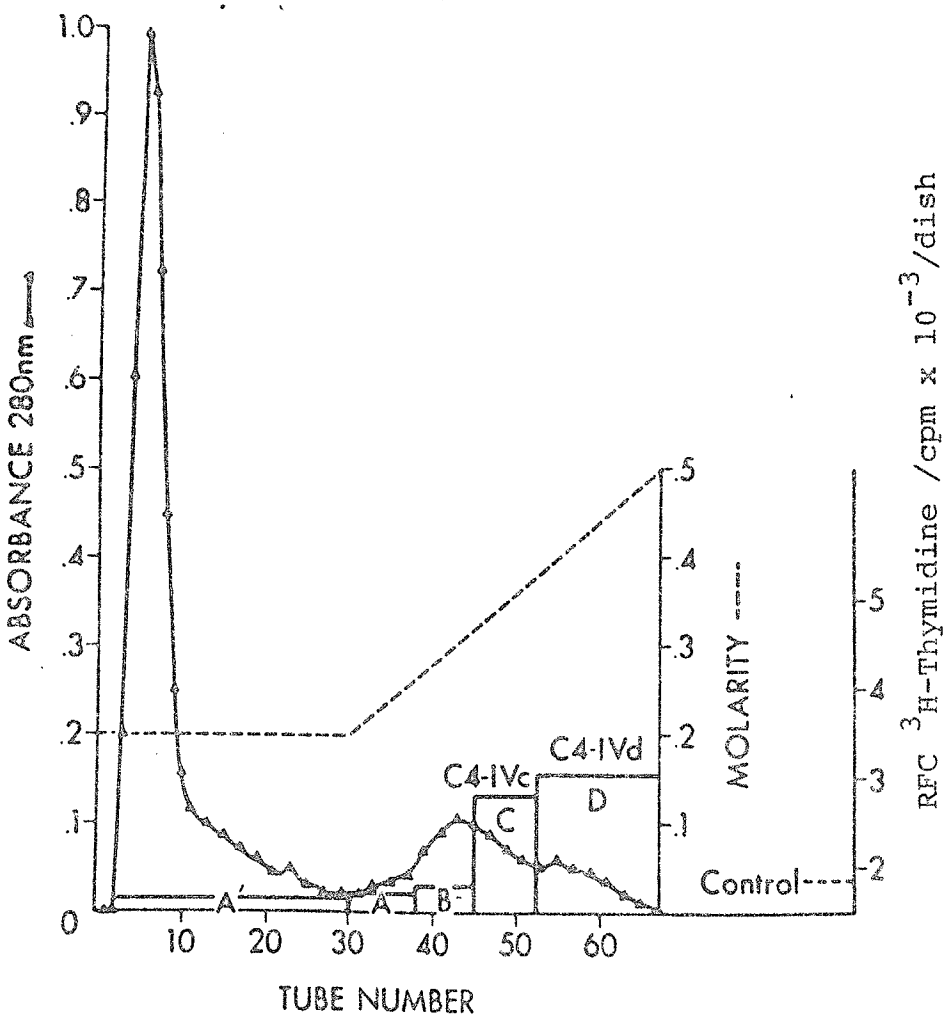
as the indicator cells. The activity was concentrated between fractions 47-50 (just before the cytochrome C marker) and these fractions were pooled, dialysed and lyophilized yielding 50 mg of protein (Fraction C4-IV).

Whatman CM52 ion exchange chromatography of C4-IV

Approximately 25 mg of C4-IV were applied to a column of carboxymethylcellulose CM52 (0.5 x 3.5 cm) equilibrated with 0.2 M ammonium formate pH 6.0. Fifteen mg of protein passed through the column unabsorbed. The column was washed with 20 ml of 0.2 M ammonium formate, eluted with a linear gradient (40 ml) of ammonium formate from 0.2 M to 0.5 M (pH 6.0). The elution profile is shown in Fig. 25 . The collected fractions were divided into five pools and bioactivity was measured using thymidine incorporation in quiescent cultures of rabbit fetal chondrocytes. The proteins which passed through the column unabsorbed (C4-IVa) and the fractions corresponding to 0.2 - 0.3 M ammonium formate (C4-IVa and C4-IVb) contained little or no bioactivity. The most active fractions, C4-IVc and C4-IVd eluted from the column corresponding to 0.3 and 0.4 M ammonium formate respectively Fig. 26.

The biological potency of these two fractions were comparable to a commercial preparation of b.pituitary FGF. The C4-IVd fraction was the most active and stimulated DNA synthesis at concentrations as low as 1 ng ml^{-1}.

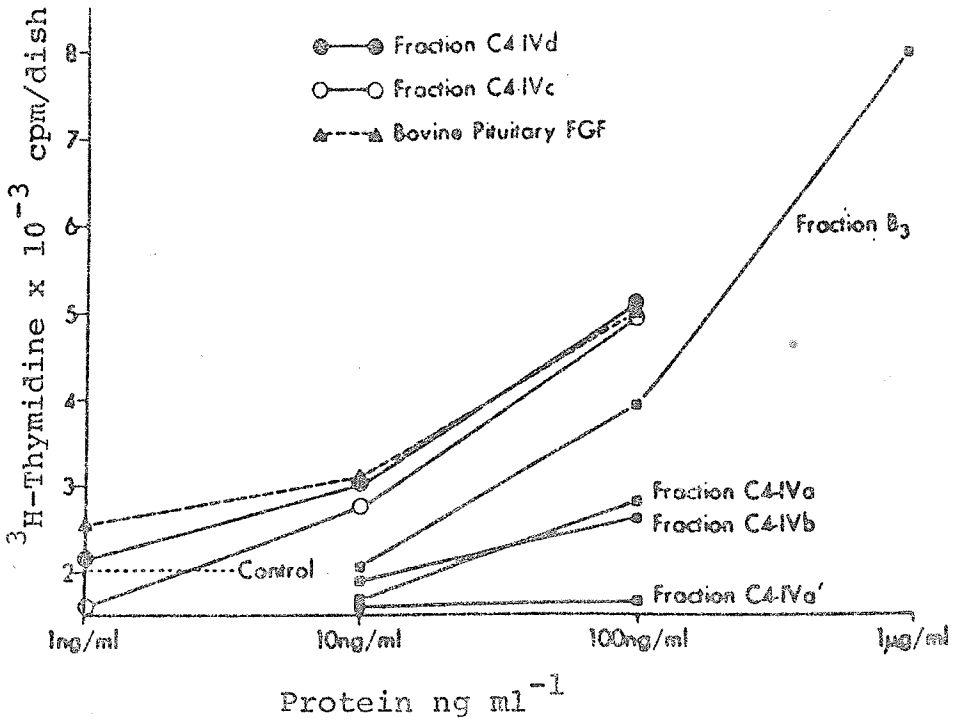
Figure 25



Chromatography of the C4-IV fraction on carboxymethyl-cellulose CM-52

Fraction C4-IV (25 mg, obtained from G-75 chromatography of fraction C4) was dissolved in 5 ml of 0.2 M ammonium formate (pH 6.0) and applied to a column (0.5 x 3.5cm) of carboxymethyl-cellulose CM-52 equilibrated in the same buffer. The column was washed with 20 ml of 0.2 M ammonium formate and eluted with a linear gradient (40 ml) of ammonium formate from 0.2 to 0.5 (pH 6.0) The eluate was collected (approx. 1.1 ml/tube) and divided into four pools and bioassayed. The histogram shows the relative activities of these pools on the stimulation of thymidine incorporation by serum starved rabbit fetal chondrocytes. Pools were tested at 10 ng/ml

Figure 26



Stimulation of DNA synthesis in rabbit fetal chondrocytes exposed to b. pituitary FGF and h.brain derived growth factors

Stimulation of DNA synthesis was measured as described in the methods. Rabbit fetal chondrocytes were exposed to increasing doses of fractions obtained from the ion exchange chromatography of fraction C4-IV. B.pit.FGF was used as a standard and fraction B_3 (C-50 ion exchange step, see Fig. 20 is shown for comparison.

The yield after lyophilization was 2.0 mg of C4-IVc and 1.5 mg of C4-IVd/70 kg h.brain.

MBP immunocrossreactivity of C4-IVc and C4-IVd

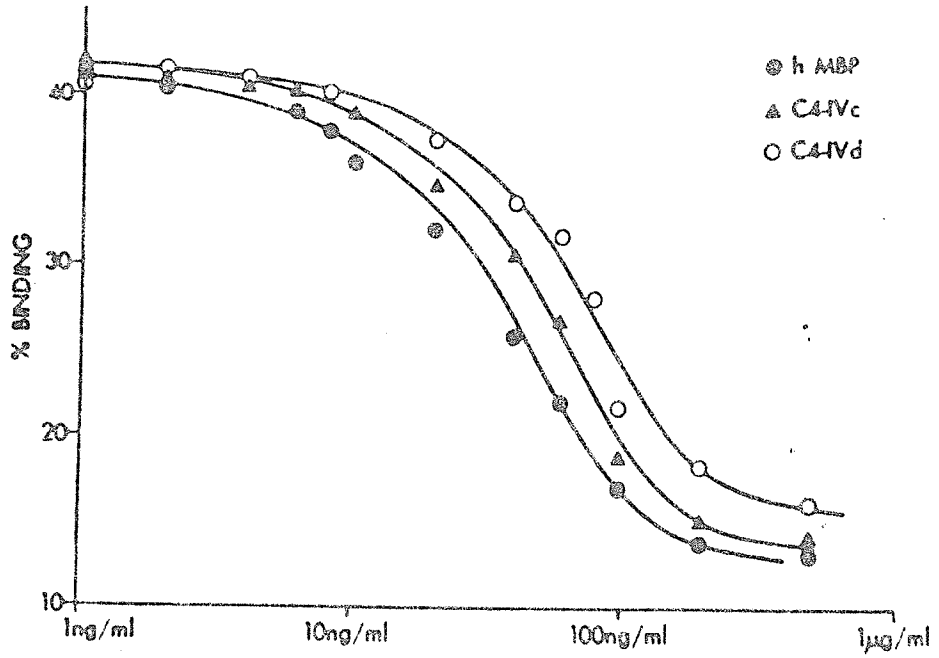
Fig. 27 shows the parallel displacement of the two h.brain derived growth factors with h.MBP Fractions C4-IVc and C4-IVd exhibited 50% crossreactivity compared to h.MBP.

However, the h.MBP failed to stimulate DNA synthesis in quiescent cultures of rabbit fetal chondrocytes. It was therefore possible that either, during purification of h.MBP using organic solvents known to destroy the biological activity of brain growth factors, the biologically active site of h.MBP was destroyed, or, that h.MBP was being co-purified as a major contaminant of the brain growth factor. In order to test the latter hypothesis, various fractions of the brain derived growth factors were subjected to isoelectric focusing. The eluted fractions were then tested for growth factor activity and h.MBP immunocrossreactivity.

Isoelectric focusing of h.brain derived growth factors

Fig. 28 shows the isoelectric focusing profile of the B_3 fraction over the range of pH 3.5 - 10. Significant bioactivity was detected in gel eluants corresponding to a pH interval of 5 - 9. The majority of bioactivity, however, was recovered in the pH interval of 7-8.0.

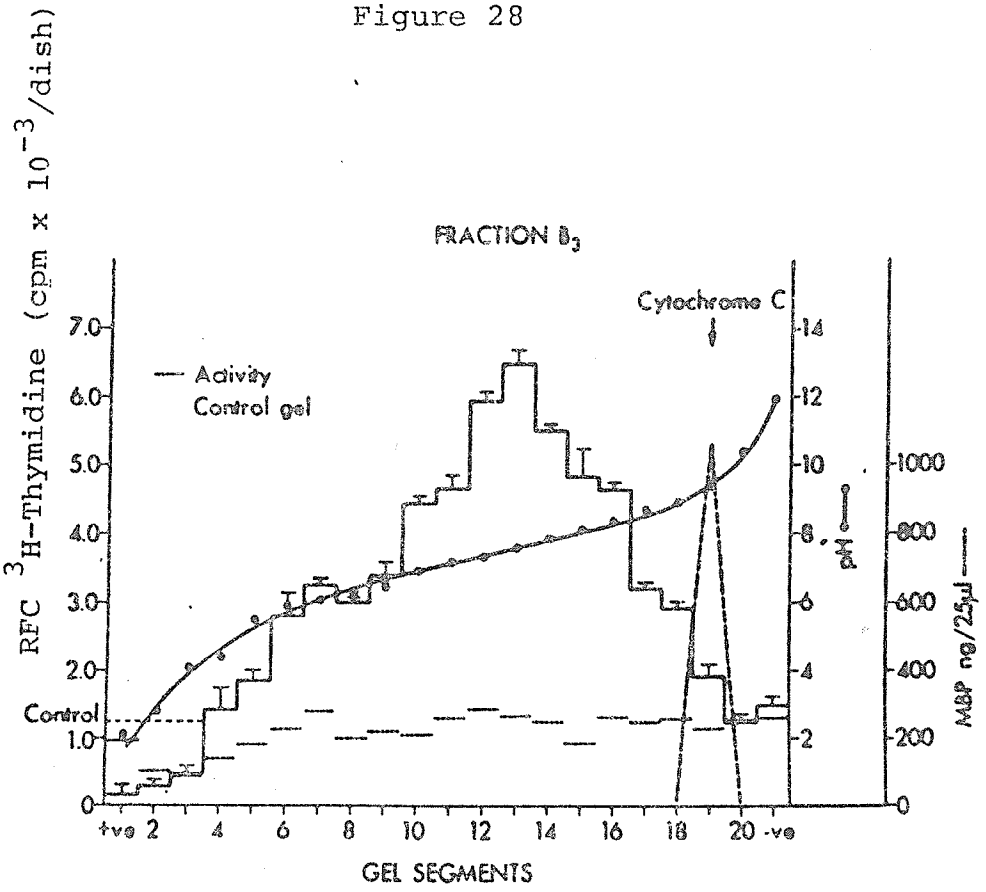
Figure 27



Crossreactivity of fraction C4-IVc and C4-IVd with h.MBP

The radioimmunoassay for h.MBP was performed as described in the methods.

Figure 28



Isoelectric focusing of fraction B_3

Fifty micrograms of fraction B_3 (obtained from the initial C-50 ion exchange chromatography of the crude extract of h.brain) was applied to slab gel polyacrylamide isoelectric focusing over the range of pH 3.5 - 10, as described in the methods. The focusing was carried out at a constant power of 4 watts for 3½ hrs. The sample channel and a control channel to which no protein had been applied were then cut into 21 gel segments which were eluted overnight in 0.5 ml PBS. Aliquots of each eluate were then assayed for growth factor activity using ^{3}H-thymidine incorporation into rabbit fetal chondrocytes, and for myelin basic protein cross-reactivity using RIA. The continuous line histogram shows the relative bioactivities in the 25 µl aliquot of each gel segment from the sample channel. MBP cross-reactivity (---) in each 25 µl aliquot is also shown.

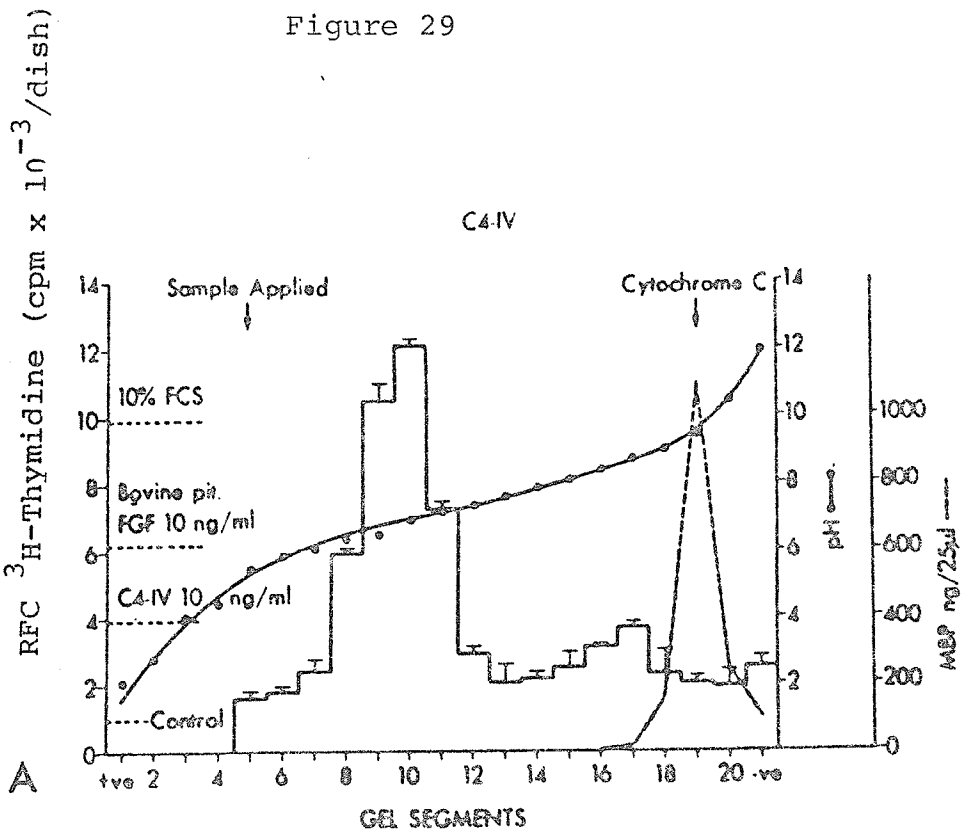
Isoelectric focusing of C4-IV, C4-IVc and C4-IVd

Fraction C4-IV represents the proteins of molecular weight 12-25,000 following fractionation of B_3 on Sephadex G-75. The majority of biological activity was detected in gel eluants corresponding to a pH interval of 6.2 - 7.2 Fig.29. The bioactivity of C4-IVc was present in gel eluants of pH 7.2 and 8.2 and in C4-IVd the bioactivity was present in gel eluants of pH 7.5 and 8.5 Fig. 30. There was no significant bioactivity in the blank gel eluants.

MBP immuno crossreactivity

In all the IEF experiments described, the immuno reactive MBP was detected in gel eluants at pH 9.5. The bioactivity detected in this region of the gel accounted for less than 5% of the total measurable activity.

Figure 29



Isoelectric focusing of fraction C4-IV

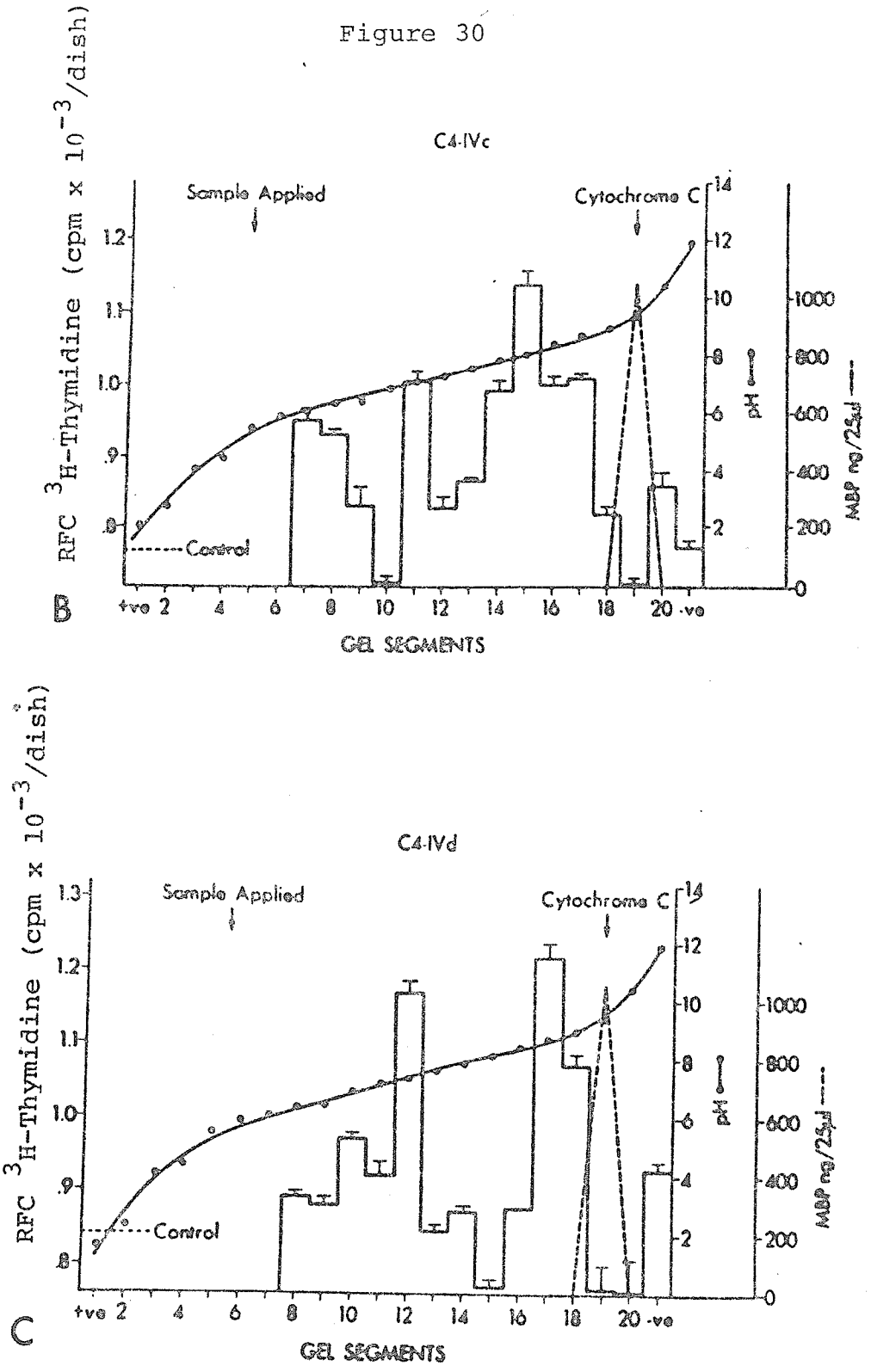
Twenty five micrograms of fraction C4-IV (obtained from gel filtration of C4 on Sephadex G-75 superfine) was applied to slab gel polyacrylamide isoelectric focusing over the range of pH 3.5-10 as described. Focusing was carried out at a constant power of 4 watts for 3½ hrs. Each sample channel was then cut into 21 gel segments which were eluted overnight in 0.5 ml PBS. Aliquots of each eluate were then assayed for growth factor activity using ^{3}H-thymidine incorporation into rabbit fetal chondrocytes and for myelin basic protein cross-reactivity using RIA. The histogram shows the relative bioactivities in the 25 ul aliquot of each gel segment. MBP cross-reactivity (---) in each 25 µl aliquot is also shown.

Figure 30:

Isoelectric focusing of fractions C4-IVc and C4-IVd

Twenty five micrograms of fractions C4-IVc and C4-IVd (obtained by chromatography of fraction C4-IV on CM52) was applied to slab gel polyacrylamide isoelectric focusing as described for fractions C4-IV. The histogram shows the relative bioactivity and MBP cross-reactivity (------) in each gel eluant.

Figure 30


C4-IVc
Sample Applied
Cytochrome C
RFC ^{3}H-Thymidine (cpm x 10^{-3}/dish)
Control
pH
MBP ng/25µl
+ve 2 4 6 8 10 12 14 16 18 20 -ve
GEL SEGMENTS
B
C4-IVd
Sample Applied
Cytochrome C
RFC ^{3}H-Thymidine (cpm x 10^{-3}/dish)
Control
pH
MBP ng/25µl
+ve 2 4 6 8 10 12 14 16 18 20 -ve
GEL SEGMENTS
C

SDS-polyacrylamide electrophoresis of fraction C4-IVc and C4-IVd

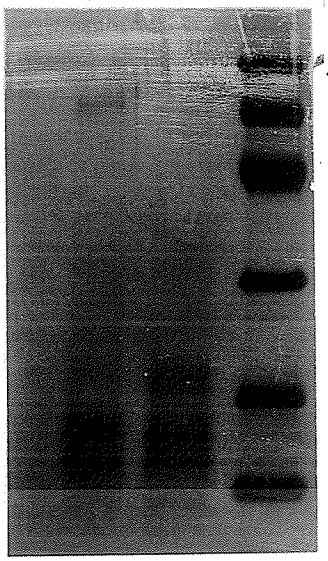
The electrophoretic mobility of fractions C4-IVc and C4-IVd are shown in Plate 3. Two major protein bands were seen in fractions C4-IVc with a molecular wt. of 16,000 and 18,000 daltons. Two faint bands were also observed corresponding to 13,000 and 80,000 daltons. Fractions C4-IVd showed 4 bands of similar intensity in approximately the same position with an additional diffuse band corresponding to a molecular weight of 22,000.

Recovery of h.brain derived growth factors

The recovery of the most potent preparations of h.brain growth factors was less than 1%, Table 8 . Significant activity was found to be present in all pooled fractions following gel chromatography of the B_3 fraction. The protein fraction, C4, corresponding to a molecular weight of 12-18,000 daltons, represents only 4% of the initial activity present in the crude extract. Further chromatography of the C4 fraction using Sephadex G-75 superfine resulted in only a 33% recovery of the applied protein, and 25% of the applied activity.

Plate 3

SDS-polyacrylamide electrophoresis of fraction C4-IVc and C4-IVd



Lane 1 2 3

Human brain derived growth factors

Lane 1 C4-IVc

Lane 2 C4-IVd

Lane 3 Protein Standards

Electrophoresis was carried out as described in the methods.

Table 8

	Protein (mg)	Activity Units/mg	Total Activity	Activity Recovered %
Bovine Pituitary FGF		1150		
Fraction A1	700,000	1	700,000	100.0
Fraction B3	3,000	65	195,000	28.0
Void Volume Gel Filtration (Fraction C1)	2,000	65	130,000	19.0
Fraction C4	150	200	22,500	4
Fraction C4-IV	50	150	7,500	1
Fraction C4-IVc	4	1150	4,600	0.7
Fraction C4-IVd	3	1150	3,450	0.5

Recovery mitogenic activity from human brain

SECTION VII

DISCUSSION

It has been postulated that tumor vascularisation and the proliferation of stromal fibroblasts may be mediated by the release of tumor cell mitogens (47, 102,165) The process of recruiting new blood vessels (angiogenesis) from the host has been shown to be indispensable for the growth of solid neoplasms (16). The data presented in this section provides evidence that a potent growth factor identified in the solid h.mammary tumors may mediate some of these effects. The partially purified h.mammary tumor derived growth factor (CM4 fraction) was shown to stimulate 3 mesodermal derived cells. These include chondrocytes, human fibroblasts and Balb/c3T3 cells reported to be of endothelial origin (14, 123). Partial purification and characterisation of the h.MTGF was, however, performed routinely using rabbit fetal chondrocytes as the target cell. These cells were easily obtainable in high yield and shown to be responsive to other growth factors such as EGF and FGF at concentrations as low as 50 pg ml^{-1} and 1 ng ml^{-1}, respectively.

These findings were of particular interest to us in view of the reported angiogenic properties of FGF (67)

and the family of sarcoma growth factors described by Todaro (154) which were shown to be biologically related to, but immunologically distinct from, EGF.

The crude extracts of h.mammary tumors were shown to stimulate DNA synthesis in quiescent cultures of RFC, and to promote several rounds of cell division when the cells were maintained in medium containing 10% FBS. Preliminary data showed that approximately 75% of the h.mammary tumors stimulated a 2-4 fold increase in cell growth using cultured RFC as the bioassay. The remaining tumor samples failed to induce a 2 fold increase in cell growth. It has been postulated by Ludford and Barlow (102) that the injurious effect of certain cells on fibroblasts was mediated by certain metabolic products released by the carcinoma cells. More recent reports have described growth inhibitors in bovine mammary glands (59), the conditioned medium of cultured h.mammary carcinoma cells (81), and a variety of tissue extracts, Lozzio (101). The magnitude of the growth response in RFC might therefore be a reflection of the relative amounts of growth stimulants and growth inhibitors present in the tumor extract.

The preliminary gel filtration studies at neutral pH of the h.mammary tumor extract revealed the existence of growth factors of different molecular weight. Discrete peaks of activity were demonstrable with proteins of molecular weight 80-150,000 and $\sim$20,000

daltons. The ineffectiveness of ammonium sulphate to selectively concentrate the growth factor supports the notion of a heterogeneous population of growth factors in the tumor extract. Size heterogeneity of polypeptide growth factors is well documented for a variety of growth factors which include NGF (164), EGF (148), IGF's I and II (57), serum-derived mitogens (80,141) and polypeptide hormones (13,88). The molecular weight heterogeneity is commonly attributable to either the presence of discrete proteins showing structural and biological relatedness or due to aggregation of low molecular sub-units, and the interaction of the sub-units with a larger molecular weight binding or carrier protein, yielding low and high molecular weight species respectively.

Preliminary experiments using ammonium sulphate fractionation or gel filtration on Sephadex G-100, designed to separate the majority of the biological activity from the bulk of the inactive proteins, met with limited success. In contrast, extraction of the h.mammary tumor homogenate at acid pH followed by chromatography using a CM52 ion exchange resin, significantly enriched the biological activity. The growth factor preparation obtained by this technique showed comparable activity with the two major protein growth factors derived from the human brain.

The h.mammary tumor growth factor (CM4 fraction) was

recovered as a single peak of activity following isoelectric focusing (pI~9.0).

Assuming an approximate 10 fold increase in purity after isoelectric focusing (based on protein staining of the gel) the specific activity of this factor increased ~10,000 fold compared to the activity demonstrated in the original tumor supernatant prepared routinely for the determination of estrogen receptors.

The investigation of the effect of pH during the extraction revealed some interesting findings. At pH 3 and 4 there was a marked (10 fold) increase in the specific activity of the growth factor. In contrast, the extraction when carried out at pH 10 resulted in the detection of little or no growth factor activity. Full biological activity was recovered following the exposure of the inactive pH 10 supernatant to pH 3.5.

The precise mechanism underlying the activation of growth factor activity is presently unknown. However, in view of the numerous reports regarding the enhanced biological potency of certain polypeptide hormones by limited proteolysis (see introduction) coupled with the observation that processing of many secretory proteins (141,25)involves multiple specific proteolytic events might suggest the existence of a proteinase-mediated activation of the h.MTGF.

The presence of a variety of proteinases have been identified in normal, benign and malignant breast tissue.

These include collagenase, plasminogen activator,

cathepsin B-like and cathepsin D-like proteinase (122,172). Of the proteinases described, elevated levels of cathepsin B-like proteinase were demonstrated in the culture medium of h.mammary carcinoma explants compared to benign and normal breast tissue explants.

The pH optimum of cathepsin B in the culture medium form the carcinoma explants was shown to be between pH 6-7, in contrast to cathepsin D with optimal activity at pH 3.5.(122). Both enzymes were inactive above pH 7.0.

These findings are consistent with the observed increase in bioactivity of the h.MTGF recovered when the extraction is carried out at acid and neutral pH with little or no activity demonstrable under alkaline conditions. Further studies using leupeptin and antipain which are specific inhibitors of cathepsin D (10) and cathepsin B (156,157)respectively during the extraction procedure may provide an estimate of the relative putative role of each proteinase in the generation of growth factor activity in crude mammary tumor extracts.

It is interesting to note that the promotion of tumors in mouse skin can be inhibited by proteinase inhibitors (155) as well as the growth and mestatasis of several experimental tumors (156,157).In contrast, agents which promote tumor formation of mouse skin such as the phorbol ester TPA (11) and the polypeptide epidermal growth factor (130) also stimulate the production of proteinases such as plasminogen activator (94).

A correlation between the production of plasminogen activator and various properties of the transformed phenotype, such as cell locomotion, morphology and loss of anchorage-dependent growth have been described (113,121), although no conclusive evidence exists for a relation between secretion of plasminogen activator and cancer (26,109,131). Hence the presence of growth factors in the local environment of the tumor may therefore arise due to the limited proteolytic degradation of proteins/ hormones in the extracellular environment. Alternatively, the mammary tumor growth factor may represent a more primitive polypeptide hormone, which has been hypothesised to have evolved from the lysosomal proteases (72). The unopposed action of this mitogen might therefore play an important role in the stimulation of tumor growth. Concomitant with its presence in the tumor host interzone, it may mediate the inductive proliferation of neighboring cells. A deficiency of proteinase inhibitors in the tumor cell or its environment would allow a degree of autonomous proliferation characteristic of malignant tumors. The proposal of a proteinase/proteinase inhibitor imbalance has been described for a variety of pathological conditions such as emphysema (44) arthritis (39) glomerulonephritis (38) muscular dystrophy (85) tumor invasion (144) and possibly its vascularisation (105).

At which point in the cell cycle the h.MTGF exerts its biological effect has yet to be determined. Future

studies using purified preparations of the h.MTGF would permit the investigations regarding the site of action of this growth factor in the cell cycle. However, preliminary studies using a sub-optimal dose of h.MTGF in combination with a maximal dose of EGF produced an additive effect in the final cell density of RFC, compared to the presence of each of these mitogens when added alone.

The additive effect of these two agents would suggest an independent pathway of cell regulation leading to the stimulation of cell proliferation. The mechanism of action of the h.MTGF might therefore be analagous to that described for the platelet derived growth factor (PDGF) and b.pituitary FGF using the Balb/c3T3 cell system (142). These factors have been proposed to render the Balb/c3T3 cell competent in the G0-G1 phase of the cell cycle, and therefore to be responsive to the action of progression factors such as the somatomedins and EGF. In addition, the induction of the EGF cell surface receptor by PDGF (118) has been proposed as an additional mechanism by which cells can overcome their density inhibition of growth. Whether the h.MTGF can modulate the EGF receptor levels in the target cells described here, is the object of future investigations.

The local production of the h.MTGF within the tumor tissue and its potential ability to render cells competent to the endogenous effect of plasma factors such

as EGF (and presumably SGF) and possibly the somatomedins, would suggest an extremely important role for this growth factor in the regulation of mammary tumor growth *in vivo*. The demonstration that the partially purified h.MTGF (CM4 fraction) can stimulate the proliferation of at least one endothelial cell type (Balb/c3T3 cell line) and human fibroblasts would support the hypothesis that these factors may be partly responsible for the mediation of tumor angiogenesis and the proliferation of stromal fibroblasts at the site of the tumor host interzone. The formation of a capillary network *in vitro* has been recently described using h.capillary endothelial cells from a variety of sources (47). These cells have been reported to grow best in tumor conditioned medium and migrate in response to tumor derived mitogens (48). Whether the h.MTGF described here would induce angiogenesis *in vivo* and/or stimulate an endothelial capillary network *in vitro* remains an avenue of future research that promises to be both exciting and informative.

Comparison of the human mammary tumor growth factor (h.MTGF) with other growth factors

The h.MTGF is a cationic (pI 9.0), heat and acid labile polypeptide. These properties distinguish it from other cationic growth factors such as platelet derived growth factor (PDGF) (2), the family of insulin-like growth factors (12) and the anionic peptide EGF (32). The mitogenic activity of the h.MTGF in the presence of 10% FBS would suggest that it is probably different from many of the major serum derived growth factors which have been described.

The activity of the h.MTGF (fraction CM4) was compared to the various growth factors purified from human brain. The purification of the h.brain derived growth factors was monitored independently by the stimulation of DNA synthesis in quiescent cultures of rabbit fetal chondrocytes. Furthermore, fractions were also tested for immunocrossreactivity with myelin basic protein (MBP). The most purified fractions of h.brain growth factors C4-IVc, C4-IVd were found to have comparable activity compared to the h.MTGF (CM4). All fractions stimulated a dose-dependent increase in rabbit fetal chondrocytes when maintained in the presence of 10% FBS. The biological activity of these fractions was estimated to be ~10% as active compared to a commercial preparation of b.pituitary FGF. The biological relatedness between the

h.MTGF and h.brain growth factors, and pituitary FGF is presently unclear.

Immunoreactive MBP constituted 80% of the proteins by weight of the h.brain fraction C4-IVc and C4-IVd. Separation of the biological activity from the MBP was accomplished by isoelectric focusing. In support of our findings, Thomas et al (150) reported that b.brain FGF purified by the method described by Gospodarowicz, was not retained on a column of chicken anti-bovine MBP coupled to sepharose. The principal mitogenic activity was found to have an isoelectric point between 4.8 - 5.8. The authors report that a further purification step of 50-100 is required to obtain purified FGF. Hence the reported relationship between b.brain FGF and MBP described by Westall et al (168) might therefore be due to the presence of small contaminating quantities of "authentic" FGF in their final preparation, which contained myelin basic protein fragments.

Further purification of the h.MTGF (CM4) and h.brain growth factors (C4-IVc and C4-IVd) using isoelectric focusing, clearly separates the biological activity from the major contaminating proteins which focus at the cathode. In the case of the h.brain growth factors, the major contaminating protein was found to be MBP. The identity of the major contaminating protein in the h.MTGF (CM4), which focused at the cathode, has not been determined.

It is of interest here to note that a family of cationic polypeptides have been extracted from solid human mammary tumors which induce symptoms closely resembling experimental allergic encephalomyelitis (46). The demonstration of cancer basic protein (CaBP) associated with a variety of human neoplasms has been demonstrated (24). The presence of these CaBP on the surface of malignant cells has been proposed to mediate the lymphocyte sensitisation in vivo in patients with malignant disease.

The method for determining lymphocyte sensitisation has been described and used extensively. Briefly, the peripheral lymphocytes of patients with diagnosed malignant neoplasia are challenged in vitro to encephalitogenic factor (EF) isolated from human brains. The lymphocytes release lymphokines into the medium, which have properties of slowing the migration of macrophages in vitro. (Macrophages are obtained from the peritoneum of guinea-pigs after I.P. injection of liquid paraffin). The degree of macrophage migration inhibition is an index of the degree of lymphocyte-antigen interaction, and an indication of sensitisation which occurred in vivo. Utilising this technique it has been demonstrated that CaBP extracted from a variety of human neoplasms can cause macrophage inhibition in vitro. Utilising EF as a reference standard, CaBP was shown to be more antigenic than EF when lymphocytes from patients with malignant

disease were used as the test. However, when lymphocytes were taken from patients with diagnosed destructive CNS lesions, the converse was demonstrated. The EF (obtained from normal brains) was more antigenic than the CaBP. Investigations have shown a chemical similarity and immunological crossreactivity between CaBP and EF (19). The nature and significance of this crossreactivity is unknown, and has been proposed to be due to the presence of neo-antigens at the surface of tumor cells. Furthermore, expression of these antigens and sensitisation of lymphocytes has been proposed to precede the clinical manifestation of neoplastic disease.

Using a transplantable SV-40 tumor cell line, Flavell (45) reported a positive response by the macrophage migration inhibition (MMI) assay when spleen cells of tumor bearing animals were challenged with encephalitogenic factor (EF). Hence, these cells were expressing antigens related to EF. At this point one could speculate that the loss of growth control in SV-40 transformed cells was associated with the expression of neo-antigens related to EF, and a loss of dependency for exogenous growth factors.

In summary, the role of the h.MTGF described here may be to mediate the proliferative changes of connective tissue cells at the tumor host interzone. The relationship between the expression of CaBP and the appearance of tumor growth factors is presently unclear.

The relationship between the major cationic polypeptide growth factor derived from the mammary tumors, with other growth factors described, must await further investigations. The development of specific antibodies and receptor assays to this major tumor growth factor will be useful in determining its immunological and biological relationship with other polypeptide growth factors.

Future proposals

1) Further purification of the human tumor derived growth factor and the development of specific antibodies and a radio-immunoassay.

2) Determine the immuno-relatedness of the h.MTGF with other growth factors and hormones.

3) Develop a specific radio-receptor assay for the determination of biological relatedness with other growth factors and hormones.

4) Determine the serum levels and tissue distribution of the h.MTGF, in normal and pathological conditions, most notably in breast cancer.

The crude h.mammary tumor extract (h.MTE) was also found to promote the growth of at least 3 h.mammary carcinoma cell lines. The growth factor present in the h.MTE which stimulated the growth of T-47D cells was thermolabile, acid stable and trypsin sensitive. The stability of the T-47D growth factor in 1 M acetic acid partly distinguishes it from the growth promoting activity in the h.MTE which stimulated the proliferation of rabbit fetal chondrocytes. These findings might suggest the presence of two populations of growth factors present in the crude h.MTE capable of supporting the proliferation of epithelial and mesodermal cells.

The response of T-47D cells to the exogenous effects of insulin and MSA over a wide concentration was minimal and neither one could sustain the continued cell replication compared to that of the crude tumor extract. It could be argued that the growth of T-47D cells in low serum concentrations requires the presence of a multiplicity of factors known to be required for normal cell growth. The presence of one factor in combination with a low serum concentration may therefore be inadequate to stimulate multiple rounds of cell division.

The presence of trace quantities of these factors present in the tumor extract might therefore be sufficient for the continued proliferation of these cells in 0.1% BS. In order to circumvent these problems, Barnes and Sato (9) have developed defined media for a variety of

cell cultures. The nutrient media usually includes 5 defined components (growth factors, hormones, attachment factors, etc.). Utilising this strategy, Barnes & Sato (8) has reported the growth of an h.mammary carcinoma cell line M-CF7 maintained in the presence of insulin, transferrin, EGF, prostaglandin, $F_2\alpha$, and cold insoluble globulin. The growth of these cells was found to be comparable to those maintained in 10% FBS. Omission of insulin alone dramatically reduced the proliferative capacity of these cells. Omission of the other factors had a less dramatic effect. These findings might suggest that the growth promoting effect of insulin (0.1 $\mu g\ ml^{-1}$) may operate through the somatomedin receptor. Whether the factor described here is related to the somatomedins is presently unknown. The partial growth response of the T-47D cells observed when incubated in the presence of insulin and M.S.A. might suggest that these cells are responsive to the somatomedin family of growth factors. Compatible with this notion is the observed dependency of the MCF-7 cell line for insulin. Somatomedin-like polypeptides have recently been identified in culture media of mammary tumors transformed by the chemical carcinogen DMBA (7,12-Dimethylbenz[a]-anthracene) (91). Chromatography of the conditioned medium under acid conditions revealed a broad range of bioactivity corresponding to a molecular weight of 7-20,000. These factors competed for ^{125}I-MSA on the surface of chick embryo fibroblasts at a concentration

that correlated with the biological activity of MSA. The MSA competing activity described by Todaro had apparent molecular weights of approximately 7,000, 10,000, 15-18,000 and several peaks greater than 24,000. The heterogeneity of the molecular size of MSA-like activity in these two reports is compatible with the T-47D cell growth promoting activity obtained following fractionation of extracts from solid h.mammary tumors.

The factors present in the solid h.mammary tumors described here and their relationship to the somatomedin family of growth factors, remains obscure. Further purification of this factor is required (perhaps using the approach described by Sato) to define the biological relatedness of this factor with other mitogens. The lack of a mitogenic effect of EGF (0.005 - 50 ng ml^{-1}) on T-47D cells described here, is compatible with its lack of effect on MCF-7 cell growth (8). Furthermore, the content of EGF in the tumor supernatant as measured by radioreceptor assay (Imai personal communication) was less than 10 pg/30 ug of tumor protein. The effect of the tumor extract at this dose was to produce a 3 fold increase in cell number. These findings raise the question concerning the nature of the mitogenic effect observed when T-47D cells were incubated in the presence of dialysed h.milk (5%v/v). The activity detected however, was 100 fold less compared to the amount in tumor extracts.

The mitogenic effect initially reported by Klagsbrun (90) was found subsequently to be inhibited by antibodies raised to h.EGF (23). However, these authors also reported that h.milk stimulated DNA synthesis in an EGF receptor-defective 3T3 derivative. The stimulating effect of h.milk on these cells was not neutralised by antibodies to EGF. Whether the effect of h.milk on T-47D cell growth reported here, and the stimulation of DNA synthesis as reported by Carpenter (23) are due to the same factor is unclear. The retention of activity after extensive dialysis of the h.milk prior to the assay using T-47D cells, suggests that this factor may be macromolecular in nature.

In summary, therefore, it remains to be shown whether any of these growth factors reported here will show a structural and functional relationship to other growth factors in a manner analagous to the relationship described, for example, between the pancreatic hormones and the insulin-like family of growth factors (12).

Summary of human brain derived growth factors

The purification of h.brain growth factors has been described, using a conventional protein purification procedure. The principal biologically active component was easily extracted and separated from the crude brain extract by chromatography on Sephadex C-50 ion exchange. The active fraction B_3 was a potent growth factor for Balb/c3T3 and rabbit fetal chondrocytes. Following chromatography of the B_3 fraction on Sephadex G-75, biological activity was detected in all fractions, with a slight increase in activity associated with proteins of molecular weight ~15,000 daltons. Isoelectric focusing of the B_3 fraction further indicated substantial heterogeneity of the brain growth factor. Biological activity could be detected over a pH interval of 5-9.

These findings might suggest the presence of a family of related protein growth factors in the brain. Alternatively, proteolytic modification of proteins during post-mortem may have added significantly to the heterogeneity of apparent molecular size and charge of the growth factor.

Recently, Maciag (103) has reported the presence of a high molecular weight ~75,000 dalton endothelial growth factor in bovine hypothalamic extracts. This factor was reported to be different from the FGF described by Gospodarowicz (66). The results described

here may therefore suggest the presence of other growth factors in the human brain. The purification of the 15,000 molecular weight fraction, (C4-IV) using CM52 ion exchange yielded 2 fractions of significant biological potency (C4-IVc and C4-IVd). The specific activity of these fractions was estimated to be ~1,150 times greater than the crude extract of h.brain. The poor recovery of the biological activity (~0.5% of total) might further suggest the presence of other growth factors. Gospodarowicz has speculated that myelin basic protein fragments may mediate glial cell proliferation in patients with multiple sclerosis. The evidence presented here indicates that the principal mitogenic activity in the human brain is unrelated to MBP. The non-identity of the brain growth factors with MBP may suggest that the proliferative changes in the CNS may be mediated by factors such as the brain FGF described here.

SECTION VIII

CONCLUDING REMARKS

Clinical significance

The presence of two populations of growth factors in solid h.mammary tumors has been described. The acid labile factors are cationic polypeptides that are potent mitogens for a variety of mesodermal cells. The role of these factors has been postulated to mediate the proliferation of stromal fibroblasts and tumor vascularisation in vivo. Alterations of the connective tissue structure and hyperplasia of stromal fibroblasts have been proposed to be a pre-malignant manifestation of disease (25).

These findings might suggest that the cationic growth factor described here may potentially be useful as a diagnostic tool for the early detection of breast cancer. The appearance of another family of cationic polypeptides have been described in extracts of solid h.mammary tumors (45). These basic proteins produce symptoms closely resembling experimental allergic encephalomyelitis. The identification of a family of cancer basic proteins (CaBP) following acid extraction of a variety of h.neoplasms has been reported (45). The expression of CaBP at the cell surface has been proposed, thereby mediating the sensitisation of peripheral lymphocytes. The manifestation of sensitivity to these cationic polypeptides has

been postulated to precede the appearance of clinically detectable neoplastic disease.

The relationship between the potent cationic growth factors described here, and the proposed concomitant expression of CaBP with the neoplastic process, warrants a closer investigation.

Evidence is also presented in this thesis for the presence of a heterogeneous population of acid stable polypeptide growth factors in solid mammary tumors. These factors are capable of stimulating at least one h.breast cancer cell line. These findings may suggest that specific therapeutic intervention in the control of production of these growth factors, has the potential to yield significant results in the control of breast cancer growth.

It is postulated that the expression of these h.mammary tumor derived growth factors, plays a fundamental role in the pathogenesis of human mammary neoplastic disease.

SECTION IX

DECLARATION OF ORIGINAL RESEARCH

The identification of novel growth factors in human mammary tumors was investigated solely by the writer. Some biochemical properties of these growth factors have been described. A scheme for the partial purification of one of these human mammary tumor growth factors was established.

The identification, purification and characterisation of novel growth factors from human brain was a collaborative project with Drs. J. Kellett and T. Tanaka.

SECTION X

BIBLIOGRAPHY

1. Angeletti, R.M., Bradshaw, R.A. (1971)
Proc. Natl. Acad. Sci. U.S.A. 68: 2417

2. Antoniades, H.N., Scher, C.D., Stiles, C.D.
Proc. Natl. Acad. Sci. U.S.A. 76: 1809

3. Argyris, T.S. (1965)
Adv. Biol. Skin 7: 55

4. Armelin, H.A. (1973)
Proc. Natl. Acad. Sci. U.S.A. 70: 2702

5. Bala, R.M., Bohnet, H.G., Carter, J.N., Friesen, H.G. (1976)
Clin. Res. 24: 655A abs.

6. Balk, S.D. (1971)
Proc. Natl. Acad. Sci. U.S.A. 68:1207

7. Balk, S.D., Whitfield, J.F., Joudale, T., Braun, A.C. (1973)
Proc. Natl. Acad. Sci. 70: 675

8. Barnes, D., Sato, G. (1979)
Nature, 281: 388

9. Barnes, D., Sato, G. (1980)
Anal. Biochem. 102: 255

10. Barrett, A.J. (1980)
in The cellular proteinases - a broad view. In proteinases and tumor invasion. Strauli et al, eds. Raven Press, N.Y.

11. Berenblum, I. (1975)
in Cancer 1. Becker, F.F. ed. Plenum, N.Y.

12 Blundell, T.L., Humbell, R.E.
Nature, 287: 781

13. Blundell, T.L., Wood, S.P. (1975)
Nature, 257, 197

14. Boone, C.W., Takeichi, N., Paranjpe, M., Gilden, R. (1976)
Cancer Res. 36: 1626

15. Bourne, H.R., Rozengurt, E. (1976)
Proc. Natl. Acad. Sci. U.S.A. :4555

16. Brem, S., Brem, H., Folkman, J., Finkelstein, D., Patz, A. (1976)
Cancer Res. 36: 2807

17. Bueker, E.D. (1948)
Anat. Rec. 102: 369

18. Burstein, P.J., Schalch, D.S., Heinrich, U.S., Johnson, C.V. (1978)
Clin. Res. 26

19. Carnegie, P.R., Caspary, E.A., Field, E.J. (1973)
Br. J. Cancer. Suppl. I. 219

20. Carpenter, G., Cohen, S. (1978)
in Biochemical actions of hormones. Academic Press, N.Y.
5:203

21. Carpenter, G., Cohen, S. (1978)
in Biochemical actions of hormones. Litwack, G. ed.
5:203 Academic Press, N.Y.

22. Carpenter, G., Cohen, S. (1979)
Ann. Rev. Biochem. 48: 193

23. Carpenter, G. (1980)
Science, 210: 198

24. Caspary, E.A., Field, E.J. (1971)
Br. Med. J. ii, 613

25. Chaudhuri, S., Koprowska, I., Putong, P.B., Townsend, O.E.R (1974)
Can. Res. 34: 1335

26. Chen, L.B., Buchanan, J.M. (1975)
Proc. Natl. Acad. Sci. U.S.A. 72: 1132

27. Chou, I.N., Prezyna, C., Black, P.H. (1979)
J. Biol. Chem. 254: 10588

28. Clark, J.L., Jones, J.L., Gospodarowicz, D., Sato, H.H. (1972)
Nature, New Biol. 236: 180

29. Clemmons, D.R., Van Wyk, J.J., Pledger, W.J. (1980)
Proc. Natl. Acad. Sci. 77: 6644

30. Cohen, S. (1960)
Proc. Natl. Acad. Sci. U.S.A. 46: 302

31. Cohen, S.J. (1962)
J.Biol. Chem. 237: 1555

32. Cohen, S., Savage, C.R. (1974)
Recent Prog. Horm. Res. 30: 551

33. Cohen, S., Haigler, H.T., Carpenter, G., King, Jr., McKanna, J.A. (1979)
in Hormones and cell culture, Sato, G.H., Ross, R. Cold Spring Harbour Conference on cell proliferation, vol. 6 131

34. Costin, G., Kogut, M.D., Phillips, L.S., Daughaday, W.H. (1976)
J. Clin. Endocrinol. Metab. 42: 370

35. Daughaday, W.H., Reeder, C. (1966)
J. Lab. Clin.Med. 68: 357

36. Daughaday, W.H., Hall, K., Raban, M.S., Salmon, W.D., van den Brande, J.L., van Wyk, J.J. (1972)
Nature 235: 107

37. Davey, M.G., Lauscher, E.F. (1966)
in Platelet proteins in biochemistry of blood platelets, Kowalski, E., Niewiarowski, S., eds. p. 922 Academic Press, N.Y.

38. Davies, M., Barrett, A.J., Travis, J., Sanders, E., Coles, G.A. (1978)
Clin. Sci. Mol. Med. 54: 233

39. Dingle, J.T. (1978)
Ann. Intern.Med. 88: 821

40. Dulak, N.C., Temin, H.M. (1973)
J. Cell. Physiol. 81: 153

41. Dulak, N.C., Temin, H.M. (1973)
J. Cell. Physiol. 81: 161

42. Eisen, M., Vapp, H.E., Vock, R. (1975)
J. Urology 114: 93

43. Elders, M.J., Wingfield, B.S., McNatt, M.L.,Clarke, J.S., Hughes, E.R. (1975)
Am. J. Dis. Child 129: 1391

44. Eriksson, S. (1978)
Acta. Med. Scand. 203: 449

45. Flavell, D.V., Goepel, J., Potter, C.W., Carr, I. (1978)
Br. J. Cancer 37: 818

46. Flavell, D.J., Goepel, J., Wilson, A.P., Potter, C.W. (1979)
Br. J. Cancer 40: 424

47. Folkman, J. (1974)
Adv. Cancer Res. 19: 331

48. Folkman, J., Haudschild, C. (1980)
Nature, 288: 551

49. Fox, F.C., Das, M. (1979)
J. of Supramolecular Structure

50. Francis, M.J.O., Hill, D.J. (1975)
Nature 255: 167

51. Froesch, E.R., Muller, W.A., Burgi, M., Wdvogel, M., Labhart, A. (1966)
Biochim. Biophys. Acta 121i: 360

52. Froesch, E.R. (1967)
Rec. Prog. Horm. Res. 23: 565

53. Fryklund, L., Skottner, A., Sievertsson, H., Hall,K. (1976)
in Growth hormone and related peptides. Pecile A. Muller, ed. pp 156. Excerpta Medica, Amsterdam.

54. Fryklund, L., Uthne, K., Sievertsson, H. (1974)
Biochem Biophys. Res. Commun. 61: 957

55. Gasic, G., Gasic, T., Jimenes, S. (1977)
Thromb. Res. 10: 33

56. Gey, G.O., Thalhimer, W. (1924)
J. Am. Med. Assoc. 82:1609

57. Ginsberg, B.H., Kahn, C.R., Roth, J., Megyesi, K., Baumann, G. (1979)
J. Clin. Edn. Met. 48: 43

58. Golde, D.W., Berch, N., Li, C.H. (1980)
Science 196:1112

59. Gonzalez, R., Verly, W.G. (1976)
Proc. Natl. Acad. Sci., 73: 2196

60. Gospodarowicz, D. (1974)
Nature 249:123

61. Gospodarowicz, D. (1975)
J. Biol. Chem. 250: 2515

62. Gospodarowicz, D., Weseman, J., Moran, J. (1975)
Nature 256:216

63. Gospodarowicz, D., Moran, J.S. (1976)
Ann. Rev. Biochem. 45: 531

64. Gospodarowicz, D., Moran, J.S., Bialecki, H. (1976)
Third Excerpta Medica, 141

65. Gospodarowicz, D.,Bialecki, H., Greenburg, G. (1978)
J. Biol. Chem. 253: 3736

66. Gospodarowicz, D., Greenburg, G., Bailecki, H.,
Zetter, B.R. (1978)
In vitro 14:85

67. Gospodarowicz, D. (1979)
Mollecular and cellular biochemistry 25: 79

68. Gospodarowicz, D., Taubner, J.P. (1980)
Endocrine Rev. 1: 201

69. Greene, L.A, Tischler, A.S. (1976)
Proc. Natl. Acad. Sci. 73: 2424

70. Gregory, H. (1975)
Nature, 257: 325

71. Haigler, H., Ash, J.F., Singer, S.J., Cohen, S. (1978)
Proc. Natl. Acad. Sci. 75: 3317

72. Hales, N. (1978)
FEBS Lett. 94: 10

73. Hall, K. (1972)
Acta Endocrinol. 163(Suppl) 1-52

74. Heldin, C.H., Westermark, B, Wasteson, A. (1981)
Biochem. J. 193: 907

75. Heldin, C.H., Westermark, B., Wasteson, A. (1979)
Proc. Natl. Acad. Sci. U.S.A. 76: 3722

76. Hendry, I.A. (1972)
Biochem. J. 128: 1265

77. Hendry, I.A., Iverson, L.L. (1973)
Nature, 243: 500

78. Hintz, R.L., Clemmons, D.R., Underwood, L.E., Van Wyk, J.I.
(1972)
Proc. Natl. Acad. Sci. U.S.A. 69: 2351

79. Holley, R.W, Kiernan, J.A. (1968)
Proc. Natl. Acad. Sci. U.S.A. 60: 300

80. Houck, J.C., Cheng, R.F. (1973)
J. Cell. Physiol. 81: 257

81. Howard, E.F., Cheng, C.Y., Howard, J.S. (1980)
Can. Res. 40: 4385

82. Hunter, W.H., Greenwood, F.G.C. (1962)
Nature (London) 194: 495

83. Imai, Y. Tsushima, T., Sasaki, N., Matsuzaki, F. (1980)
in Growth and growth factors. Proc. Int. Symp. on
Growth and growth factors. Shizume, K., Takano, K.,
eds. 275-288

84. Kano-Sueoka, T., Cohen, D.M., Yamauzumi, Z.,
Nishimura, S., Mori, M., Fujiki, H. (1979)
Proc. Natl. Acad. Sci. U.S.A. 76: 5741

85. Kar, N.C., Pearson, C.M. (1979)
Clin. Biochem. 12: 37

86. Kasper, S. (1981)
MSc. Thesis. University of Manitoba, Winnipeg, Canada.

87. Kellett, J.G., Tanaka, T., Rowe, J.M., Shiu, R.P.C.,
Friesen, H.G. (1981)
J. Biol. Chem. 256: 54

88. Kemper, B., Habener, J.F., Mulligan, R.C., Potts. J.T.,
Rich, A. (1974)
Proc. Natl. Acad. Sci. U.S.A. 71: 3731

89. Kibrick, E.A., Becks, H., Marx, W., Evans, H.M. (1941)
Growth 5: 437

90. Klagsbrun, M. (1978)
Proc. Natl. Acad. Sci. U.S.A. 75: 5057

91. Knauer, D.J., Iyer, A.P., Banerjerr, M.R., Smith, G.L.
(1980)
Cancer Res. 40: 4368

92. Kohler, N., Lipton,A (1974)
Exp. Cell Research 87: 297

93. Ledet, T. (1976)
Diabetes, 25:1011

94. Lee, L.S., Weinstein, I.B. (1978)
Nature (London) 274: 696

95. Leung, C.K.H., Shiu, R.P.C. (1981)
Can. Res. 41: 546

96. Levi-Montalcini, R., Hamburger, R. (1951)
J. Exp. Zool. 116: 321

97. Levi-Montalcini, R., Angelletti, P.U. (1961)
in Regional neurochemistry, p.362. Kety, S.S., Elkes, J. eds. Pergamon Press, N.Y.

98. Levi-Montalcini, R., Angelletti, P.U. (1968)
Phsyiol. Rev. 48: 534

99. Levi-Montalcini, R. (1976)
Prog. Brain Res. 45:235

100. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951)
J. Biol. Chem. 193: 265

101. Lozzio, B.B., Lozzio, C.B., Bamberger, E.G., Lair, S.V. (1975)
in Regulators of cell division. p. 1-47

102. Ludford, R.J., Barlow, H. (1944)
Can. Res. 4:694

103. Maciag, T., Gerundolo, J., Ilsley, P.S., Kelley, P.R., Forand, S.R.
(1979)
Proc. Natl. Acad. Sci. U.S.A.76:5764

104. Maciag, T., Nemore, R.E., Weinstein, R., Gilchrist, B.A. (1981)
Sci. 211: 1452

105. Maugh, T.H. (1981)
Sci. 212: 1374

106. Maupin, B. (1969)
in Blood platelets in man and animals.
Pergamon Press, London. Vol. 1. 117-121, 130-133

107. Megyesi, K. (1974)
Biochem. Biophys. Res. Commun. 57: 307

108. Moses, A.C., Nissley, S.P., Cohen. K.L.
Rechler, M.W., (1976)
Nature, 263: 137

109. Mott, D.M., Fabisch, P.H., Sani, B.P., Sorof, S. (1974)
Biochem. Biophys. Res. Comm. 61: 571

110. Murphy, R.A., Saide, J.D., Blanchard, M.H., Young, M. (1977)
Proc. Natl. Acad. Sci. U.S.A. 74:2330

111. Nair, B.K., De Ome, K.B. (1973)
Cancer Res. 33: 3222

112. Osinchak, J. (1964)
J. Cell Biol. 21: 35

113. Ossowki, L., Quigley, J.P., Kellerman, G.M., Reich, E. (1973)
J. Exp. Med. 138: 1056

114. Phillips, L.S., Orawski, A.T. (1977)
Diabetes, 26: 864

115. Phillips, L.S., Herington, A.C., Daughaday, W.H. (1975)
Endocrinology, 97: 798

116. Phillips, L.S., Young, H.S. (1976)
Endocrinology, 99: 304

117. Phillips, L.S., Vassilopoulou, Sellin, R. (1980)
New. Engl. J. of Med. 302: 371

118. Phillips, P.D., Cristofalo, V.J. (1981)
In vitro, 17: 213

119. Pierson, R.W.Jr., Temin, H.M. (1972)
J. Cell Physiol. 79: 319

120. Pledger, W.J., Stiles, C.D., Antoniades, H.N. Scher, C.D. (1977)
Proc. Natl. Acad. Sci. U.S.A. 74: 4481

121. Pollack, R., Risser, R., Conlon, S., Rifkin, D. (1974)
Proc. Natl. Acad. Sci. U.S.A. 71: 4792

122. Poole, A.R., Recklies, A.D., Mort, J.S. (1980)
in Proteinases and tumor invasion, Strauili et al, eds.
Raven Press, N.Y.

123. Porter, K.R., Todaro, G.J., Fonte, V. (1973)
J.Cell Biol. 59: 633

124. Ptashne, K., Hsueh, H.W., Stockdale, F.E. (1979)
Biochemistry, 18: 3533

125. Rechler, M., Posskalny, J.M., Goldfine, I.D., Wells, C.A. (1974)
J. Clin. Endocrinol. Metabl. 39: 512

126. Rees, L.H., Ratcliffe, J.G. (1974)
Clin. Endocrinol. (Oxf.) 3: 263

127. Rinderknecht, E.,Humbel, R.E. (1976)
Proc. Natl. Acad. Sci. U.S.A. 73: 4379

128. Rinderknecht, E.,Humbel, R.E. (1978)
FEBS Lett. 89: 283

129. Rinderknecht, E., Humbel, R.E. (1978)
J. Biol. Chem. 253: 2769

130. Rose, S.P., Stahn, R., Passovoy, O.S.,
Hershman, H. (1976)
Experientia, 32 : 913

131. Rosenthal, S.L, Zucker, D., Davidson, R.L. (1978)
J. Cell. Physiol. 95: 275

132. Ross, R.J., Glomset, B., Kariya, B., Harker, L. (1974)
Proc. Natl. Acad. Sci. 71: 1207

133. Ross, R., Glomset, J.A. (1976)
New Engl. J. Med. 295: 369, 420

134. Rudland, P.S., Eckhart, W., Gospodarowicz, D.,
Seifert, W. (1974)
Nature, 250: 337

135. Salmon, W.D.Jr., Daughaday, W.H. (1957)
J. Lab. Clin. Med. 49: 825

136. Salmon, W.D.Jr., Mosse, B.R. (1971)
Proc. Soc. Exp. Biol. Med. 136: 805

137. Salmon, W.D.Jr., DuVall, M.R. (1970)
Endocrinology, 87: 1168

138. Savage, C.R., Cohen, S. (1972)
J. Biol. Chem. 247: 7609

139. Schlessinger, J., Schechter, M.C., Willingham, A.
Pastan, I. (1978)
Proc. Natl. Acad. Sci. 75: 2659

140. Sirbasku, D.A., Benson, R.H. (1979)
in Hormones and cell culture. Cold Spring Harbour
conference on cell proliferation. Ross, R., Sato,
G.H., eds. p. 477-497.

141. Steiner, D.F. (1976)
in Peptide hormones, p. 49-64. Parsons, J.A. ed.
University Park Press, London.

142. Stiles, C.D., Capne, G.T., Scher, C.D. Antoniades, H.M., Van Wyk, J.J., Pledger, W.J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76: 1279

143. Stiles, C.D., Pledger, W.J., Van Wyk, J.J. Antoniades, H., Sher, C.D. (1979) in Hormones and cell culture. Cold Spring Harbour on cell proliferation. Vol. 6. Book A. Sato, G.H., Ross, R. Eds. p. 425-439.

144. Strauli, P. (1980) Proteinases and tumor invasion. Strauli, P. et al eds. Raven Press, N.Y.

145. Svoboda, M.E. (1980) Biochemistry, 19: 790

146. Swanson, T.L., Moore, G.E.(1979) Biochem. Biophys. Res. Commun. 90: 1166

147. Tanaka, T., Shiu, R.P.C., Gout, P.W., Beer, C.T. Noble, R.C., Friesen, H.G. (1980) J. Clin. Endocrinol. Met. 51: 1058

148. Taylor, J.M., Cohen,S., Mirchell, W.M. (1970) Proc. Natl. Acad. Sci. 67: 171

149. Taylor, J.M., Mitchell, W.M., Cohen, S. (1972) J. Biol. Chem. 247: 5928

150. Thomas, K.A., Riley, M.L., Lemmon, S.K., Baglan, N.C., Bradshaw, R.A. (1980) J. Biol. Chem. 255: 5517

151. Todaro, G.J., De Larco,J.E., Nissley, S.P., Rechler, M.M. (1977) Nature (London) 267: 526

152. Todaro, G.J., De Larco,J.E. (1980) in Control mechanisms in animal cells. Jimenez de Asua, L, et al eds. Raven Press, N.Y.

153. Todaro, G.J., De Larco,J.E., Marquardt, H., Bryant, M.L., Sherwin, S.A., Sliski, A.H. (1979) in Hormones and cell culture. Cold Spring Harbour conferences on cell proliferation. Sato, G.H., Ross, R., eds. p. 113

154. Todaro, G.J., De Larco, J.E. (1978) Cancer Res. 38: 4147

155. Troll, W., Klassen, A., Janoff, A. (1970) Science, 169: 1211

156. Umezawa, H. (1972)
in Enzyme inhabitors of microbial origin, Umezawa, H. ed. Univ. of Tokyo Press, pp 15

157. Umezawa, H. (1976)
Methods in Enzymol. 55: 678

158. Uthne, K. (1973)
Acta Endocrinol. Copenh. Suppl. 175: 135

159. Van Buul-Offers, Van den Brande, J.L. (1979)
Acta Endocr. (Kbh) 92: 242

160. Van Den Brande, J.L., Van Buul, S., Heinrich, U., Van Roon, F., Zurchart, Van Steirtegem, A.C. (1975)
Adv. Metab. Disord. 8:171

161. Van Wyk, J.J., Svoboda, M.E., Underwood, L.E. (1980)
J. Clin. Endocr. Metab. 50: 206

162. Van Wyk, J.J., Underwood, L.E., Hintz, R.L. (1974)
Rec. Prog. Horm. Res. 30: 259

163. Varon, S., Nomura, J., Shuster, E.M. (1967)
Biochemistry, 6: 2202

164. Varon, S., Nomura, J., Shuster, E.M. (1968)
Biochemistry, 7: 1296

165. Vasiliev, J.M. (1958)
Br. J. Cancer, 12:524

166. Vogel, A., Raines, E., Kariya, B., Riverst, M.J., Ross, R. (1978)
Proc. Natl. Acad. Sci. U.S.A. 75: 2810

167. Weber, K., Osborn, M. (1969)
J. Biol. Chem. 244: 4406

168. Westall, F.C., Lennon, V.A., Gospodarowicz, D. (1978)
Proc. Natl. Acad. Sci. U.S.A. 75: 4675

169. Westermark, B., Wastesan, A. (1975)
Ad. Metab. Disord. 8: 85

170. Westermark, B., Wasteson, A. (1975)
Exp. Cell Res. 98: 170

171. Wiedemann, E., Schwartz, E. (1972)
J. Clin. Endocrinol. Metab. 34: 51

172. Woolley, D.E., Tetlow, L.C., Mooney, C.J., Evanson, J.M. (1980)
in Proteinases and tumor invasion, Strauli, P. et al eds. Raven Press, N.Y.

173. Young, F.G. (1940)
J. Endocrinol. 26: 345

174. Zapf, J., Schoenle, E., Froesch, E.R. (1978)
Eur. J. Biochem. 87: 285